Influence of Dilution on the Physical State of Model Bile Systems: NMR and Quasi-Elastic Light-Scattering Investigations†

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ABSTRACT: Multinuclear (^1H and ^{31}P) nuclear magnetic resonance (NMR) spectroscopy and quasi-elastic light scattering have been used to characterize molecular aggregates formed in dilute sodium taurocholate–egg lecithin solutions. When mixed micelles (1.25 g/dL) are diluted with 150 mM aqueous sodium chloride, light-scattering measurements suggest a transformation from mixed micelles to unilamellar vesicle species. Decreased ^1H NMR line widths for bile salt resonances are consistent with predominance of a monomer form. The concurrent appearance of a second phospholipid choline methyl resonance indicates two types of phospholipid environment in slow chemical exchange: this behavior is consistent with small unilamellar vesicles. The appearance of bilayer vesicles in dilute model bile solutions is confirmed by addition of a lanthanide shift reagent (Pr^{3+}), which splits the ^1H or ^{31}P head-group peak into two components with distinct chemical shift sensitivities. These mixed micelle and vesicle aggregates are also distinguished by their susceptibility to the lipolytic enzyme phospholipase A_2 from cobra venom.

Bile salts and structurally related lipids play a variety of roles in bile formation and fat digestion. Together they emulsify and alter the enzymatic susceptibility of triglyceride substrates (Sêmêriva & Desnuelle, 1979), and they also provide a means of efficient transport of digestive products to the absorptive cells of the small intestine (Carey et al., 1983). The physiological activity of bile salts arises from their ability to form micelles, small molecular aggregates which can solubilize otherwise insoluble amphiphiles including phospholipids, acylglycerols, fatty "acid soaps", and cholesterol (Carey & Small, 1970). Lipolytic enzymes are activated strongly by the presence of micellar interfaces, though the influence of substrate structure, added ions (either soluble metal ions or amphiphilic ionic detergents), and aggregate surface characteristics is not well understood.

A variety of physical and spectroscopic studies have contributed to a molecular understanding of lipid solubilization. Particular attention has been focused recently on the structure of aggregates formed in bile salt–lecithin (BS–L)[1] mixtures for which the BS concentration is greater than its critical micelle concentration (cmc) and comparable to the lecithin concentration. The overall dislike shape of such aggregates (Small, 1967) is supported by quasi-elastic light-scattering (QLS) (Mazer et al., 1980) and X-ray (Muller, 1981) studies; the mixed micelles are thought to consist of small portions of L bilayer protected from the aqueous solvent by a perimeter layer of BS molecules. QLS data have also implicated BS incorporation within the L bilayer. Other groups have challenged this mixed disk model (Claffey & Holzbach, 1981; Spink et al., 1982), but difficulties exist with the interpretation of each series of experiments. For example, the study of Spink et al. (1982) was in a region of the phase diagram where vesicles and not micelles are the dominant aggregate. In addition to investigations aimed at the macroscopic arrangement of mixed micellar components, a number of recent nuclear magnetic resonance (NMR) experiments have addressed questions of bilayer asymmetry (Lichtenberg & Zilberman, 1979; Brouillette et al., 1982) and the dynamic behavior of key molecular moieties in BS–L mixtures (Stark et al., 1983; Stark & Roberts, 1984).

The present study extends the high-field ^1H NMR approach used for solutions of varying BS:L ratio (Stark & Roberts, 1984) to mixtures for which aggregate properties change as a function of total lipid concentration. With the excellent sensitivity and resolution obtained at 500 MHz, individual bile salt and lecithin resonances may be monitored independently. Trends in BS molecular mobility and L head-group packing derived from NMR line widths may be evaluated and compared with QLS measurements of overall aggregate size. The two methods confirm the formation of unilamellar vesicles from BS–L mixed micelles when dilution brings the BS below its intermicellar concentration. These dilution vesicles are better substrates for cobra venom phospholipase A_2 than so-

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[1] Abbreviations: BS, bile salt; L, lecithin; TC, taurocholate; DOC, deoxycholate; QLS, quasi-elastic light scattering; NMR, nuclear magnetic resonance; cmc, critical micelle concentration; SUV, small unilamellar vesicle(s).

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nicated vesicles. Taken together, these physical and spectroscopic results provide important new information about organized molecular assemblies of likely significance in fat digestion and transport.

Experimental Procedures

Materials. Taurocholate (Calbiochem-Behring, San Diego, CA), deoxycholate (Sigma Chemical Co., St. Louis, MO), and egg lecithin (Lipid Products, Redhill, Surrey, England) were purified as described previously (Stark & Roberts, 1984), cosolubilized in organic solvents, and then dried under N_2 at reduced pressure. Lipid films were hydrated with H_2O or D_2O (Aldrich Chemical Co., Milwaukee, WI) containing 150 mM NaCl (Stark & Roberts, 1984). The pH of aqueous solutions was 7–7.5. Dilutions were from micellar solutions at a total lipid concentration of 1.25 or 3.75 g/dL, with 30, 40, 50, or 60 mol % L. Each sample was equilibrated for 24 or 48 h as indicated in the text. Aliquots of $Pr(NO_3)_3$ stock solutions (10 or 100 mM) were added to assess vesicle formation (Bergelson, 1978).

Unilamellar egg lecithin vesicles were prepared by sonication (5 W, 5 min with a Branson probe sonifier) of 50 mM aqueous dispersions of egg lecithin. Sample temperature was kept below 10 °C with an ice–water bath. Titanium fragments and multilamellar aggregates were removed by centrifugation at 5000*g* for 5 min at 25 °C. Concentrated aqueous solutions of bile salts and $Pr(NO_3)_3$ were added sequentially to the vesicle preparations to give 1.2 mM BS and 0.63 mM Pr^{3+}. Phospholipase A_2 from cobra venom was purified as described previously (Roberts et al., 1977).

Methods. ^1H NMR measurements at 500 MHz were conducted at 20 °C (unless otherwise noted) with a home-built spectrometer at the Francis Bitter National Magnet Laboratory (M.I.T., Cambridge, MA). Typical spectral parameters include the following: 90° pulse width, 15 μs; sweep width, 3400 Hz, recycle time, 1 s; number of transients, 100–1000; sample size, 300 μL. Line widths and relative peak areas were derived from spectra with 0.5–1.0-Hz line broadening. Detection and measurement of multiple *N*-methylcholine resonances were facilitated with convolution difference spectra (Campbell et al., 1973).

^1H NMR data at 90 MHz and ^{31}P NMR data at 32 MHz were obtained at 20 °C with a JEOL FX-90Q spectrometer. Typical parameters include the following: 90° pulse widths, 80 (^1H) and 30 μs (^{31}P); sweep widths, 1000 (^1H) and 5000 Hz (^{31}P); recycle time, 1.5 s; number of transients, 200–20000; sample size, 1.5 mL. Chemical shifts were measured with respect to an internal D_2O lock signal with a precision of 2 Hz.

QLS measurements were made at 20 °C with an experimental apparatus described previously (Missel et al., 1983). The intensity autocorrelation function of scattered light was fitted with two cumulants (Koppel, 1972) to a high degree of precision, yielding a mean diffusion coefficient ($\bar{D}$) for each micellar solution. Mean hydrodynamic radii ($\bar{R}_h$) were obtained from the relationship $\bar{R}_h = kT/6\pi\eta\bar{D}$ where k is the Boltzmann constant, T is the temperature (in degrees kelvin) and η is the viscosity of the solvent. It is assumed in this calculation that intermicellar interactions may be neglected [see Mazer et al. (1976)]. Each $\bar{R}_h$ reflects multiple measurements on two or more samples prepared independently. Errors estimated with this procedure are 3%.

Phospholipase A_2 activity was assayed at 25 °C in duplicate by the pH-stat method (Dennis, 1973) with an end point of pH 8. Lipid mixtures cosolubilized and hydrated as described above were equilibrated for 24 or 48 h at room temperature.

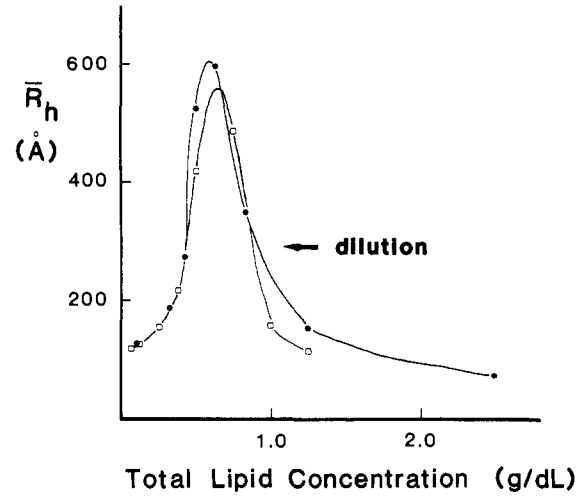

FIGURE 1: Mean hydrodynamic radii ($\bar{R}_h$) of 50:50 mol % TC–L solutions as a function of total lipid concentration. QLS results are shown in D_2O [(□) this work] and H_2O (*). The arrow indicates the direction of dilution. Mixtures were equilibrated for 48 h prior to QLS analysis.

Table I: Effect of $Pr(NO_3)_3$ on Aggregate Size in Dilute TC–L Mixtures[a]

sample	composition (g/dL)	$[Pr^{3+}]$ (mM)	[L] (mM)	$\bar{R}_h$ (Å)
50:50	0.063		0.48	125[b]
50:50	0.063	0.07	0.48	120[b]
40:60	0.31		2.7	184
40:60	0.31	0.07	2.7	178
40:60	0.31	0.20	2.7	190

[a] Prepared as described under Experimental Procedures with 48-h equilibration. [b] A determination on a duplicate sample gave 118 Å (Figure 1).

Immediately before the addition of phospholipase A_2 (typically 0.5–10 μg), $CaCl_2$ was added to the assay mix to a final concentration of 5 mM. After perchloric acid digestion, phospholipid concentrations were checked by phosphate assay (Turner & Rouser, 1970).

Results

QLS Studies. Mean hydrodynamic radii ($\bar{R}_h$) for 50:50 TC–L solutions are plotted as a function of total lipid concentration in Figure 1. Starting with mixed micelle solutions (1.25 g/dL in D_2O, $\bar{R}_h$ = 113 Å; 2.5 g/dL in H_2O, $\bar{R}_h$ = 70 Å), dilution with 150 mM NaCl initially causes a 5-fold growth in size and then a decrease in radius (to $\bar{R}_h$ = 118 Å at 0.063 g/dL). The large ($R_h \sim$ 500–600 Å) particles (micelles and/or vesicles) also have a broad size distribution, whereas the polydispersity index (V) is less than 30% in the most dilute solutions. As in a related study of glycocholate–lecithin mixtures (Schurtenberger et al., 1984), these results suggest the formation of unilamellar vesicles. The similarity of dilution curves in D_2O and H_2O solvents also ensures the validity of comparisons between NMR and QLS data. Finally, Table I shows that the aggregate size remains constant if Pr^{3+} is added to dilute TC–L solutions (see NMR results below).

^1H NMR Studies. A typical 500-MHz ^1H NMR spectrum for TC–L (50:50 mol %) mixed micelles is shown in Figure 2, along with structures of TC and 1-palmitoyl-2-oleoylphosphatidylcholine (the major component in egg lecithin). Discrete BS and L peaks are clearly seen at this field strength (Stark & Roberts, 1984). Shown in Figure 3 are the choline *N*-methyl and TC $CH_2(25)$ and $CH_2(26)$ regions for these 50:50 mol % TC–L mixtures recorded as a function of total

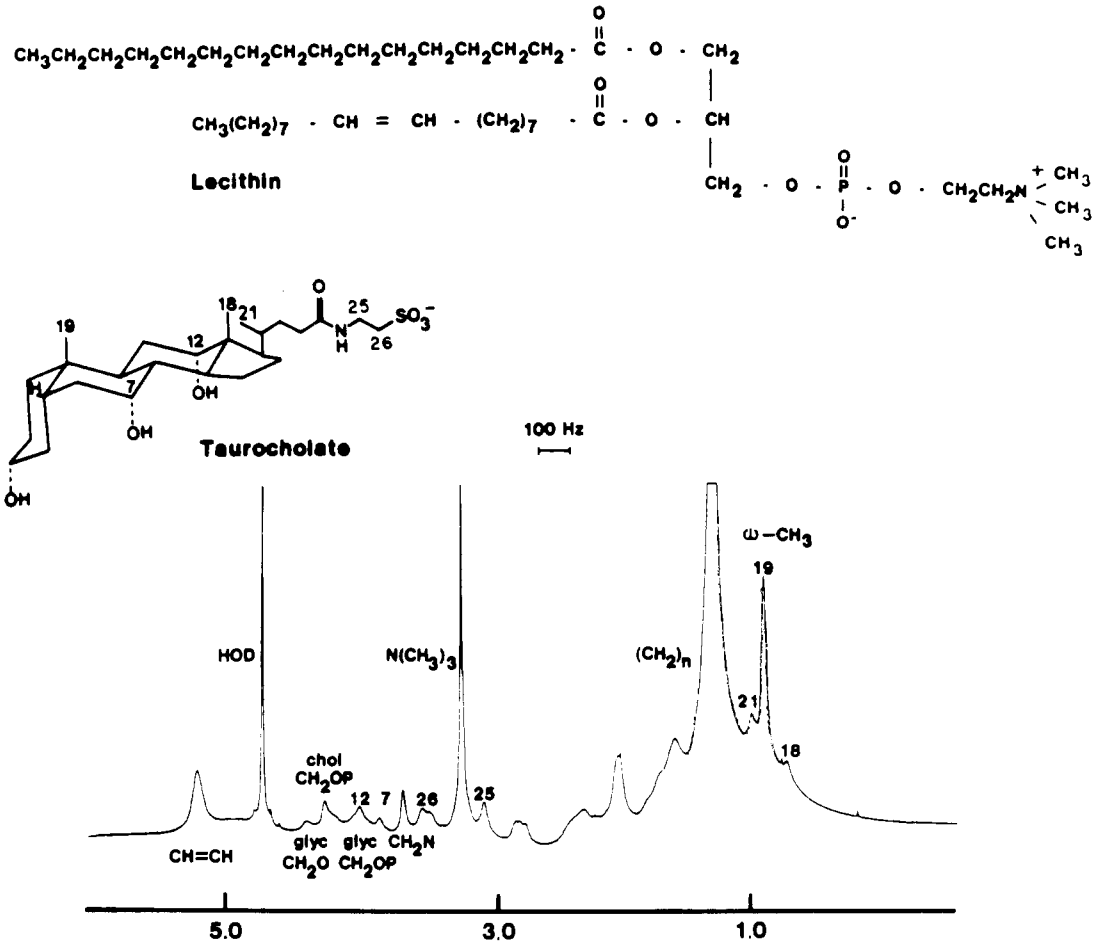

FIGURE 2: 500-MHz ^{1}H NMR spectrum (100 transients, 1-s recycle time) of a taurocholate–egg lecithin (TC–L) mixture. Structures of TC and 1-palmitoyl-2-oleoylphosphatidylcholine (the major component of egg lecithin) are shown for ease in peak identification. The sample composition is 50:50 mol % TC–L, with a total lipid concentration of 1.25 g/dL. Resonance assignments are made by reference to published spectra (Barnes & Geckle, 1982; Hauser et al., 1976). The following resonances overlap but are resolvable from other protons: lecithin CH=CH and CHO; CH(12) of TC and the glycerol CH_2OP; steroid skeleton CH, CH_2, and fatty acyl $(CH_2)_n$; CH_3(19) and the lecithin terminal CH_3.

Table II: ^{1}H NMR Line Widths (Hz)[a] for 40:60 mol % Taurocholate–Lecithin Solutions[b]

	total lipid concn (g/dL)					
peak assignment	1.25	0.31	0.08	0.02	0.005	0.042, pure TC[c]
CH=CH	49	38	42	–[d]	–	
glyc CH_2O (d)[e]	32	26	–	–	–	
chol CH_2OP	34	21	23	–	–	
CH_2N	17	14	17	–	–	
$N(CH_3)_3$ downfield	6	6	9	16	–	
$N(CH_3)_3$ upfield	14	12	16	17	–	
7	21	13	9	6	7	7
26[f]	26	19	16	16	16	13
25[f]	32	17	15	13	13	12
21	24	20	18	11	9	9
18	21	15	10	5	3	1

[a] Line widths at half-height; observed values corrected for artificial broadening during data processing and for contributions from magnetic field inhomogeneity. Error limits based on repeated measurements are ~20%. [b] Prepared as described under Experimental Procedures with 48-h equilibration. Structural assignments are noted in Figure 2. [c] Monomeric solution. [d] (–) entries indicate broadening of peaks beyond an observable limit of ~100 Hz. [e] The downfield doublet of an AB spin system. [f] Data for 50:50 mixtures suggest that these signals probably broaden first between 1.25 and 0.31 g/dL and then sharpen as shown above.

lipid concentration (in grams per deciliter). Similar spectra are obtained at 25 °C. As the 50:50 mixed micelle solution

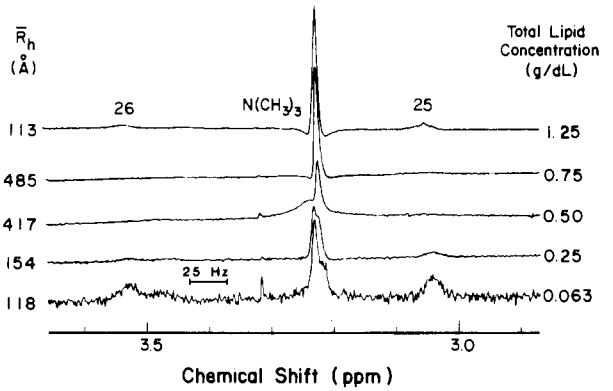

FIGURE 3: 500-MHz ^{1}H NMR spectra of the $N(CH_3)_3$ and TC CH_2(25), CH_2(26) region of 50:50 mol % TC–L mixtures as a function of total lipid concentration and hence mean hydrodynamic radius, after 48-h equilibration. Convolution difference spectra are displayed to enhance resolution of the choline methyl peaks.

is diluted (with 150 mM NaCl), the taurocholate side-chain CH_2(25) and CH_2(26) resonances broaden initially (1.25–0.75 g/dL) and then become narrow (0.25 and 0.063 g/dL). A second resonance also becomes visible in the lecithin choline methyl region upon dilution past the largest $\bar{R}_h$ value. Other bile salt and lecithin resonances also show line-width changes upon dilution; (1) there is a progressive loss of high resolution for all resonances upon dilution from 1.25 to 0.50 g/dL; (2)

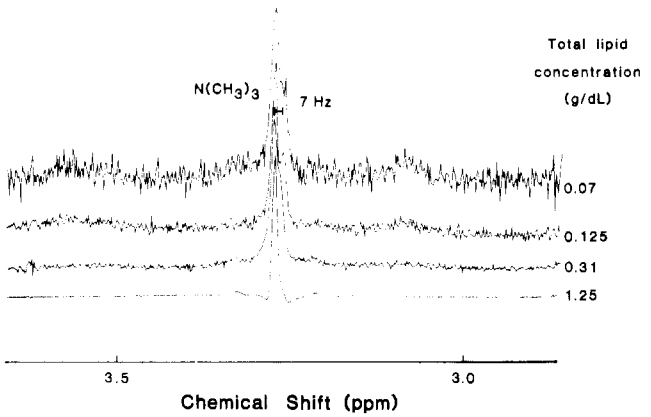

FIGURE 4: 500-MHz ^1H NMR spectra of 40:60 mol % TC–L mixtures as a function of total lipid concentration after 24-h equilibration.

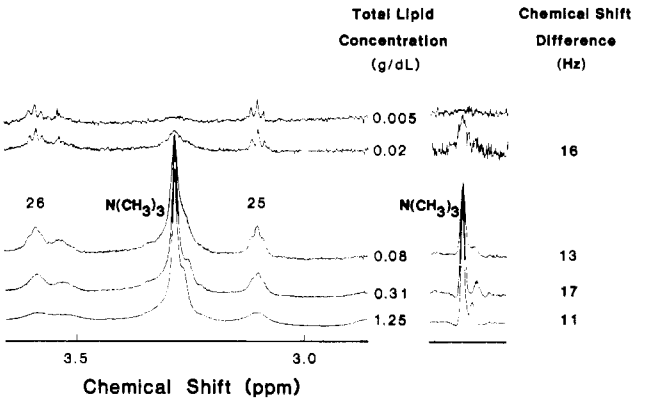

FIGURE 5: 500-MHz ^1H NMR spectra of 40:60 mol % TC–L mixtures with varying total lipid concentration after 48-h equilibration. (Left spectra) Data processed with exponential broadening of free induction decays (artificial broadening = 0.5 Hz); (right spectra) data processed by using the convolution difference technique for resolution enhancement. Error limits of 1–2 Hz are estimated for chemical shift differences between lecithin $N(CH_3)_3$ resonances.

the TC resonances, but not L resonances, become much sharper in more dilute solutions (see Table II). Similar phenomena (i.e., line-width changes and *N*-methyl splitting) are observed for serial dilutions with 70:30, 60:40, 50:50, and 40:60 mol % TC–L mixtures. Lecithin-rich mixed micelles (40:60 TC–L) require much less dilution to give spectra where the second choline peak is detectable than do TC-rich mixed micelles (70:30 TC–L).

With the benefit of the QLS results, the NMR spectral changes exhibited by both TC and L components in the dilution series may be given a more precise structural interpretation. The initial broadening of TC side-chain resonances suggests preferential motional restriction of bile salt moieties in large mixed micelles, a phenomenon observed recently for TC–L mixtures of varying relative composition (Stark & Roberts, 1984). TC resonances such as $CH_2(25)$ and $CH_2(26)$ are expected to become narrow with further dilution of the mixture as bile salts are removed from the disklike aggregate to maintain the intermicellar or in this case intervesicle concentration. In fact, the line widths for very dilute TC–L mixtures and TC monomers are comparable (see Table II).

Whereas a single resonance is observed for $N(CH_3)_3$ protons of the phospholipid head group in mixed micelle solutions, the formation of vesicle structures upon dilution below 0.75 g/dL is indicated by two peaks chemically shifted by up to 17 Hz (0.03 ppm). The broader downfield component observed at 0.50 g/dL (Figure 3) may be attributed to larger vesicles in a polydisperse distribution or perhaps to a sliver of a hexagonal liquid-crystalline phase (Small et al., 1984). For the 120- and 150-Å aggregates found in more dilute solutions, however, separate signals in the NMR spectrum are likely to reflect differences in head-group packing for the inner and outer vesicle monolayers (Eigenberg & Chan, 1980). The suggestion that these spectral changes derive from reorganization of the aggregate structure as well as changes in size is supported by two observations: (a) NMR spectra obtained for aggregates with comparable mean hydrodynamic radii have dramatically different line widths (for example, the 1.25 and 0.063 g/dL mixtures in Figure 3 have $\bar{R}_h$ = 120 Å) but dramatically different ^1H NMR spectra in terms of line widths and relative intensity of TC and L resonances; (b) the $N(CH_3)_3$ resonance remains a single peak and requires greater dilution when the proportion of bile salt is larger (enough presumably to maintain a mixed micelle structure).

The time allowed for sample equilibration prior to NMR observation has very noticeable effects on the ^1H NMR spectra of dilute TC–L mixtures. Comparison of Figures 4 (24-h equilibration) and 5 (48-h equilibration) shows that lengthening the equilibration time accentuates $N(CH_3)_3$ chemical shift differences for 40:60 solutions [17 Hz (48 h) vs. 4 Hz (24 h) at 0.31 g/dL; 11 Hz (48 h) vs. unobservable (24 h) at 1.25 g/dL]. For side-chain resonances [$CH_2(25)$ and $CH_2(26)$] of TC, dilution-induced narrowing is quite modest after 24 h but much dramatic after 48 h. These results are not unexpected in light of long equilibration times previously reported for other "dilution vesicle" systems (Schurtenberger et al., 1984). A particularly interesting phenomenon is the broadening of L resonances in very dilute solutions. Since only homogeneous-sized vesicles are present under these conditions (as determined by QLS), the broadening may reflect an altered exchange rate between inner and outer L molecules. As TC molecules are removed from the bilayer, defects are formed which accentuate the transbilayer flip-flop rate, changing it from the slow to intermediate exchange regime and hence broadening the $N(CH_3)_3$ signal.

Effect of Lanthanides on NMR Spectra. When lanthanide ion such as Pr^{3+} are added to small unilamellar lecithin vesicles, the particle size is invariant, but the $N(CH_3)_3$ signal corresponding to the outer lecithin monolayer is preferentially shifted (downfield) because it is in contact with the exterior aqueous solution containing the paramagnetic reagent (Bergelson, 1978). Because a pure lecithin bilayer is impermeable to ions and transbilayer diffusion is very slow, the inner lecithin monolayer is in contact with water containing no lanthanides and hence remains unshifted. As a result, two discrete $N(CH_3)_3$ resonances are distinguishable. The inner phospholipid resonance of pure vesicles is unshifted even after overnight equilibration (Figure 6 inset).

By contrast, in solutions at pH 7.5 containing ~10 mol % DOC (where about 20% of the unconjugated bile salt is undissociated), the Pr^{3+}-induced chemical shift difference between inner and outer phospholipid groups decreases with time. After 14 h, the peaks broaden and coalesce to the chemical shift of phospholipid exposed to Pr^{3+}. This indicates that the vesicles have become permeable to Pr^{3+} and/or exhibit an increase in the exchange rate of inner and outer lecithin populations (Figure 6, left spectra). In fact, DOC has been proposed as an ionophore in other vesicle studies (Castellino & Violand, 1979; Hunt & Jawaharlal, 1980).

A similar experiment with TC, which is completely ionized at that pH, added to preformed egg lecithin vesicles (Figure 6, right spectra) shows no loss of resolution for inner and outer phospholipids, suggesting that separate inner monolayer and outer monolayer resonances should be observed in the spectra

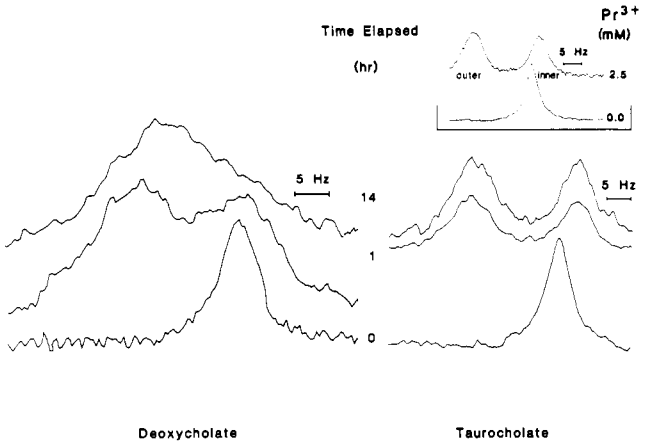

FIGURE 6: 90-MHz ^1H NMR spectra (choline methyl region) of 10 mM sonicated egg lecithin vesicles. Each spectrum required ~4 min of acquisition time; 4K data points were used to define a spectral region of 1000 Hz. Head-group protons in the inner and outer vesicle monolayers are identified by analogy with previous studies (Hutton et al., 1977). Inset: 40 mM vesicles before and after addition of the paramagnetic shift reagent. (Left spectra) Time-dependent spectra for vesicles containing 0.63 mM $Pr(NO_3)_3$ and 1.2 mM DOC; (right spectra) time-dependent spectra for vesicles containing 0.63 mM $Pr(NO_3)_3$ and 1.2 mM TC.

whenever small unilamellar vesicles are present in TC–L solutions.

Similar lanthanide shift experiments (^1H or ^{31}P) with TC–L dilution mixtures are consistent with the presence of vesicles. Though the particle size remains invariant under these circumstances (Table I), Pr^{3+}-induced shifts can be measured for each choline methyl resonance in dilution mixtures and compared with Pr^{3+}-induced shifts in suitable vesicle and mixed micelle reference systems. When a 0.125 g/dL 40:60 TC–L solution has 0.06 mM Pr^{3+} added, the ^1H chemical shift of the downfield $N(CH_3)_3$ resonance changes by ~7 Hz while the upfield resonance position is invariant. A more quantitative analysis of the 500-MHz ^1H spectra is difficult because of concurrent broadening effects.

^{31}P NMR observations of lecithin phosphate resonances in vesicle and mixed micelle solutions when Pr^{3+} is added reveal the following (Table III): (a) the single phosphorus peak observed in 40:60 TC–L solutions which contain some mixed micelles and some vesicles splits into two components upon the addition of 0.07 mM Pr^{3+}, where each component has characteristic paramagnetic-induced chemical shifts suggestive of inner monolayer and outer monolayer environments for lecithin; (b) the downfield component identified with phospholipids exposed to bulk Pr^{3+} shows a paramagnetic-induced shift which is comparable to 50:50 TC–L mixed micelles, as expected if the outer monolayer is exposed to the bulk aqueous solution; (c) Pr^{3+}-induced chemical shifts vary somewhat with vesicle size (which is determined by the starting total lipid concentration) and are larger than values found for sonicated vesicles of pure lecithin; and (d) chemical shifts attributed to inner monolayer lecithins are unaffected by the addition of Pr^{3+} only for sonicated vesicles, suggesting that the presence of TC in vesicles formed by dilution promotes some exchange of monolayer populations or partial permeability to Pr^{3+}.

Susceptibility of TC–L Aggregates to Phospholipase A_2. The specific activity of phospholipase A_2 is low toward both lecithin multilayers and unilamellar vesicles composed of phospholipid in the liquid-crystalline state (Verger & de Haas, 1976; Kensil & Dennis, 1979); the addition of a detergent such as TC results in interfacial activation of the enzyme, as portions of the L bilayer are solubilized in mixed micelles (Olive & Dervichian, 1968). With a further excess of bile salt present, the enzymatic activity drops again. Proposed explanations include dilution of the surface concentration of available lecithin by added detergent (Deems et al., 1975), modification of the micellar surface, or competitive inhibition by detergent species (Burns et al., 1982). For any TC–L solution of interest, then, increasing the fraction of bile salt may *either* increase or decrease the lecithin susceptibility of phospholipase A_2: the result will depend on whether the initial mixture contains vesicular or mixed micellar aggregates and how effectively the bile salt may bind to the enzyme.

Table III: Effect of $Pr(NO_3)_3$ on the Lecithin ^{31}P NMR Signal in TC–L Mixtures

sample composition[a]	change in chemical shift (Hz) induced with 0.07 mM $Pr(NO_3)_3$[b] downfield component	upfield component
50:50 1.25 g/dL micelles	45[c]	
40:60 1.25 g/dL large vesicles and micelles	53	18
40:60 0.31 g/dL small vesicles	47	28
0:100 0.54 g/dL sonicated vesicles	30	<1

[a] Prepared as described under Experimental Procedures. Identifications of the aggregates are made by methods discussed in the text. [b] Positive numbers indicate downfield shifts, referenced to an internal ^2H field-frequency lock and normalized for [L] = 10 mM. For each sample, spectra were monitored for approximately four $Pr(NO_3)_3$ concentrations in the range 0.05–0.40 mM. These spectra remain unchanged after overnight equilibration. [c] Only one resonance is observed.

For a 50:50 mol % TC–L mixture which is 9.5 mM in each component (total lipid concentration 1.25 g/dL), NMR and QLS evidence indicates the presence of mixed micellar aggregates at 20 °C. NMR spectra at 25 °C imply the same structure is present. Accordingly, we expect and find dramatically higher enzyme specific activity toward L in these aggregates (560 μmol min^{-1} mg^{-1}) as compared with enzyme activity toward the same concentration of L dispersed in sonicated unilamellar vesicles (7 μmol min^{-1} mg^{-1}). As shown in Figure 7 (■), the phospholipase specific activity decreases as TC is added to the mixed micelle system (dashed line), although this bile salt inhibited phospholipase activity is still considerably greater than that observed for pure lecithin sonicated vesicles. This result may be rationalized in terms of substrate surface dilution (Deems et al., 1975) and/or competitive bile salt inhibition (Burns et al., 1982).

When the same 50:50 mol % TC–L mixture is diluted to 0.25 g/dL (1.9 mM in each component), the spectroscopic studies (at 25 °C, the assay temperature) suggest formation of unilamellar vesicles. Phospholipase A_2 activity toward these dilution vesicles (18–28 μmol min^{-1} mg^{-1}) is significantly greater than that toward 1.9 mM egg lecithin sonicated vesicles (typically 5 μmol min^{-1} mg^{-1}) [Figure 7, (○) and (△)] but well below the activity of the enzyme toward 1–2 mM short-chain lecithins mixed with comparable amounts of taurocholate (C. D. DeBose and M. F. Roberts, unpublished results). The observed specific activity also depends on sample equilibration time after dilution. Enzymatic activity is higher toward samples incubated for 48 h. As TC is added to the dilution vesicle samples (increasing the TC concentration from 1.9 to 12 mM), enzyme activity increases. This result is consistent with a transformation of vesicles to mixed micelles. It should be noted that roughly constant (rather than decreasing) specific activity is observed for bile salt rich solutions in this series [similar to the behavior reported by Olive & Dervichian (1968)].

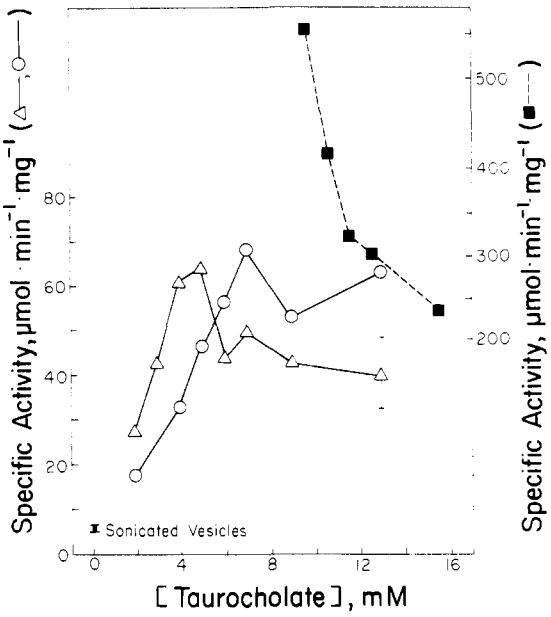

FIGURE 7: Phospholipase A_2 activity (25 °C) toward egg lecithin in the presence of taurocholate. Each substrate mixture was prepared as described under Experimental Procedures and equilibrated the indicated time before use: (■) [L] = 9.5 mM, 48-h incubation; (○) [L] = 1.9 mM, 24-h equilibration; (△) [L] = 1.9 mM, 48-h equilibration. Errors are estimated from the results of duplicate assays. The activity shown for sonicated vesicles (bottom left corner) reflects the range of values determined for 2 and 10 mM samples.

Discussion

The structural characterization of bile salt–lecithin aggregates is a key prerequisite for a molecular understanding of bile function, as demonstrated in a number of recent spectroscopic studies (Mazer et al., 1980; Muller, 1981; Stark & Roberts, 1984). Such knowledge can be particularly illuminating with regard to the dilute bile salt concentrations present during fat digestion and transport, since both enzymatic accessibility and transport efficiency are sensitive functions of the substrate aggregation state (Sêmêriva & Desnuelle, 1979). For BS–L mixtures with varying relative composition or total lipid concentration, a combination of QLS and NMR methods permits us to correlate changes in *overall* aggregate size with *site-specific* effects on mobility, packing, and intermolecular interactions. In the present study, this approach also provides evidence that a transformation from micelles to small unilamellar vesicles occurs when model bile mixtures are diluted to levels which match the concentrations found physiologically in the upper small intestine.

A careful examination of the chemistry and NMR spectra of these mixtures is essential in identifying the split choline methyl peaks with a dilution vesicle structure.[2] The observation of separate NMR signals implicates two phospholipid populations in slow chemical exchange, and this condition is fulfilled for sonicated vesicle preparations in the absence of bile salts (Sheetz & Chan, 1972). In a recent ^1H NMR study of egg lecithin unilamellar vesicles, spectral properties of the $N(CH_3)_3$ resonances were studied as a function of added deoxycholate (DOC) (Brouillette et al., 1982). Although chemical shift inequivalence was observed in pure phospholipid vesicles, the peaks coalesced and then narrowed as enough bile salt was added to approach the composition of our 40:60 and 50:50 mixtures. The failure to observe separate choline methyl peaks in DOC–L mixtures may result from the omission of an equilibration period or the fact that DOC promotes the formation of large vesicles or mixed micelles. An alternate explanation involves the possibility of enhanced transbilayer flip-flop rates (Kramer et al., 1981): even if vesicle structures remain intact, BS-accelerated exchange of lecithin molecules in inner and outer environments may result in a single NMR signal.

Though these hypotheses are difficult to test directly, the lanthanide additions to sonicated vesicles containing bile salts address the related phenomenon of vesicle permeability. Since low levels of lanthanide do not induce major alterations in structure, the NMR spectral changes should reflect properties of the model bile aggregates. For mixtures containing TC as the bile salt additive, the persistence of resolved peaks in the presence of Pr^{3+} leads us to expect distinct ^1H NMR signals from inner and outer monolayers of a small vesicle structure. Moreover, it is possible to identify inner and outer phospholipid moieties by their differing chemical shift sensitivity to added Pr^{3+}. The observation that inner phospholipid chemical shifts are not strictly invariant in dilute TC–L solutions suggests that bile salts promote some Pr^{3+} transport to the interior of the vesicle, perhaps by forming back to back dimer channels within the bilayer [as suggested by Hunt & Jawaharlal (1980)].

Provided that the two phospholipid populations do exchange slowly on the NMR time scale, what are their relative ratios, and how different are their chemical environments? For egg lecithin vesicles with a radius of 120 Å, the ratio of outer to inner peak areas should be ~1.6 (Brouillette et al., 1982). Rough estimates obtained from the spectra in Figure 5 are noticeably larger than this, but they may be biased by residual bile salt incorporation within the inner monolayer and/or an induced ^1H chemical shift on neighboring lecithin molecules. (Mixed micelle contributions to the downfield peak are unlikely, since QLS measurements show aggregates of a single size only.) $N(CH_3)_3$ shift differences in excess of 10 Hz are expected only for egg lecithin vesicles with radii smaller than 110 Å (Brouillette et al., 1982), although larger values are observed for 125–150-Å dipalmitoylphosphatidylcholine and dihexadecylphosphatidylcholine vesicles below their gel to liquid-crystalline phase transition (Sheetz & Chan, 1972; DeBose & Roberts, 1983). Chemical shift differences between inner and outer lecithin $N(CH_3)_3$ groups of 11–17 Hz are observed in dilute TC–L mixtures (Figure 5), suggesting that residual bile salt accentuates differences in chemical environment between the inner and outer phospholipid monolayers.

Identification of micelle and vesicle species in these biliary mixtures is useful in understanding their susceptibility to lipolytic enzymes. The lecithin in TC–L dilution vesicles is noticeably more accessible to phospholipase A_2 than in pure lecithin sonicated vesicles. (The discrepancy in kinetic behavior increases with equilibration time after dilution. Such a result is not surprising in light of NMR evidence for a time-dependent physical change and subsequent leakiness of the dilution vesicles.) This difference in activity could reflect (a) size differences for the two types of vesicles (unlikely given NMR and QLS results), (b) the influence of vesicle-incorporated TC on neighboring L substrate molecules or on resorption of product, or (c) possibly TC binding to phospholipase A_2 (although the binding constant, K_D, for DOC binding to the enzyme is about 10 mM) (C. D. DeBose and M. F. Roberts, unpublished results).

Enzyme activation as bile salt is added to dilution vesicles is diagnostic for the formation of micelles in these biliary

[2] For instance, while a hexagonal liquid-crystalline phase has been detected in this system (Small et al., 1984), such a structure is not uniquely identifiable by ^1H NMR methods.

mixtures, so that kinetic observations also serve to confirm structural inferences drawn from other physical techniques. Enzymatic activity may be inhibited by further TC additions, but only at high total lipid concentration. Otherwise, a plateau in activity is observed. At higher TC concentrations, pure TC micelles as well as TC–L mixed particles are probably present (Mazer et al., 1980). The kinetic results suggest that TC in pure micelles may be a less effective inhibitor of the enzyme than TC in mixed disk particles. Additional experiments are in progress to explore this possibility.

These studies demonstrate the complementarity of NMR, QLS, and enzyme assays in studies of biliary mixtures. In addition to providing dynamic structural information for micellar and vesicular aggregates relevant to fat digestion, this strategy appears very promising for mechanistic investigations of lipolytic enzymes such as phospholipase A_2.

Registry No. TC, 81-24-3; DOC, 83-44-3; phospholipase A_2, 9001-84-7.

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