Structural Analysis of Short-Chain Lecithin/Triglyceride Micellar Particles†

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ABSTRACT: Mixed micellar particles of the short-chain lecithins dihexanoyl-, diheptanoyl-, bis(5-methylhexanoyl)-, bis(4,4-dimethylpentanoyl)-, and dioctanoylphosphatidylcholine with the triglycerides tributyrin and trihexanoin have been characterized by ^{13}C and ^{31}P NMR spectroscopy and quasi-elastic light scattering. Maximum triglyceride solubilized (determined by NMR) depends only on the number of carbons in the lecithin fatty acids; lecithins synthesized with 5-methyl hexanoate and 4,4-dimethyl pentanoate show triglyceride solubilities comparable to that of the straight-chain lecithin diheptanoylphosphatidylcholine. Hydrodynamic radii for particles with saturating amounts of triglyceride are small, approximately 30–40 Å for all systems studied. The dependence of the mean hydrodynamic radius on the lecithin/triglyceride ratio shows two characteristic regions. At low triglyceride/lecithin ratios, the data are consistent with lecithin micelles coexisting with mixed lecithin/triglyceride micelles. At triglyceride concentrations approaching the saturation limit, the particle radii vary in accordance with a geometrical model of a lecithin surface coating a triglyceride core. ^{13}C NMR spin–lattice relaxation times indicate that the lecithin acyl chains are more mobile in the presence of triglyceride. The motional characteristics of tributyrin and trihexanoin are similar to those of the neat anhydrous triglyceride, suggesting a viscosity of 10–15 cP in the microemulsion particles. The aqueous "interfacial tensions" of triglycerides are estimated and suggest that at least some of the triglyceride should be present at the particle surface. This hypothesis is examined with two different NMR probes of triglyceride core/surface. Triglyceride carbon resonances are not broadened by aqueous Mn^{2+} ions, while lecithin carbons show specific interactions, indicating that the bulk of the triglyceride is not accessible to the aqueous paramagnetic agent. On the other hand, in particles with tributyrin, the ^{31}P line widths are nearly independent of particle hydrodynamic radius and are narrower than in pure lecithin micelles or lecithin/trihexanoin particles. If the line width reflects lecithin head-group/head-group interactions, this observation is consistent with a greater relative surface partitioning of tributyrin. Such behavior can be accommodated by the simple geometrical model with the estimate that about one-tenth of maximum tributyrin solubilized may be at the surface. These differences in triglyceride behavior can be related to differences in pancreatic lipase kinetics toward these microemulsions.

Mammalian lipid transport and hydrolysis involve a series of complex microemulsion particles, lipoproteins, with similar fundamental structures, a surface film of protein, phospholipid, and cholesterol and a hydrophobic core of triglyceride and cholesterol ester (Scanu & Landsberger, 1980; Pownall et al., 1979). Analyses of these naturally occurring particles have shed light on the physical state of the lipids in these systems (Shipley et al., 1972; Atkinson et al., 1977). Specific protein/lipid and lipid/lipid interactions and association to form micellar complexes are less well understood. Heterogeneity of protein and lipid components in lipoproteins is one of the primary difficulties. Micellar model systems provide an alternate approach in constructing well-defined biomimetic particles which can be used to investigate specific lipoprotein interactions. Long-chain lecithins, which form bilayer vesicles in aqueous solution, have been used successfully as membrane models (Boelar & Chan, 1978; Jain & Wagner, 1980), but detergent molecules must be added to form micellar structures with these phospholipids (Ribeiro & Dennis, 1975; Roberts & Dennis, 1978; Roberts et al., 1978). In contrast, short-chain phospholipids form micelles in aqueous solution (Tausk et al., 1974a–c). This property has made them extremely useful in studying the interfacial behavior of water-soluble phospholipases (Verger & de Haas, 1976; Wells, 1974). NMR and Raman studies have shown that these micellar lecithins retain many of the conformational features (chain packing and nonequivalence, head-group orientation, etc.) of long-chain lecithins (Burns & Roberts, 1980; Burns et al., 1982).

Their micellar form also makes short-chain lecithins a useful matrix for creating model lipoproteins. These synthetic lecithins solubilize relatively large quantities of triglyceride (Burns & Roberts, 1981a) and cholesterol (Burns & Roberts, 1981b). The mixed particles are excellent substrates for a variety of lipolytic enzymes. To understand these kinetics and further build up the mixed particle with additional lipids or protein, we need to determine the particle size, the motional behavior of individual components, and the average location of lipid solutes. The latter characteristic is the most interesting and the most difficult to measure. A variety of pure detergent micelles (Menger, 1979), binary mixed micelles (Mazer et al., 1979; Forrest et al., 1981), and bilayer systems (McIntosh et al., 1980; Heelis et al., 1979) have been examined in detail. Solute location studies in bilayers have involved X-ray and neutron scattering (Franks, 1976; Worcester & Franks, 1976; Franks & Lieb, 1979; White et al., 1981); in mixed micelles, these types of studies have involved solute molecules with spectroscopic probes (Narayanan et al., 1980; Menger & Bonicamp, 1981; Russell et al., 1981).

We have used quasi-elastic light scattering (QLS)[1] to

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[1] Abbreviations: QLS, quasi-elastic light scattering; diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; cmc, critical micelle concentration; Pipes, 1,4-piperazinediethanesulfonic acid; TB, tributyrin; TH, trihexanoin; TG, triglyceride; T_1, spin–lattice relaxation time; EDTA, ethylenediaminetetraacetic acid.

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measure the size and polydispersity of short-chain lecithin/triglyceride micellar particles and ^{13}C NMR to assess chain motions of individual lipids. Water-insoluble triglyceride is modeled by "core" and "surface" populations in equilibrium. ^{31}P NMR line widths of lecithin phosphorus resonances and QLS estimations of particle sizes and polydispersity as a function of triglyceride/lecithin suggest there is a different core/surface partitioning between tributyrin and trihexanoin in these micelles. The data are consistent with qualitative placement of each component on the basis of estimated interfacial tensions. The model for discrete particles with differences in core/surface partitioning of triglycerides can be used to understand lipase kinetics toward these micellar particles (Burns & Roberts, 1981a).

Experimental Procedures

Materials. Dihexanoyl-PC was obtained from Calbiochem or Avanti; diheptanoyl-PC and dioctanoyl-PC were obtained from Avanti or synthesized by the fatty acyl imidazolide method described below (Burns & Robers, 1980). Branched seven carbon chain lecithins were also synthesized with this procedure by using 5-methylhexanoic acid (Pfaltz & Bauer) and 4,4-dimethylpentanoic acid (Saber Laboratories). Detailed physical characterization of these lecithins will be presented separately. Tributyrin (Aldrich) and trihexanoin (Sigma) were used without further purification; purity analysis of these triglycerides is available elsewhere (Burns & Roberts, 1981a). Lipid purity was monitored by thin-layer chromatography in three systems (Burns & Roberts, 1980, 1981a).

The appropriate fatty acyl imidazolide is prepared by reacting 1 volume of the fatty acid with a 1.2-fold molar excess of carbonyldiimidazole (Sigma) in 1–2 volumes of $CHCl_3$ under dry air for approximately 1.5 h, or until the insoluble carbonyldiimidazole is dissolved. Solid glycerophosphocholine ($CdCl_2$ adduct; Sigma) is added directly to the fatty acyl imidazolide mixture (glycerophosphocholine:fatty acid:carbonyldiimidazole ratio of 1:5:6). This reaction is allowed to proceed for 2 days with light heating and occasional mixing under an N_2 atmosphere. The mixture is then washed 6–10 times with $CHCl_3/CH_3OH$ (3:1) and 3 M NaCl, pH 3 (lecithins with seven carbon acyl chains), or distilled H_2O, pH 3 (dioctanoyl-PC). Phases are separated by centrifugation at 6800*g* for 10 min. The organic phase is thoroughly dried, and the phospholipid is purified by two successive silicic acid columns. The overall yield is 40%, excluding repurification of contaminated column fractions. The reaction is conveniently monitored throughout the synthesis by thin-layer chromatography in $CHCl_3/CH_3OH/10.5$ N NH_4OH (60:35:8) with detection by iodine vapor.

Lecithin/triglyceride micellar particles were formed by cosolubilization of both lipids in benzene and/or chloroform, solvent removal under N_2, evacuation at low pressure for at least 12 h, addition of buffer, and incubation at room temperature for at least 4 h. For determination of maximum solubilities of triglycerides in lecithins, excess triglyceride was cosolubilized. After incubation of the aqueous mixture for 15 h, insoluble triglyceride was suctioned from the top of the sample. The optically clear solution was then used for NMR and QLS studies. Longer incubation periods did not change the hydrodynamic radii of the saturated micelles. Calculation of relative lecithin/triglyceride ratios was based on comparing ^{13}C NMR intensities of similar carbon atoms (NOEs are known and essentially equal to the maximum) in spectra taken under nonsaturating conditions. The absolute lecithin concentration was determined by phosphorus assay (Eaton & Dennis, 1976).

NMR Spectroscopy. ^{13}C NMR spectra were obtained at 67.9 MHz with a Bruker 270 spectrometer. Samples contained 80 ± 10 mM lecithin and varying amounts of triglyceride in 50 mM potassium phosphate and 1 mM EDTA, in D_2O, pD 7.4, 30 °C. Spin–lattice relaxation times were measured as described previously (Burns & Roberts, 1980). Lipid samples for Mn^{2+} line-broadening experiments were prepared in 50 mM Pipes, 1 mM EDTA, and 0.8 mM [2-^{13}C]acetate, pD 7.4. Details of measuring ΔI, the fractional intensity for specific broadening due to Mn^{2+} proximity, have been given previously (Burns & Roberts, 1981a,b). ^{31}P NMR spectra were obtained at 36.4 MHz with a JEOL FX-90Q spectrometer equipped with a Shimplex. With this system, line-width measurements are limited by $(\pi T_2)^{-1}$ of 0.05 Hz.

Interfacial Tensions. Interfacial tensions of triglyceride/water systems were determined by using the mutual solubility technique of Donahue & Bartell (1952). Using 31 organic liquid/water systems, these authors found a linear empirical relationship between interfacial tension (S) and log ($N_1 + N_2$), where N_1 is the mole fraction of water in the organic phase and N_2 is the mole fraction of the organic liquid in the aqueous phase. For the equation $S = A_1 \log (N_1 + N_2) + A_0$, these authors obtain $A_1 = -16.5$ (±0.3) and $A_0 = -3.8$ (±0.7) (coefficient of determination $r^2 = 0.99$). For the measurements done in this study, the solubility of water in the organic phase was determined by using ^3H_2O (Amersham). One milliliter of the appropriate organic liquid was incubated for 18 h (with occasional bath sonication) with 1 mL of ^3H_2O. Half of the organic phase was removed and incubated with a second aliquot of ^3H_2O. The solubility of the organic molecule in water was not determined. In the study of Donahue and Bartell, $N_1/N_{2,av} = 180$ (±200); water is on the average 100 times as soluble in an organic liquid as the organic liquid is in water. In no case was $N_2 > N_1$. Using the organic liquids 2-pentanone, ethyl acetate, diethyl ether, ethyl butyrate, benzene, toluene, and octane, values of N_1 were determined by ^3H isotopic dilution assuming neat liquid densities. Best-fit values to the equation $S = A_1 \log N_1 + A_0$ were $A_1 = -15.1$ (±0.7) and $A_0 = -9.9$ (±1.7) (coefficient of determination $r^2 = 0.99$), in reasonable agreement with the values of Donahue and Bartell (above). Values of interfacial tension obtained by this technique should be considered as approximations, but they are easy to obtain and can be used qualitatively to understand the surface activity of solutes.

Quasi-Elastic Light Scattering. The Brownian motion of micelles results in temporal fluctuations in the intensity of scattered light. The autocorrelation function, $R(\tau)$, of the scattered light intensity is given by $R(\tau)/R(0) = (\sum_{n=1}^{\infty} G_n e^{-\Gamma_n \tau})^2$. The diffusion coefficient of the nth species, D_n, is equal to Γ_n/q^2 where q is the magnitude of the scattering vector. For a monodisperse system, the decay constant Γ is simply proportional to $1/D$. The autocorrelation function of each species, $e^{-\Gamma_n \tau}$, is weighted by the fraction of light scattered by each species: $G_n = C_n M_n/(\sum C_n M_n)$ where C_n and M_n are the weight concentration and the molecular weight of the nth species, respectively. The mean translational diffusion coefficient is calculated from the average decay constant: $\bar{D} = \bar{\Gamma}/q^2$, where $\bar{\Gamma}$ is defined as $\sum G_n \Gamma$. The mean hydrodynamic radius, $\bar{R}_h$, is calculated from the mean diffusion coefficient by using the Stokes–Einstein relation $\bar{D} = k_B T/(6\pi\eta\bar{R}_h)$ where k_B is the Boltzmann constant, T is the absolute temperature, and η is the solvent viscosity. For a polydisperse system, the width of the distribution is characterized by the variance, the standard deviation of the mean decay constant $V = 100 \times (\overline{\Gamma^2} - \bar{\Gamma}^2)^{1/2}/\bar{\Gamma}$. Details of the apparatus of the data analyses

Table I: Maximum Triglyceride Solubilities in Short-Chain Lecithin Micelles[a]

lecithin	maximum mole fraction		$\bar{R}_h$ (Å)[b]	
	tributyrin	trihexanoin	tributyrin	trihexanoin
dihexanoyl-PC	0.16 (0.01)[c]			
diheptanoyl-PC	0.36 (0.02)	0.20 (0.03)	34	34
dioctanoyl-PC	0.52 (0.01)	0.30 (0.01)	34	31
bis(5-methylhexanoyl)-PC	0.36 (0.01)	0.22 (0.02)		
bis(4,4-dimethylpentanoyl)-PC	0.34 (0.02)	0.21 (0.03)		

[a] Determined from integration of ^{13}C NMR spectra in D_2O; lecithin concentration is 80 ± 10 mM by phosphate assay. [b] $\bar{R}_h$ determined by QLS as described under Experimental Procedures for the triglyceride-saturated particle. [c] Values in parentheses represent standard deviations.

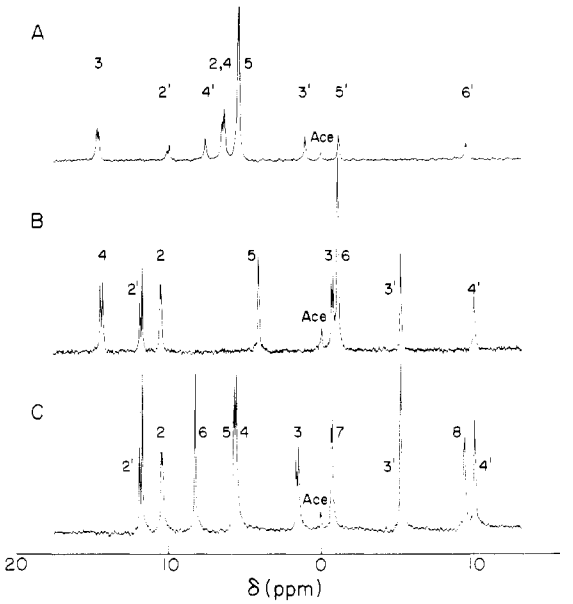

FIGURE 1: ^{13}C NMR spectra of the alkyl chain region of (A) bis-(4,4-dimethylpentanoyl)-PC (76 mM)/trihexanoin (20 mM), (B) bis(5-methylhexanoyl)-PC (72 mM)/tributyrin (41 mM), and (C) dioctanoyl-PC (88 mM)/tributyrin (95 mM). Lecithin chain carbons are numbered from the carbonyl end. Primed numbers indicate triglyceride carbons.

have been previously described (Missel, 1981; Missel et al., 1980; Mazer et al., 1976).

Solutions were pipetted into cylindrical scattering cells and centrifuged at 10000 rpm for 20 min to sediment dust. Some weakly scattering samples required up to 2 h to remove all the dust. On a single sample, measurements were repeated at least 3 times.

Results

Solubilities and Interfacial Tensions. Table I shows the maximum mole fraction of triglyceride solubilized after 15 h with 80 ± 10 mM diacyl-PC, the phospholipid concentration used throughout this study. Shorter incubation times of 2.3 h gave comparable results. The values of $\bar{R}_h$ for the saturated particles are also shown. As much as 0.5 mol fraction of triglyceride can be solubilized in this system. Figure 1 shows representative spectra for dioctanoyl-PC with tributyrin and with trihexanoin. Carbons from lecithin and triglyceride are well separated. Bis(5-methylhexanoyl)-PC and bis(4,4-dimethylpentanoyl)-PC both have seven carbon atom fatty acyl chains and show maximum solubilities identical with that of diheptanoyl-PC. No effect on triglyceride solubilization is observed by increasing the number of methyl groups in the phospholipid alkyl chains.

Table II shows aqueous "interfacial tension" for a variety of organic molecules calculated by the method of Donahue & Bartell (1952). This simple method is not appropriate for alcohols, carboxylic acids, or other organic molecules with readily exchangeable hydrogen atoms. For comparison, interfacial tensions obtained by direct measurement (Girifalco & Good, 1957) are included in Table II. Short-chain triglyceride (TB and TH) interfacial tensions are comparable to values for heptanol and heptanoic acid and intermediate between values for ethyl acetate and ethyl butyrate.

Table II: Aqueous (H_2O[a]) Interfacial Tensions (IT) of Triglycerides and Related Molecules[b]

molecule	IT, calcd (erg/cm²)	IT, measured[c] (erg/cm²)
triacetin	1 (3)[d]	
tributyrin	7 (3)	
trihexanoin	9 (4)	
ethyl acetate	6 (3)	6.8
ethyl butyrate	14 (4)	15.7
ethyl hexanoate	15 (3)	
heptanol		7.7
heptanoic acid		7.0
n-heptane		50.2
benzene		35.0

[a] Identical solubilities against H_2O and D_2O were observed for ethyl hexanoate, tributyrin, and trihexanoin. [b] Measured as described under Experimental Procedures. [c] Girifalco & Good (1957). [d] Interfacial tensions are not easily measured by this technique for interfacial tensions <2 erg/cm², as discussed in Donahue & Bartell (1952).

Table III: Lecithin Acyl Chain ^{13}C T_1 Values and the Influence of Triglyceride

carbon atom	T_1 (s)			
	dihexanoyl-PC		diheptanoyl-PC	
	+tributyrin[a]	pure PC micelle	+tributyrin[a] or trihexanoin[b]	pure PC micelle
C=O	3.95 (0.18)[c]	2.96 (0.12)	3.00 (0.16)	2.24 (0.06)
2 *sn*-2	0.43 (0.01)	0.43 (0.01)	0.33 (0.04)	0.51 (0.03)
2 *sn*-1	0.64 (0.04)	0.64 (0.04)	0.51 (0.18)	0.51 (0.18)
3 *sn*-2	0.72 (0.04)	0.72 (0.04)	0.51 (0.01)	0.36 (0.02)
3 *sn*-1	0.95 (0.08)	0.95 (0.08)	0.54 (0.06)	0.54 (0.06)
4 *sn*-2	1.31 (0.03)	0.90 (0.09)	0.72 (0.07)	0.72 (0.07)
4 *sn*-1	1.23 (0.05)	0.89 (0.08)	0.73 (0.01)	0.73 (0.01)
5	1.89 (0.04)	1.32 (0.28)	1.03 (0.07)	0.61 (0.13)
6	3.27 (0.18)	2.68 (0.27)	1.11 (0.05)	0.53 (0.16)
7			1.56 (0.04)	1.20 (0.04)
			2.40 (0.16)	2.40 (0.16)

[a] Mole fraction of tributyrin, 0.2. [b] Mole fraction of triglyceride, 0.2. [c] Values in parentheses represent errors in T_1 determined as described in Burns & Roberts (1980).

^{13}C NMR Relaxation Studies. Table III shows lecithin acyl chain spin–lattice relaxation times (T_1 values) in the presence or absence of short-chain triglycerides. Values for pure lecithin micelles are taken from an earlier study (Burns & Roberts, 1980). No significant differences in T_1 values were detected

Table IV: ^{13}C T_1 Values (s) for Neat Triglycerides and Comparisons with Micellized Triglyceride

carbon atom	T_1 (s) for heat TG	T_1(micelle)/[T_1(neat)][a,b] diC$_6$PC	diC$_7$PC	diC$_8$PC	diC$_8$PC	diC$_8$PC
tributyrin						
mole fraction of TG in particle	1.00	0.2	0.2	0.2	0.3	0.5
glyc CHO	0.42 (0.05)[c]	0.3 (0.1)	–	–	–	–
glyc CH_2O	0.26 (0.01)	0.81 (0.07)	–	–	–	–
C=O	4.7 (1.3)	–	–	–	–	–
C-2 (*sn*-2)	0.95 (0.19)	–	–	–	–	–
C-2 (*sn*-1,3)	0.97 (0.15)	1.6 (0.3)	–	–	–	–
C-3	1.87 (0.14)	1.4 (0.2)	–	–	–	–
C-4	3.46 (0.04)	1.17 (0.08)	0.65 (0.07)	0.56 (0.03)	0.91 (0.02)	–
trihexanoin						
mole fraction of TG in particle	1.00		0.2	0.2		
glyc CHO	0.23 (0.03)		–	2.8 (0.8)		
glyc CH_2O	0.16 (0.01)		–	–		
C=O	3.6 (0.2)		0.4 (0.2)	0.7 (0.1)		
C-2 (*sn*-2)	0.43 (0.01)		–	2.8 (0.7)		
C-2 (*sn*-1,3)	0.50 (0.01)		1.7 (0.2)	–		
C-3	0.73 (0.01)		1.5 (0.1)	–		
C-4	1.03 (0.06)		1.6 (0.2)	–		
C-5	1.51 (0.07)		1.4 (0.1)	–		
C-6	2.56 (0.06)		1.3 (0.1)	–		

[a] Abbreviations: TG, triglyceride; diC$_n$PC, diacylphosphatidylcholine. [b] When the T_1(neat)/[T_1(micelle)] ratio = 1, i.e., when a given carbon T_1 is unaltered by micellization with lecithin, a dash is shown in the table; this emphasizes differences in neat and micellized triglyceride. [c] Values in parentheses give errors in T_1 determinations.

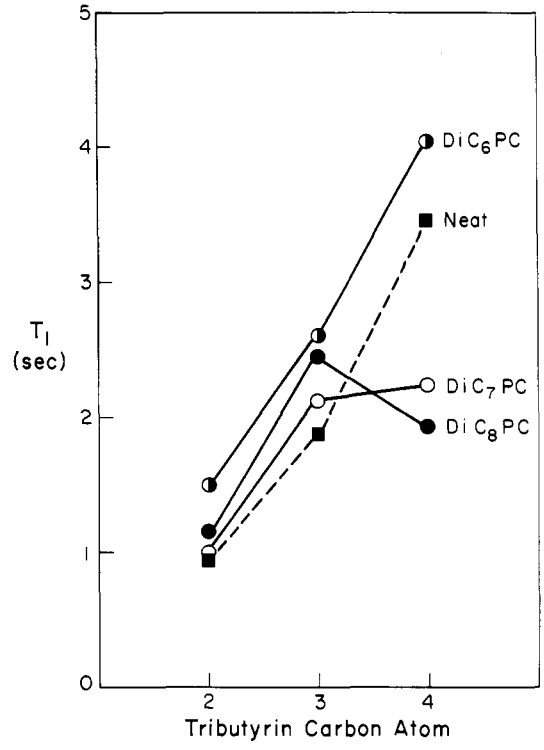

FIGURE 2: ^{13}C T_1 acyl chain profile for 0.2 mole fraction of tributyrin in 80 ± 10 mM of the indicated lecithins and neat tributyrin.

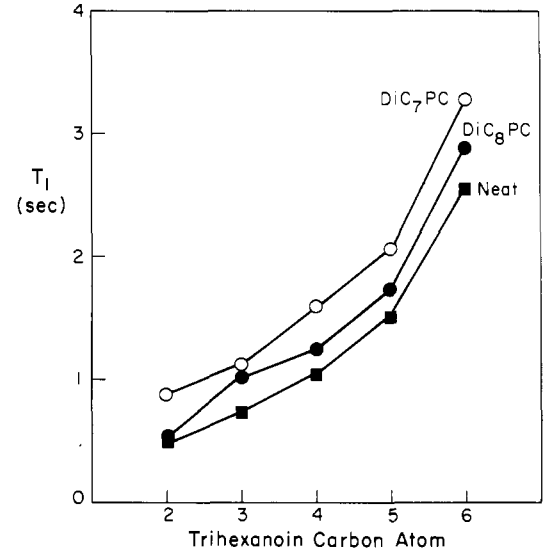

FIGURE 3: ^{13}C T_1 acyl chain profile for 0.2 mole fraction of trihexanoin in 80 ± 10 mM of the indicated lecithins and neat trihexanoin.

for dioctanoyl-PC or for backbone and head-group resonances for *any* of the three short-chain lecithins examined (data not shown). Differences in ^{13}C T_1 relaxation times occur for dihexanoyl-PC carbonyl resonances (33% increase with TB), for acyl chain methylene carbon atoms (40% increase in the T_1 of C-4, 43% increase in the T_1 of C-5), and at the terminal methyl end of the chain (22% increase with TB). Similar increases in chain T_1 values occur for diheptanoyl-PC solubilizing either tributyrin or trihexanoin: the carbonyl T_1 increases 34%; the *sn*-2 α-methylene carbon shows a significant decrease (−35%), while other chain methylenes show T_1 increases from 40 to 110%. With this lecithin matrix, the terminal methyl T_1 is unaltered with the inclusion of triglyceride.

Table IV shows ^{13}C T_1 relaxation times for neat anhydrous tributyrin and trihexanoin. ^{13}C T_1 values for acyl chain carbons in neat tributyrin are considerably longer than those for comparable carbons in trihexanoin. This suggests that the number of acyl chain carbons in these molecules controls the chain–chain interactions and segmental motions. Also shown in this table are triglyceride carbons whose T_1 values when solubilized by lecithin are *different* from the neat relaxation times [i.e., T_1(micelle)/[T_1(neat) ≠ 1]. When T_1 ratios are nearly equivalent, i.e., less than two combined uncertainties from the neat T_1 value, no entry is shown in the table (as an indicator of general data quality, the average percent uncertainty for all triglyceride T_1 values was 13%). Representative triglyceride chain T_1 profiles are also shown in Figures 2 and 3. (The dihexanoyl-PC/trihexanoin system cannot be examined by ^{13}C NMR because of extensive overlap between hexanoyl carbons of the lecithin and triglyceride.) These data show the following: (i) in systems where the phospholipid and

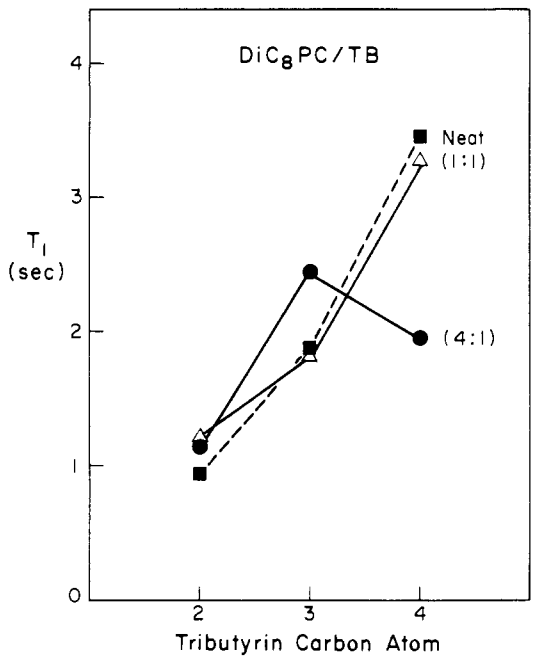

FIGURE 4: ^{13}C T_1 and chain profile for different amounts of tributyrin solubilized in 80 ± 10 mM dioctanoyl-PC.

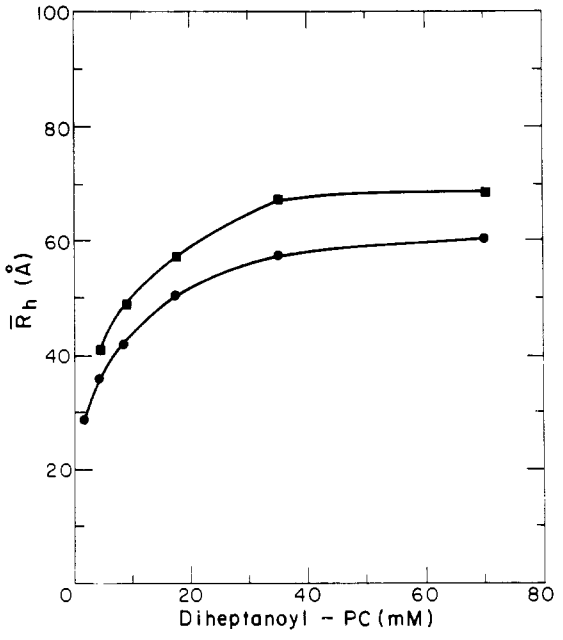

FIGURE 5: Mean hydrodynamic radius, $\bar{R}_h$, of diheptanoyl-PC micelles as a function of lecithin concentrations at 5 (■) and 29 °C (●).

triglyceride chain lengths are similar (dihexanoyl-PC/TB, diheptanoyl-PC/TH), the triglyceride chain T_1 values tend to be *longer* than the corresponding neat T_1 values (with glyceryl backbone and carbonyl values possibly shorter); however, the chain T_1 profile is similar to that of neat triglyceride; (ii) in diheptanoyl-PC or dioctanoyl-PC systems with subsaturating TB, the terminal methyl group T_1 values are *shorter* than the neat value [in the dioctanoyl-PC/TB system, this T_1 difference becomes smaller as increasing amounts of tributyrin are placed in the mixed micelle (Figure 4)]; (iii) the majority of T_1 values for micellized triglyceride are very similar to the values for neat triglyceride.

QLS of Lecithin Micelles. Measurements of $\bar{R}_h$ of diheptanoyl-PC were made at several concentrations from 70 to 2.2 mM, at two temperatures, 5 and 20 °C. The experimental values of $\bar{R}_h$ are shown in Figure 5. The size of diheptanoyl-PC micelles is a strong function of both temperature and concentration. At the cmc, the extrapolated size is approximately 26 Å. Using classical light scattering and equilibrium ultracentrifugation, Tausk et al. (1974b) determined the mean micellar weight at the cmc to be approximately 40 monomers. They proposed a spherocylinder model with a hydrocarbon core. The hydrodynamic radius of such a micelle with an aggregation number of 40 is calculated to be about 26 Å, which compares extremely well with the observed $\bar{R}_h$. In contrast, Tausk et al. (1974b) have shown that dihexanoyl-PC micelles do not grow with increasing lecithin concentration. This lipid forms small, spherical micelles. Dioctanoyl-PC shows a complex dependence of $\bar{R}_h$ on dioctanoyl-PC concentration (Tausk et al., 1974c), whose interpretation is difficult.

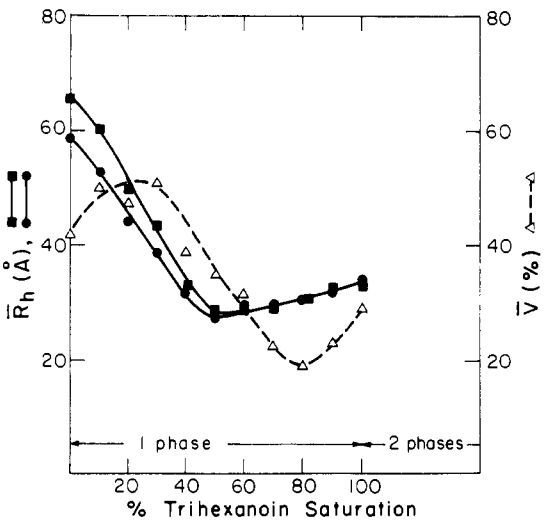

FIGURE 6: Mean hydrodynamic radius, $\bar{R}_h$, of diheptanoyl-PC/trihexanoin mixed micelles as a function of the percent trihexanoin saturation (defined by Table I as 0.2 mole fraction) at 5 (■) and 20 °C (●). The polydispersity, $\bar{V}$, at 20 °C is also shown.

QLS of Mixed Lecithin/Triglyceride Micelles. Aliquots of lecithin solutions and triglyceride-saturated lecithin solutions were mixed in varying proportions to produce a series of solutions with constant lecithin concentration and varying triglyceride concentration. The $\bar{R}_h$ and V of the diheptanoyl-PC/trihexanoin series at two different temperatures are shown in Figure 6. As the triglyceride concentration increases, $\bar{R}_h$ initially decreases and then increases slightly. In aqueous saturated TB and TH solutions, the scattering intensity was less than 1% of that of diheptanoyl-PC at the cmc. Therefore, triglyceride micelles were not detected and could not have contributed to the $\bar{R}_h$ measured in mixed TG/PC samples.

The growth of rod-shaped micelles, such as diheptanoyl-PC, is temperature sensitive. However, the $\bar{R}_h$ of the saturated mixed micelles is constant over a range from 5 to 40 °C. The temperature dependence of $\bar{R}_h$ at various TH/PC ratios is shown in Figure 6.

The $\bar{R}_h$ values of tributyrin/diheptanoyl-PC mixtures are shown in Figure 7. Two qualitative differences are apparent between tributyrin and trihexanoin. First, there is an initial increase in $\bar{R}_h$ as tributyrin is added and then a decrease as was seen for trihexanoin. Second, the slope of the $\bar{R}_h$ vs. TG/PC curve from 50–60% triglyceride saturation to saturation is less than that in the trihexanoin case. Possible explanations for this are discussed below.

Mn^{2+}-Induced ^{13}C NMR Line Broadening. Table V shows Mn^{2+}-induced ^{13}C NMR peak intensity changes for a diheptanoyl-PC/TB sample. This procedure uses ^{13}C peak height changes in an Mn^{2+} titration to separate specific Mn^{2+}-induced

Table V: Mn^{2+}-Induced Intensity Changes for Carbon Atoms of Micellar Diheptanoyl-PC and Solutes

carbon atom	ΔI (+TB)	ΔI_{av} (+TB)[a]	ΔI_{av} (+TH)[b]	ΔI_{av} (+cholesterol)[c]
diheptanoyl-PC				
$N(CH_3)_3$	−0.42 (0.13)			
CH_2N	−0.25 (0.11)			
choline CH_2OP	−0.55			
glycerol CH_2OP	−0.50	−0.38 (0.13)	−0.41 (0.10)	−0.35 (0.17)
CHO	−0.26 (0.16)			
CH_2O	−0.27 (0.07)			
C=O	−0.20 (0.06)			
C-2 *sn*-2	−0.32 (0.13)			
C-2 *sn*-1	−0.19 (0.15)			
$C\text{-}3_{av}$	−0.17 (0.07)	−0.18 (0.06)	−0.19 (0.06)	+0.01 (0.05)
$C\text{-}4_{av}$	−0.18 (0.08)			
$C\text{-}5_{av}$	−0.12 (0.11)			
$C\text{-}6_{av}$	−0.21 (0.11)			
$C\text{-}7_{av}$	−0.16 (0.09)			
triglyceride				
CHO	−0.03 (0.19)			
CH_2O	−0.08 (0.09)			
C=O	+0.02 (0.17)			
		−0.07 (0.06)	−0.05 (0.10)	
C-2 *sn*-2	−0.02 (0.14)			
C-2 *sn*-1,3	−0.12 (0.19)			
C-3	−0.12 (0.14)			
C-4	−0.12 (0.12)			
cholesterol C-4				
Pipes				−0.30 (0.04)
$N(CH_2)$	−0.16 (0.14)			
ring	−0.12 (0.09)	−0.18 (0.08)	−0.19 (0.15)	−0.03 (0.1)
CH_2SO_4	−0.27 (0.16)			
% intensity change due to lock broadening (per mM Mn^{2+})		−0.10 (0.04)	−0.07 (0.03)	−0.15 (0.04)

[a] At 0.3 mole fraction of tributyrin. [b] At 0.2 mole fraction of trihexanoin (Burns & Roberts, 1981a). [c] At 0.05 mole fraction of cholesterol (Burns & Roberts, 1981b).

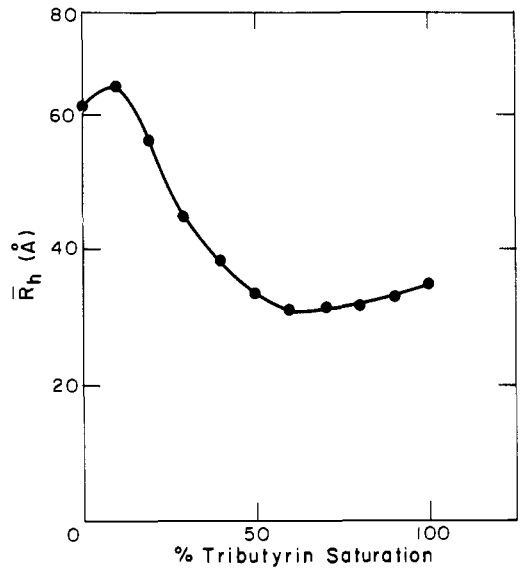

FIGURE 7: Mean hydrodynamic radius, $\bar{R}_h$, of tributyrin/diheptanoyl-PC mixed micelles as a function of tributyrin saturation at 20 °C.

line broadening from line-broadening contributions due to broadening of the internal D_2O lock frequency. The specific ΔI values are quite similar to those observed previously for the diheptanoyl-PC/TH micelle (Burns & Roberts, 1981a). Average values of ΔI for various parts of the lipid molecules in two systems as well as for diheptanoyl-PC/cholesterol micelles (Burns & Roberts, 1981b) are summarized in Table V. Although the uncertainty in the average ΔI values for backbone and head-group resonances appears large, the individual values show that systematic variations occur (i.e., carbon atoms around the phosphate are most broadened).

Table VI: ^{31}P NMR Line Widths and QLS Hydrodynamic Radii

lecithin	triglyceride	mole fraction of triglyceride	T_2^{-1} (s^{-1})	$\bar{R}_h$ (Å)[a] D_2O	$\bar{R}_h$ (Å)[a] H_2O
diC_6PC			2.5	24	
diC_7PC			4.7	70	65
diC_8PC			13	125	130
diC_6PC	TB	0.2	4.1	26	
diC_7PC	TB	0.2	4.7	43	34
diC_8PC	TB	0.2	7.2	153	83
		0.3	5.0	77	38
		0.5	4.7	34	34
diC_7PC	TH	0.2	4.7	37	35
diC_8PC	TH	0.1	6.3	62	55
		0.2	4.1	42	31

[a] $\bar{R}_h$ values determined at 25 °C; values corrected for D_2O solvent.

Significant differences occur between triglyceride and cholesterol ΔI_{av} values and the phospholipid acyl chains in these binary systems. The cholesterol C-4 near the surface-active hydroxyl group is more strongly affected by Mn^{2+} than any of the triglyceride carbons. The differences in buffer ΔI_{av} values and the percent intensity change due to lock broadening are not significant.

^{31}P NMR Line Widths. Table VI shows ^{31}P NMR relaxation rates determined from line widths and QLS hydrodynamic radii of the pure and mixed micellar samples. NMR and QLS measurements were made on the same D_2O solutions (Figure 8). ^{31}P NMR line widths for both pure lecithin micelles and mixed micelles with trihexanoin show a marked dependence on particle size. In the presence of tributyrin, the lipid ^{31}P line width is less sensitive to $\bar{R}_h$ and becomes narrower. A different motional characteristic must be dominating ^{31}P line widths for lipid in tributyrin-containing particles than pure

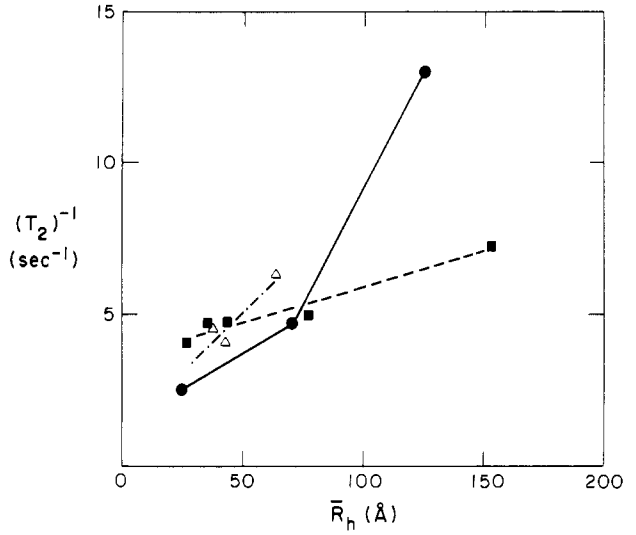

FIGURE 8: ^{31}P NMR T_2^{-1} values for the lecithin phosphorus in pure lecithin micelles (●) and microemulsion particles with trihexanoin (▲) and tributyrin (■) in D_2O. Compositions corresponding to certain sizes are shown in Table VI.

lipid micelles or lipid/trihexanoin micelles, possibly a contribution from a surface pool of tributyrin.

Discussion

Many of the topics currently under investigation in the field of micelle chemistry are directly related to the question of the molecular dynamics of surfactant molecules. The motions of amphiphiles in the micelles determine the association of the micelle interface with a specific portion of the amphiphile molecule, the definition of "surface and core pools" of a molecule solubilized in a micelle, and the penetration of water into the micelle. The interaction of surface-active enzymes such as lipases and phospholipases with micelles undoubtedly depends on these parameters. In order to understand enzyme kinetics, we have characterized micellar particles of short-chain lecithins and triglycerides. Of particular interest are particle size distribution, motions of individual lipid molecules, and the distribution of triglyceride at either (or both) the surface or particle core.

Particle Coexistence Model and Lipase Kinetics. The interaction of lecithins and triglyceride could take place in at least two ways. The triglyceride could be present at the surface of a diheptanoyl-PC micelle, and it could form a core of triglyceride with a surface of diheptanoyl-PC. We can hypothesize the formation of a spherical microemulsion-type particle with a triglyceride core, and then by knowing the molecular volumes of the triglycerides and the hydrocarbon part of the lecithin and the surface area per lecithin head group, we can estimate the size of the particle. For diheptanoyl-PC and maximum TH, and using an area of 65 Å^2/head group (Tausk et al., 1974b), we arrive at a radius of 33 Å, very close to the experimental value of 34 Å. In each particle there would be about 30 molecules of TH and 150 molecules of diheptanoyl-PC.

If the area per lecithin head group remains constant, then we can calculate the dependence of the particle radius on the triglyceride/lecithin ratio. This is a simple geometric relation between volume, area, and surface. The surface area and volume of the hydrocarbon core composed of TG and lecithin chains are related as follows:

$$R_{\text{hydrocarbon}} = 3\left(\frac{V_{PC}N_{PC} + V_{TG}N_{TG}}{A_{PC}N_{PC}}\right)$$

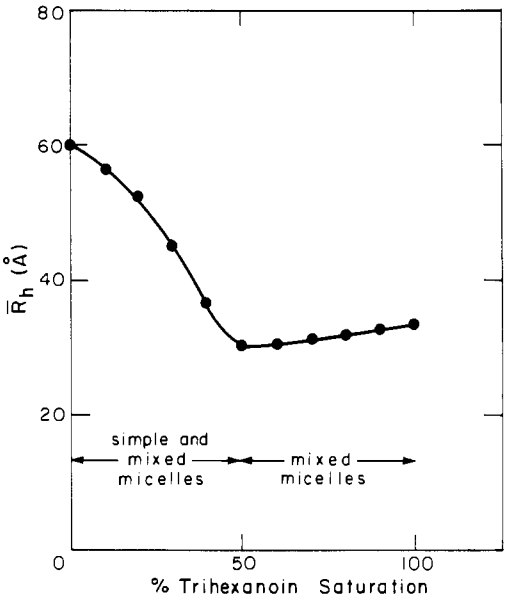

FIGURE 9: Mean hydrodynamic radius, $\bar{R}_h$, as a function of trihexanoin saturation predicted by the simple geometric model. The coexistence region where both simple and mixed micelles are present extends from 0 to 50% saturation. Above 50% saturation, only mixed micelles are present.

where V_{PC} and V_{TG} are the molecular volumes of the hydrocarbon part of diheptanoyl-PC and TG, respectively, and N_{PC} and N_{TG} are the number of molecules in each particle. The total radius is the radius of the hydrocarbon part plus the head group, estimated to be about 10 Å. According to this geometric model, the radius of the mixed micelle should increase (compared with the limiting size for pure lecithin micelles) with increasing triglyceride/lecithin ratio. This is observed experimentally for TH/PC molar ratios greater than 0.1 (or at about half saturation of the particle).

However, there must be a minimum size for the mixed micelle. This implies that for low TG/PC ratios there are two types of micelles coexisting, diheptanoyl-PC micelles and mixed micelles. As TG is added to PC micelles, some of the diheptanoyl-PC is used up to solubilize it. There is less diheptanoyl-PC remaining in the simple micelles, so the $\bar{R}_h$ of these sample micelles decreases according to the previously described concentration dependence. We can calculate an average aggregation number for each $\bar{R}_h$ on the basis of Tausk and Overbeek's model of rod-shaped micelles. From the definition of D, we can calculate a hypothetical dependence of $\bar{R}_h$ on the TG/PC ratio; this is shown in Figure 9. It is derived from the model based on the following assumptions: (i) there is a coexistence region of rod-shaped diheptanoyl-PC micelles and mixed micelles, and (ii) the radius of the mixed micelles is determined by geometrical considerations (the ratio of volume to surface area). It is to be emphasized that the qualitative features of the dependence of $\bar{R}_h$ on the TG/PC ratio do not depend on the precise estimates used for molecular volumes, surface area, and aggregation numbers of diheptanoyl-PC micelles. Therefore, with this simple model of diheptanoyl-PC micelles and mixed micelles in coexistence, we can qualitatively predict the experimental curve. We can also predict that the polydispersity will reach a maximum in the coexistence region. This is observed experimentally in Figure 6. This picture of mixed micelles in coexistence with simple micelles is very similar to that proposed for the bile salt/lecithin system investigated by Mazer et al. (1980). Also as predicted by the coexistence model, there is a marked temperature dependence in the coexistence region from 0 to 10% TH, whereas in the

region where only mixed micelles are present, $\bar{R}_h$ is not a function of temperature.

Given this model, we can understand pancreatic lipase hydrolysis rates of triglycerides in short-chain lecithin mixed micelles. At lecithin concentrations sufficient to solubilize and clarify triglycerides, a fixed activity is observed, no matter how much excess lecithin is added (Burns & Roberts, 1981a). "Surface dilution" kinetics are not observed because the triglyceride concentration of the mixed micellar particle is not diluted as excess lecithin is introduced. A thermodynamically stable mixed particle with a unique composition is formed, and excess phospholipid segregates into pure lecithin micelles.

Surface and Core Distribution of Triglyceride: QLS Evidence. The simple geometric model derived from QLS data for short-chain lecithin solubilization of triglycerides assumes that all the triglyceride is in a hydrophobic core and excludes the possibility that some might be present at the surface. We can predict how a surface pool of triglyceride would affect that model. If the total surface area is increased by adding a percentage of the total triglyceride at the surface, then the prediction for $\bar{R}_h$ as a function of triglyceride/lecithin (TG/PC) must be modified by adding a contribution to the area from the triglyceride:

$$R_{\text{hydrocarbon}} = 3\left(\frac{V_{PC}N_{PC} + V_{TG}N_{TG}}{A_{PC}N_{PC} + A_{TG}N_{TG}S_{TG}}\right)$$

where S_{TG} is the percentage of triglyceride at the surface; A_{TG} is taken to be 100 Å^2. The effect of this is to decrease the slope of the curve of $\bar{R}_h$ vs. TG/PC. This is experimentally observed when diheptanoyl-PC solubilization of tributyrin and trihexanoin for the mixed micelle region is compared (greater than 50% of triglyceride saturation), suggesting that more tributyrin is at the surface.

A more quantitative estimate of the tributyrin present at the surface can be made by knowing particle sizes and maximum triglyceride solubilities. In Table I, the relative solubilities of trihexanoin and tributyrin in various chain length lecithins are shown. If the triglycerides are interacting nonspecifically, then one would predict that approximately equal volumes of each would be solubilized at the phase limit. The ratio of molar volumes for trihexanoin to tributyrin is 1.33, but the ratio of maximal solubilities is 1.8. Relatively more tributyrin than trihexanoin is solubilized. The presence of tributyrin at the micellar surface would increase the surface area per volume of hydrocarbon core and allow more tributyrin than trihexanoin to be solubilized. An estimate of the percentage of tributyrin at the surface from our simple geometrical model using the measured $\bar{R}_h$ values at maximum solubility

$$34\ \text{Å} = \frac{V_{PC}N_{PC} + V_{TB}N_{TB}}{A_{PC}N_{PC} + A_{TB}N_{TB}S_{TB}}$$

and

$$35\ \text{Å} = \frac{V_{PC}N_{PC} + V_{TH}N_{TH}}{A_{PC}N_{PC}}$$

gives the fraction of tributyrin at the surface as 7%. Although this is only an estimate, there appears to be a detectable amount of tributyrin at the surface. A greater amount of TB at the surface compared to TH is also supported by the $\bar{R}_h$ vs. percent triglyceride saturation curves (Figures 6 and 7). Addition of 10% of maximum solubilized TB slightly increases, rather than decreases, $\bar{R}_h$; with that amount of TH, $\bar{R}_h$ clearly decreases. This is consistent with an initial amount of TB solubilized at the surface in the larger rod-shaped diheptanoyl-PC micelles. The effects of TB and TH on dioctanoyl-PC micelles are similar: there is a large increase in $\bar{R}_h$ upon addition of 10–20% of the saturating TB concentration. For longer chain triglycerides, we would expect even smaller surface populations of triglyceride. For comparison, 2.8% triolein is solubilized in egg lecithin bilayers (Hamilton & Small, 1981). With a given chain length lecithin matrix, the triglyceride surface population appears to decrease with increasing triglyceride chain length. When the lecithin chain length increases, more of a given chain length triglyceride is solubilized, though how the surface population varies is not clear.

Motions of Components: NMR Evidence for a Surface Population of Tributyrin. ^{13}C NMR T_1 relaxation studies indicate that phospholipid dynamics in these micellar particles are slightly altered from those in pure lipid micelles: headgroup and backbone carbons are unaffected, while acyl chain carbons in general show increases in T_1 as triglyceride is solubilized. The local motion of the C–H bond seems to dominate ^{13}C relaxation. The acyl chains may become somewhat fluidized. The lack of effect of the triglyceride on lecithin carbon chemical shifts is extremely significant and indicates that the lipid chains remain highly disordered. No change in chain "trans" conformers occurs when triglyceride is incorporated as when monomers aggregate to form micelles (Burns et al., 1982) or when cholesterol is incorporated in these micelles (Burns & Roberts, 1981b). ^{13}C T_1 values for triglyceride in short chain lecithin micelles are similar to the corresponding neat triglyceride values. While it is tempting to speculate that this similarity occurs due to the formation of a "core" of triglyceride in the microemulsion particle, it may instead indicate that the "microviscosity" of the particle is similar to that of the neat triglyceride.

Levy et al. (1974) have shown that for simple ^{13}C dipolar relaxation, T_1 should be inversely proportional to viscosity. Comparing the T_1 data for neat tributyrin [viscosity = 11.6 (0.2); Weast, 1980) with those for tributyrin in methyl hexanoate and CD_3OD (data not shown) [similar viscosities, average = 0.7 (0.2) cP] shows which carbons are sensitive to viscosity changes. Specifically, the function

$$\log \frac{T_{1,\text{org}} - T_{1,\text{neat}}}{1/N_{\text{org}} - 1/N_{\text{neat}}} = \log N$$

should equal 1 if the simple dipolar relaxation law is being obeyed (Levy et al., 1974). This function is less than 1 (i.e., a lower than first power dependence is observed) in micellar samples. Backbone CHO and CH_2O carbon atoms give values of 0.0 (0.5) and −0.1 (0.4), respectively, while chain carbon atoms 1–4 yield values of 0.83 (0.12), 0.45 (0.09), 0.40 (0.10), and 0.32 (0.11). Due to larger T_1 changes observed for chain carbon atoms, they serve as a more sensitive monitor of viscosity than backbone carbon atoms. As observed in an earlier study (Levy et al., 1974), the value of this function steadily decreases down the acyl chain toward the terminal methyl group.

For subsaturating levels of triglyceride, different relaxation behavior is noted for tributyrin and trihexanoin. At the same mole fraction of tributyrin (0.2), the methyl T_1 decreases dramatically as the lecithin chain length increases (Figure 2). In dioctanoyl-PC, the tributyrin methyl T_1 increases and approaches the value observed for neat triglyceride as particle saturation is reached (Figure 4); trihexanoin carbons do not exhibit this effect; at subsaturating concentrations, T_1 values parallel to those of neat TH are observed. The observed decreased methyl T_1 for tributyrin could reflect a population

of tributyrin at the surface of the particle with the terminal methyl group in an environment more like lecithin than neat triglyceride; i.e., the observed T_1 would be a weight average of shorter T_1 for surface TB (around 1.5 s as for the lecithin methyl group) and a longer value for the saturated particle (similar to neat TB). The plots used to generate T_1 values in these subsaturating systems show no obvious evidence of multiple contributions to the measured T_1, although such deviations might be difficult to pick up. With this type of analysis of the observed T_1, the 0.2 mole fraction of TB in the dioctanoyl-PC particle would have about 50% of its TB at the surface; hence, 10% of the maximum TB solubilized could exist at the surface. For the 0.3 mole fraction of TB, the same treatment predicts that about 5% of the maximum TB solubilized by dioctanoyl-PC is at the surface. Therefore, the ^{13}C NMR data are consistent with 5–10% of the saturating amount of TB at the surface and the rest in a core. The T_1 data for trihexanoin carbons suggest that much less than 5% triglyceride is at the surface.

The interpretation that some of the triglyceride is at the particle surface is reasonable in view of the interfacial tension analysis. This parameter is related to the "surface excess" parameter used by Mukerjee & Cardinal (1978). The interfacial tension is the excess free energy per square centimeter of interface formed between an organic liquid and water (each saturated with the other). The amount of the molecule present at the surface is related to the energetic cost of building the organic/water interface. Obviously, a variety of factors such as micelle packing (which can expose only parts of the solute favorable for interaction with water or change the surface area per molecule) and local charge can strongly affect this parameter. Inspection of Table II shows that these values are consistent with current ideas on "surface and core" populations; heptanol and heptanoic acid (believed to be present at the interface) show low interfacial tension (7 erg/cm^2) while molecules which are believed to be at least partially in cores (such as heptane and benzene) show higher values (>35 erg/cm^2). The interfacial tensions estimated for short-chain triglycerides are low, implying that some of the triglyceride should be present at the surface. Even if the triglyceride values are as low as those for the simple esters (14–15 erg/cm^2), we would still expect some triglyceride at the surface.

Given this indirect evidence for a population of tributyrin at the surface, how do we rationalize the two indirect but conflicting NMR probes of surface/core triglyceride? The Mn^{2+}-induced broadening of accessible carbons would only affect one-tenth of the tributyrin on the average. If the ester linkages of the triglyceride are less efficient chelation sites for Mn^{2+} (compared to the lecithin phosphate moiety), then detection of an exchanging surface population of triglyceride would be extremely difficult. A nonspecific paramagnetic probe would be needed to detect surface tributyrin.

Lecithin ^{31}P NMR line widths of the microemulsions are consistent with the surface population of tributyrin. With subsaturating trbutyrin, the ^{31}P line width is insensitive to average particle size (this is most pronounced for particles with dioctanoyl-PC). From light-scattering studies of dioctanoyl-PC and triglycerides, we would expect a mixture of pure lecithin micelles (T_2^{-1} = 13 s^{-1}, $\bar{R}_h$ = 125 Å) and saturated microemulsions (T_2^{-1} = 4.7 s^{-1}, $\bar{R}_h$ = 34 Å). For 0.2 mole fraction of tributyrin (40% tributyrin "saturation"), the predicted weight-averaged T_2^{-1} is 11 s^{-1}; the experimental value is 7 s^{-1} and $\bar{R}_h$ = 153 Å. If a small amount of triglyceride is incorporated in the surface of rod-shaped micelles, $\bar{R}_h$ might be unaltered or increased. T_2^{-1} might decrease if lecithin/lecithin intermolecular interactions (electrostatic interactions of one lecithin phosphate with a neighboring choline) which contribute to the ^{31}P relaxation are disrupted (Yeagle et al., 1977). Since tributyrin particles are less sensitive to size than trihexanoin particles, we would propose a larger amount of tributyrin at the surface.

Lipase Specific Activity toward Different Particles. The expectation that triglycerides will exist at the micelle interface may explain the enzymatic activity of water-soluble lipoprotein lipases. Triglycerides are believed to be solubilized predominantly in lipoproteins in a hydrophobic core. This would make them inaccessible to water-soluble lipases and would require penetration of the enzyme through the phospholipid monolayer to reach its substrate. If some of the triglyceride is solubilized in the lecithin surface layer, the enzyme acquires access to the substrate. The actual particle specific activity, which depends on the acyl chain length of both components, could then reflect variations in the amount of surface triglyceride. Kinetic and physical studies with modified lecithin/triglyceride particles are in progress to examine this hypothesis.

Acknowledgments

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Registry No. TH, 621-70-5; TB, 60-01-5; dihexanoyl-PC, 34506-67-7; diheptanoyl-PC, 39036-04-9; dioctanoyl-PC, 19191-91-4; bis(5-methylhexanoyl)-PC, 84237-70-7; bis(4,4-dimethylpentanoyl)-PC, 84237-71-8.

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Studies of Calmodulin Structure: Laser Raman Spectroscopy of Biomolecules†

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ABSTRACT: The structure of bovine brain calmodulin was probed by using laser Raman spectroscopy to elucidate cation-induced conformational changes in the protein. Local changes, most likely reflecting metal binding but not rearrangement of the peptide backbone, were observed in the presence of calcium or magnesium. A conformational change involving the peptide backbone and secondary structure content of calmodulin was observed only in the presence of calcium. The calcium-induced conformational change in the peptide backbone involves increased α helix and β sheet. This was the only major calcium-specific change observed in the Raman spectrum, which suggests that the flexibility of the backbone conformation may play a critical role in the physiological activity of calmodulin.

Calmodulin is a small, acidic, intracellular calcium-binding protein that influences the activity of a number of enzymic and structural systems in the cell in a calcium-dependent manner [for reviews, see Klee et al. (1980), Cheung (1980), Brostrom & Wolff (1981), and Means et al. (1982)]. The protein, ubiquitous in eukaryotes, is one of the principal targets for intracellular calcium released following external stimuli. Calmodulin becomes activated when it binds calcium, undergoes conformational change, and complexes with its target protein.

Several metal ions, including Mg^{2+} and Mn^{2+}, have been shown to compete with calcium for binding sites on calmodulin but typically cannot induce biological activity in the protein (Wolff et al., 1977; Haiech et al., 1981). Conformational changes in calmodulin on binding various cations have been investigated in studies involving NMR[1] (Forsén et al., 1980; Seamon, 1980; Seamon & Moore, 1980), fluorescence (Kilhoffer et al., 1980, 1981; LaPorte et al., 1980, 1981; Tanaka & Hidaka, 1980; Kohse & Heilmeyer, 1981), CD (Dedman et al., 1977; Klee, 1977; Wolff et al., 1977), and ORD (Liu & Cheung, 1976). These studies generally agree that unique conformational states are associated with the binding of different metal ions.

Although some side chain groups and regions of the polypeptide backbone have been reported as undergoing cation-dependent structural changes, there is at present no precise identification of the physiologically relevant rearrangement. Laser Raman spectroscopy is a useful spectroscopic probe for investigation of both main chain and side chain groups in

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3',5'-phosphate; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; pH*, uncorrected pH meter reading in deuterated solvent.

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