Biochimica et Biophysica Acta, 770 (1984) 115–121
Elsevier

BBA 71986

500 MHz ^{1}H-NMR STUDIES OF BILE SALT-PHOSPHATIDYLCHOLINE MIXED MICELLES AND VESICLES

EVIDENCE FOR DIFFERENTIAL MOTIONAL RESTRAINT ON BILE SALT AND PHOSPHATIDYLCHOLINE RESONANCES

RUTH E. STARK [a,*] and MARY F. ROBERTS [b]

[a] *Department of Chemistry, Amherst College, Amherst, MA 01002 and* [b] *Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139 (U.S.A.)*

(Received August 31st, 1983)

Key words: Bile salt-phospholipid interaction; Taurocholate; Phosphatidylcholine; ^{1}H-NMR; (Mixed micelle)

^{1}H nuclear magnetic resonance (NMR) spectra at 500 MHz have been obtained for taurocholate/egg phosphatidylcholine mixtures of varying composition. The excellent chemical shift dispersion permits identification of most resonances for each component. This high-resolution character of the NMR spectra is retained until the phosphatidylcholine (PC) mole fraction exceeds 60–70% (the exact limit depends on ionic strength). ^{1}H linewidths have been monitored as a function of solute composition in order to evaluate trends in local molecular mobility of each component as the distribution of aggregate particles is varied, and to examine the effects of added NaCl in altering micellar size and shape. Although prior light scattering studies (Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) Biochemistry 19, 601–615) and our own work indicate a 6-fold increase in particle hydrodynamic radius from pure taurocholate micelles to 1:1 taurocholate/PC mixtures containing 150 mM NaCl, both lipid components retain substantial motional freedom and exhibit narrow NMR signals in this compositional region. As the solubilization limit for PC is approached (approx. 2:1 PC:taurocholate), differential behavior is observed for the two components: the motion of taurocholate becomes preferentially restricted, while polar portions of the PC remain mobile until large multilayers predominate.

Introduction

Bile salts play a crucial role in the secretion of membrane lipids into bile [1] and in lipid digestion and absorption [2]. Common defects in bile salt functions include cholesterol gallstones and dietary fat malabsorption. The principal physiological activity of bile salts derives from their ability to form micelles, small molecular aggregates which effectively solubilize otherwise insoluble lipids such as fatty 'acid soaps', monoacylglycerols, phospholipids and cholesterol [3]. During the last 15 years, studies employing physical and chemical spectroscopic techniques have contributed much toward understanding these solubilization phenomena on a molecular level [2–4]. A disk-like model for bile salt-PC mixed micelles was proposed by Small [4], in which small sections of phospholipid bilayer are protected from exposure to the aqueous solvent by a shell of bile salt molecules. More recent laser-light scattering [5] and X-ray scattering [6] studies have suggested

* To whom correspondence should be addressed.
Abbreviations: PC, phosphatidylcholine; QLS, quasielastic light scattering.

that a high proportion of bile salts resides within the phospholipid bilayer of these particles. Additional structural and dynamic information on this system is needed, since there is controversy [5–9] as to the physical state of bile salt-PC micellar systems at low PC mole fraction, i.e., the physiological range of lipid ratios in bile. More information is also needed on the physical state of bile salt-PC systems at high dilution [5] such as may occur physiologically at the hepatic sites of bile formation and in the upper small intestine during fat digestion.

The potential of NMR for providing molecular information on these systems was first demonstrated in 1969 [10], when ^1H linewidth measurements were used to confirm back-to-back hydrophobic association in bile-salt micelles and to investigate bile salt-PC interactions. More recent NMR studies of the mixed micelles have addressed the relationship between ^1H spectral features and phospholipid bilayer curvature [11], the influence of bile salts on PC headgroup motions [12], and the use of ^2H spin relaxation to focus on acyl-chain packing and dynamics [13].

The high-field (500 MHz) ^1H-NMR approach employed in the present study benefits from both enhanced sensitivity and spectral resolution. In solutions of varying bile salt-PC composition a wide variety of aggregate assemblies may be surveyed rapidly, and in each case a good fraction of bile salt and PC resonances are monitored (without isotopic enrichment) to provide site-specific structural information. NMR linewidths are used to assess local molecular mobility as a function of lipid composition and evaluated in light of known changes in overall aggregate size [5]. The NMR results are shown to complement structural information derived from other physical studies, and analysis of the linewidth trends imposes geometric constraints on the arrangement of the lipid constituents within the mixed micelles.

Experimental procedures

Materials

The sodium salt of taurocholate (3α,7α,12α-trihydroxy-5β-cholanoyltaurine) was purchased from Calbiochem-Behring (San Diego, CA). After recrystallization by the method of Pope [14] and freeze-drying from water, its purity by TLC [15] and HPLC [16] was greater than 98%. Purified bile salt was stored over anhydrous $CaCl_2$. Egg yolk phosphatidylcholine (PC) was a Grade I product obtained from Lipid Products (Redhill, Surrey, U.K.) as a $CHCl_3/CH_3OH$ (2:1, v/v) solution. Its purity was judged to be greater than 99% by a number of chromatographic methods [15]. PC samples were stored in the dark at −20°C under N_2.

Mixed taurocholate/PC samples for NMR were prepared as follows [11,17]. Solutions of taurocholate in CH_3OH and PC in $CHCl_3/CH_3OH$ (2:1, v/v) were mixed in the desired molar ratios, dried under a stream of N_2 at 40°C, desiccated overnight under reduced pressure, and hydrated with 99.96 atom% ^2H_2O (Aldrich Chemical, Milwaukee, WI) to a final concentration of 1.25 g/dl. Duplicate samples were prepared in NaCl-free or 150 mM $NaCl/^2H_2O$ media, pH 7. Solutions were allowed to equilibrate at least 24 h and then vortexed prior to the NMR measurements. Samples were layered with nitrogen and refrigerated (4°C) after use; any samples which became yellow were discarded.

NMR spectroscopy

^1H-NMR measurements were made at 20°C with a home-built 500 MHz spectrometer at the Francis Bitter National Magnet Laboratory (M.I.T., Cambridge, MA). Linewidths at half height are corrected for artificial broadening during data processing and for contributions from magnetic field inhomogeneity; error limits based on repeated measurements are approx. 20%. Peak assignments are made by reference to published spectra [18,19].

Quasielastic light scattering (QLS)

Measurements were made at 20°C with an experimental apparatus described previously [20]. The intensity autocorrelation function of scattered light was fit to a high degree of precision with two cumulants [21], yielding a mean diffusion coefficient, $\bar{D}$, for each micellar solution. Mean hydrodynamic radii, $\bar{R}_h$, were obtained from the relation $\bar{R}_h = kT/6\pi\eta\bar{D}$, where k is Boltzmann's constant, T is the absolute temperature (K), η is the viscosity of the solvent, and it is assumed that intermicellar

interactions may be neglected [20]. Errors of 3% are estimated for reported $\overline{R}_h$ values, based on repeated runs on the same sample and for nominally identical solutions prepared independently.

Bulk viscosity and density measurements

Kinematic viscosities were measured at 25°C with Cannon-Manning semi-micro viscometers. Viscometers of differing bore were employed so that flow times were over 200 s and kinetic energy corrections could be avoided. All measurements were made in triplicate for both micellar and multibilayer solutions. Flow times measured by stopwatch were generally within 1 s of each other. Solution densities were measured at the same temperature using a Mettler-Anton peak density meter (Model DMA45) to an accuracy of $\pm 1 \cdot 10^4$ g/ml. Solution viscosities were derived from the product of kinematic viscosity and solution density and were not normalized for the density of the solvent (150 mM NaCl in ^2H_2O).

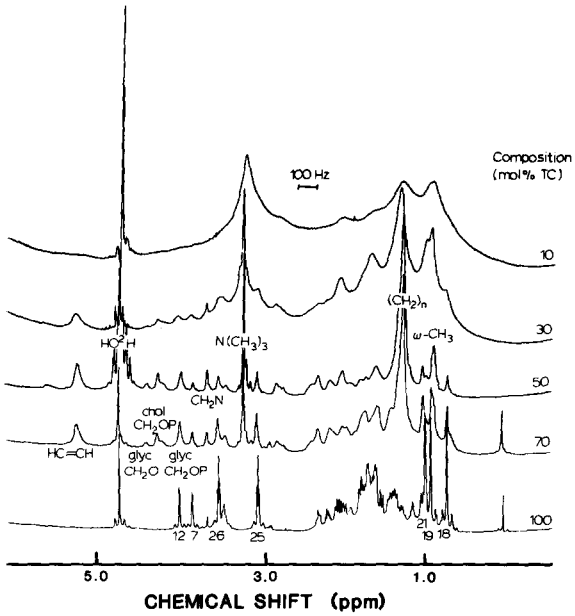

Fig. 1. 500 MHz ^{1}H-NMR spectra of taurocholate/PC mixtures with varying relative composition, at a total lipid concentration of 1.25 g/dl. (Concentrations are 23 mM and 16 mM for solutions of pure taurocholate and PC, respectively.) Resonance assignments are made by reference to published spectra [18,19], using the numbering scheme shown in Fig. 2.

Results

The 500 MHz ^{1}H-NMR spectra of taurocholate/PC mixtures, recorded as a function of solution composition (mol% taurocholate), are shown in Fig. 1. As observed previously [10], the presence of bile salts produces dramatic narrowing of the PC peaks. Conversely, the high-resolution character of the spectrum is diminished in PC-rich mixtures (10–30% taurocholate), where the micellar phase limit is exceeded [15] and the cloudiness of the dispersions suggests the formation of large bilayer aggregates. Similar spectral trends are observed if the mixtures contain 150 mM NaCl, but

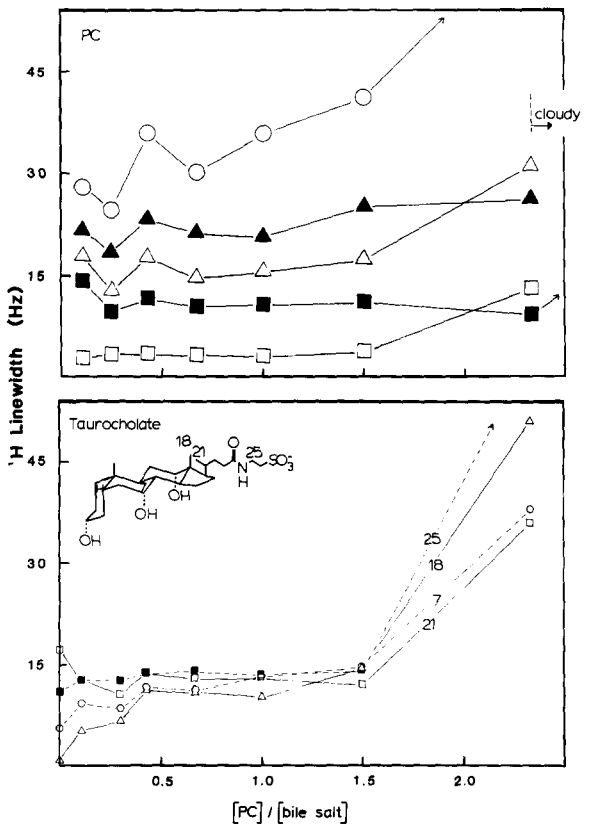

Fig. 2. Proton NMR linewidths as a function of PC : taurocholate molar ratio, in 1.25 g/dl NaCl-free solutions. Several signals (glyc CH_2O, 25,26) exhibit resolved scalar couplings in bile salt-rich mixtures; the linewidth of the entire multiplet has been measured to facilitate comparisons with broader envelopes observed at other compositions. Arrows indicate broadening of peaks beyond an observable limit of approx. 100 Hz. ○, HC=CH; ▲, glyc CH_2O; △, chol CH_2OP; ■, CH_2N; □, $N(CH_3)_3$.

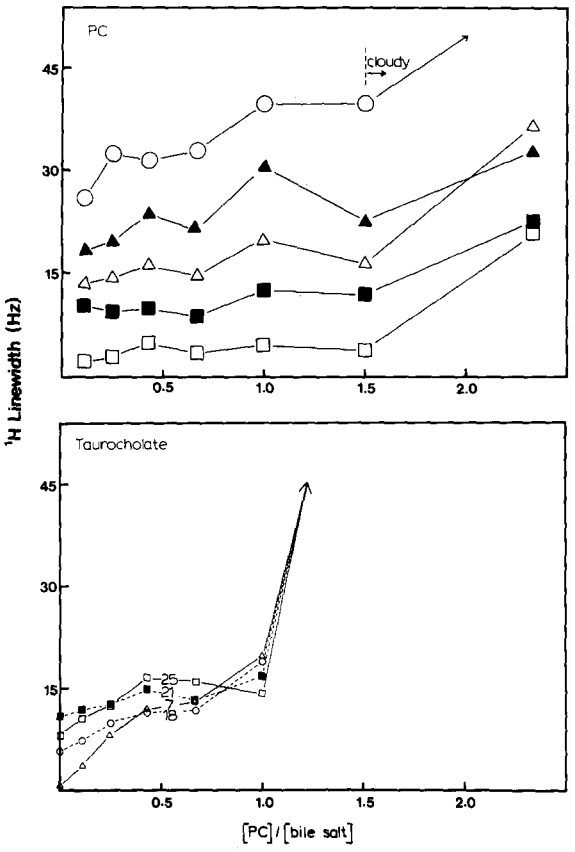

Fig. 3. Proton NMR linewidths as a function of PC : taurocholate molar ratio, in 1.25 g/dl solutions containing 150 mM NaCl. Arrows indicate broadening beyond observable limits. Symbols as in Fig. 2.

in this series overall broadening occurs at 60 mol% PC, consistent with the phase relations [15] at high NaCl concentrations.

Analyses of linewidths for the individual resonances are summarized in Figs. 2 and 3. Here contrasting behavior is apparent for taurocholate and PC, as well as among the PC functional groupings. All lines remain narrow up to PC : bile salt ratios of 1 : 1 to 1.5 : 1, indicating retention of local molecular mobility even as the mixed micelles grow 6-fold in hydrodynamic radius (vide infra). The addition of NaCl reduces the PC : bile salt ratio where line broadening for the TC resonances occurs, and in both sets of mixtures the first increase in linewidths is noted for the bile salt component. Thus, motional restriction occurs preferentially at bile-salt sites as the proportion of PC

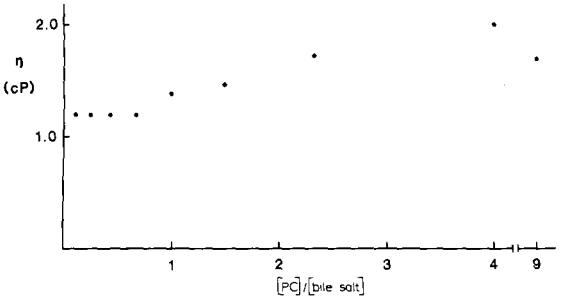

Fig. 4. Bulk viscosities (η) of taurocholate/PC solutions at 1 : 25 g/dl total lipid concentration and 25°C. All mixtures were prepared in ^2H_2O and contained 150 mM NaCl.

reaches 50–60%, and the effect is remarkably similar for hydrophilic (7,CH_2(25), CH_2(26)) and hydrophobic (CH_3(18), CH_3(21)) moieties. For PC, most linewidths remain small and invariant (motional freedom is retained) until the phase limit is exceeded and the formation of large vesicle structures is signalled by the appearance of cloudy solutions; PC resonances then broaden substan-

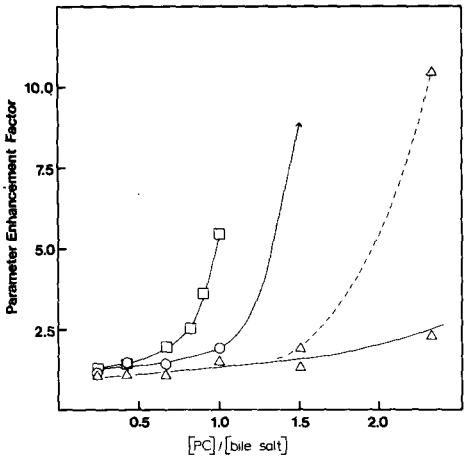

Fig. 5. Enhancement of hydrodynamic radii and NMR linewidths as a function of PC : taurocholate molar ratio, in 1.25 g/dl solutions containing 150 mM NaCl. $\bar{R}_h$ values obtained from QLS measurements in ^2H_2O ($\bar{R}_h/\bar{R}_h^0$, □) follow closely the trends observed for taurocholate/PC mixtures in H_2O [5]. PC NMR data ($\Delta\nu/\Delta\nu^0$, △) are averaged for HC=CH, glyc CH_2O, chol CH_2OP, and CH_2N resonances, which show similar behavior ($N(CH_3)_3$ is plotted separately, △------△). Taurocholate NMR data ($\Delta\nu/\Delta\nu^0$, ○) are averaged for 7, 21, 25 and 26 sites; the methyl group at position 18 is omitted from the analysis, since the narrow linewidth in the 90 : 10 reference spectrum is not well determined. The arrow indicates enhancement of the linewidth beyond an observable limit of approx. 100 Hz.

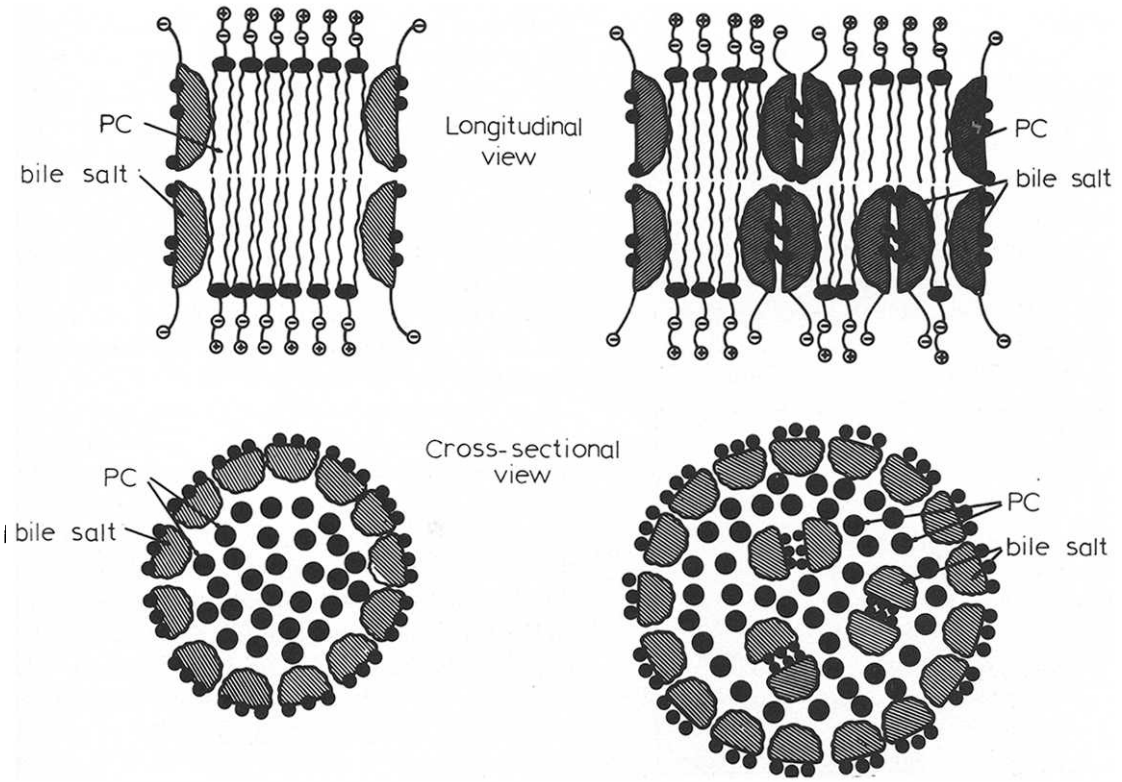

Fig. 6. Schematic models (plain and mixed disks) for the bile salt-PC mixed micelle, shown in longitudinal and cross section. The closed circles and ovals represent the nonionic polar groups of the molecules, and the open circles with negative and positive signs represent the ionic polar parts. Reprinted from Ref. 5, by permission.

tially, but some resolved structure remains in the NMR spectrum until the PC content exceeds 80–90%. The bulk solution viscosity exhibits only modest changes for this series of mixtures (Fig. 4), so that this property must be discounted as a major determinant of the observed trends in NMR linewidths.

NMR estimates of local molecular mobility may also be contrasted with other physical measurements of overall aggregate size. Mean hydrodynamic radii ($\bar{R}_h$) deduced from QLS measurements of taurocholate/PC mixtures with varying relative composition exhibit a divergence in size at a 1 : 1 PC : taurocholate ratio [5]. If these $\bar{R}_h$ values are normalized to the radius of 90 : 10 taurocholate/PC micelles ($\bar{R}_h^0$), and $\Delta\nu$ values for both lipid moieties are referenced similarly to those of $\Delta\nu^0$, it is possible to compare QLS and NMR results directly. Enhancements of both parameters are plotted as a function of PC : taurocholate ratio in Fig. 5. Although the hydrodynamic radius of the particle increases by a factor of 6 as the PC : taurocholate ratio approaches 1 : 1, retention of local motional freedom is indicated by small and largely invariant NMR linewidths for both taurocholate and PC in this compositional regime. And though $\bar{R}_h$ and $\Delta\nu$ for taurocholate resonances both diverge at roughly the same phase limit, PC resonances remain narrow well beyond a 1 : 1 PC : taurocholate ratio. If local reorientation times were controlled by overall particle size, then NMR parameter enhancements should have paralleled and been even more dramatic than the QLS trends [22]. Moreover, resonances for both lipid components would exhibit identical broadening trends vs. PC : bile salt ratio. Instead, differing linewidth enhancements are observed for PC and bile salt constituents of the aggregate, clearly implicating site-specific variations in motional freedom.

Discussion

The present study uses high-field ^{1}H-NMR to survey micellar structure and dynamics in bile salt/PC mixtures. Variations in spectral linewidth are good probes of molecular mobility for a variety of sites on each lipid. When combined with independent estimates of overall aggregate size, the local information obtained by NMR methods serves to test and refine structural hypotheses about these physiologically important mixed aggregates.

Both the present work and prior studies of taurocholate/PC mixtures by quasielastic light scattering (QLS) techniques [5] indicate dramatic growth of disk-like aggregates as the solubilization limit is approached, but the retention of high-resolution NMR spectra implies that molecular reorientation is not restricted concurrently within the aggregates. Small linewidths are observed for polar portions of the PC (glycerol backbone and phosphorylcholine headgroup resonances), which are presumably located near the aqueous interface of the mixed micelle. Molecular mobility is expected to persist if loose aggregate packing in this region allows local reorientation to occur without hindrance from neighboring molecules. By contrast, unsaturated acyl chain sites exhibit a somewhat greater degree of motional restriction in all solutions (Figs. 2 and 3) as expected if they are constrained in a bilayer arrangement [13].

One surprising trend, observed with or without added NaCl, is the onset of broadening for all bile-salt moieties before broadening occurs for PC resonances. Previous workers reported preferential restriction at bile-salt hydrophobic sites (in particular CH_3(18)) even in bile salt-rich cholate/PC solutions [10]. An alternate explanation of this observation is supplied by the present work. At low field, the CH_3(18) resonance could simply be obscured by modest broadening of the PC bulk methylene peak. In our studies at 500 MHz, no differences are observed in the behavior of hydrophobic moieties (steroid nucleus, methyl groups) and hydrophilic bile salt sites (C<u>H</u>OH, taurine side-chain). Our NMR results are consistent with the proposal [4] of a belt of bile-salt molecules around the disk perimeter (Fig. 6). As more PC is solubilized in the interior, the disk may swell and induce motional constraints on bile salts which cover the perimeter (such growth is accelerated in the presence of NaCl [5]). Bile salts are incorporated in larger vesicle structures beyond the micellar phase limit, so that dramatic broadening of the NMR signals may also reflect motional restrictions which accompany this transition. Proximity of the PC acyl chains is expected to restrict motion of hydrophobic taurocholate moieties, but the loss of molecular mobility for hydrophilic and side-chain sites is unexpected in light of trends observed for polar portions of the PC molecule. For these latter taurocholate sites, motional restriction may be attributed to tight molecular packing (at the micellar disk perimeter or at vesicle-incorporated sites) and/or interactions with interfacial water molecules. An intermediate chemical exchange rate of bile salt from intermicellar sites to the large PC-rich structures is an alternative (although less likely) explanation of preferential bile-salt line broadening as the phase limit is approached. Exchange rates for monomer units in a micelle are typically greater than 10^4 s^{-1} [23]. For the taurocholate-PC particles we have examined, the change in particle size would have to reduce the exchange rate 10^3-fold (to approx. 10 s^{-1}) in order to alter the NMR exchange regime from fast to intermediate and contribute to enhanced line-broadening of taurocholate resonances.

The proposal of a mixed disk structure [5] (Fig. 6) remains difficult to assess by ^{1}H-NMR methods. The most dramatic line-broadening effects occur in PC-rich solutions, for which bile salt incorporation within the disk would be minimal in any case. At the other compositional extreme, spectral properties may be averaged over interior and perimeter mixed micelle sites and also pure bile-salt micelles. Detailed structural interpretation is difficult in such solutions, for which fast exchange occurs among a variety of sites.

In summary, ^{1}H-NMR linewidths provide a rich source of structural and dynamic information in taurocholate/PC mixtures. NMR estimates of local molecular motion for each lipid functional group may be compared with QLS determinations of overall aggregate size. The complementary nature of this comparison is illustrated by the observation that motional freedom is retained for both constituents even as the mixed-particle radius increases 6-fold. As the micellar phase limit is

approached in PC-rich solutions, motional restriction occurs first for all taurocholate moieties, suggesting tight bile salt packing in large aggregates as well as possible interactions with PC acyl chains and with interfacial water molecules. By contrast, portions of the PC located near the aqueous interface continue to reorient freely in large mixed micelles and are constrained only when multibilayer structures are formed.

Acknowledgments

This work was supported by grants from the National Science Foundation (7912611-PCM, M.F.R.) and National Institutes of Health (GM-26762, M.F.R.). R.E.S. acknowledges the support of an Amherst College Trustee-Faculty Fellowship for 1982-3. The Francis Bitter National Magnet Laboratory is supported by the National Institutes of Health (RR-00995) and the National Science Foundation (C-670). We thank Martin Carey and Joanne Donovan for reading the manuscript, and we wish to acknowledge Grace Ko for performing the viscosity and density measurements.

References

1 Carey, M.C. (1982) in The Liver: Biology and Pathobiology (Arias, I.M., Popper, H., Schachter, D. and Shafritz, D.A., eds.), pp. 429–465, Raven Press, New York
2 Carey, M.C., Small, D.M., and Bliss, C.M. (1983) Annu. Rev. Physiol. 45, 651–677
3 Carey, M.C. and Small, D.M. (1970) Am. J. Med. 49, 590–608
4 Small, D.M. (1967) Gastroenterology 52, 607–610
5 Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) Biochemistry 19, 601–615
6 Muller, K. (1981) Biochemistry 20, 404–414
7 Claffey, W.J. and Holzbach, R.T. (1981) Biochemistry 20, 415–418
8 Spink, C.H., Muller, K. and Sturtevant, J.M. (1982) Biochemistry 21, 6598–6605
9 Mazer, N.A. and Carey, M.C. (1983) Biochemistry 22, 426–442
10 Small, D.M., Penkett, S.A. and Chapman, D. (1969) Biochim. Biophys. Acta 176, 178–189
11 Lichtenberg, D., Zilberman, Y., Greenzaid, P. and Zamir, S. (1979) Biochemistry 18, 3517–3525
12 Castellino, F.J. and Violand, B.N. (1979) Arch. Biochem. Biophys. 193, 543–550
13 Stark, R.E., Manstein, J.L., Curatolo, W. and Sears, B. (1983) Biochemistry 22, 2486–2490
14 Pope, J.L. (1967) J. Lipid Res. 8, 146–147
15 Carey, M.C. and Small, D.M. (1978) J. Clin. Invest. 61, 998–1026
16 Armstrong, M.J. and Carey, M.C. (1982) J. Lipid Res. 23, 70–80
17 Yedgar, S., Hertz, R. and Gatt, S. (1974) Chem. Phys. Lipids 13, 404–414
18 Barnes, S. and Geckle, J.M. (1982) J. Lipid Res. 23, 161–170
19 Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature 261, 390–394
20 Missel, P., Mazer, N.A., Benedek, G.B. and Carey, M.C. (1983) J. Phys. Chem. 87, 1264–1277
21 Koppel, D.E. (1972) J. Chem. Phys. 57, 4814–4820
22 MacKay, A.L., Burnell, E.E., Nichol, C.P., Weeks, G., Bloom, M. and Valic, M.I. (1978) FEBS Lett. 88, 97–100
23 Fendler, J.H. and Fendler, E.J. (1975) Catalysis in Micellar and Macromolecular Systems, pp. 25–30, Academic Press, New York