papers on methodology

Use of ^{1}H-NMR to determine the distribution of lecithin between the micellar and vesicular phases in model bile[1]

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Abstract Biliary cholesterol/phospholipid vesicles play an important role in the pathogenesis of gallstone disease. A prerequisite for the study of the lipid composition and stability of these vesicles is a reliable method to quantify the amount of vesicular lipid. In the present report we show that NMR can be used to determine the distribution of biliary lecithin between the micellar and vesicular phases. The relatively large size of the vesicles leads to such a broadening of the lipid resonances that they are no longer visible in high resolution ^{1}H-NMR spectra. Since micelles are much smaller, lipid present in the micellar phase does give rise to sharp peaks in ^{1}H-NMR spectra. Micellar lecithin can easily be quantified in these spectra. The resonances of cholesterol are masked by the closely related bile acid that is present in a much higher concentration. By determining the difference between chemically and NMR estimated lecithin, the distribution of this phospholipid between the micellar phase and vesicular phase can be assessed. We have compared the results of NMR with gel permeation and density gradient ultracentrifugation. Using standard fractionation conditions, both gel permeation and density gradient ultracentrifugation lead to an underestimation of vesicular lecithin, the difference being minor at relatively high total lipid concentrations (10 g/dl) but large in diluted model bile. We conclude that ^{1}H-NMR can be used to determine the distribution of lecithin in model bile. The method can be used either to determine vesicular lecithin directly or as a gold standard to standardize other fractionation methods such as gel permeation or density gradient ultracentrifugation. —**Groen, A. K., B. G. Goldhoorn, P. H. M. Egbers, R. A. F. M. Chamuleau, G. N. J. Tytgat, and W. M. M. J. Bovée.** Use of ^{1}H-NMR to determine the distribution of lecithin between the micellar and vesicular phases in model bile. *J. Lipid Res.* 1990. **31:** 1315–1321.

Supplementary key words vesicles • cholesterol • micelles

In bile, lecithin and cholesterol can be solubilized in two distinctly different types of particles: mixed micelles and vesicles. In mixed micelles, the two lipids are surrounded by bile salt molecules. In the vesicular phase, lecithin and cholesterol form unilamellar vesicles with a diameter of 50–100 nm (1–4). The mixed micellar phase is thermodynamically stable but the vesicular phase can be unstable depending on the conditions prevailing in the biliary tree. The stability of the vesicles is mainly determined by the cholesterol/lecithin ratio. Halpern et al. (4) and Kibe et al. (5) have shown in model bile how the distribution of cholesterol and lecithin between the micellar and vesicular phases is determined by the concentration of the individual lipids. Under physiological conditions a very important determinant is the total lipid concentration (4).

Although the presence of the vesicles in human bile has been demonstrated unequivocally by several groups (1, 3, 6–11) it has not yet been possible to quantify the exact amount of lipid in the vesicular phase. This is due to the rapid equilibrium between the vesicular and micellar phases. When the two phases are separated by techniques such as gel permeation chromatography or density gradient ultracentrifugation, dilution of the bile sample cannot be prevented. This leads to a shift of lecithin and cholesterol from micelles to vesicles (7, 12). It has been argued that the shift can be prevented by adding bile salt to the elution buffer at an intermicellar concentration but, since there is no gold standard for the determination of vesicular lipid, this assumption has not yet been validated. Recently, Somjen et al. (13) described a method to quantify micellar lipid by using quasi-elastic light scattering (QELS). Assuming that isolation of vesicles by column chromatography does not induce changes in the size of the vesicles, they determined light scattering as a func-

Abbreviations: QELS, quasi-elastic light scattering; NMR, nuclear magnetic resonance; HRNMR, high resolution NMR; CSI, cholesterol saturation index; IMC, intermicellar concentration.

[1]This work was presented in part in abstract form at the annual meeting of the American Association for the Study of Liver Diseases, Chicago, IL, October 1988.

tion of the vesicular cholesterol/phospholipid ratio. Further assuming that vesicles in bile are monodisperse and that the cholesterol/phospholipid ratio is the only determinant for vesicular size, they were able to calculate the amounts of vesicular cholesterol and phospholipid from QELS measurements. It is clear that this method still rests on a number of assumptions.

In the present report we have chosen a different approach to determine the distribution of lipid between the vesicular and micellar phases in bile. NMR has been used to study the structure and dynamics of lipid-water mixtures for many years (14). In a model system, Stark et al. (15) demonstrated that, depending on the total lipid concentration and the ratio of taurocholate and lecithin, micelles and/or vesicles were formed. Micelles showed high resolution ^{1}H-NMR (HRNMR) spectra with narrow lines. In the spectrum of the vesicular phase the NMR lines were greatly broadened. In the present study we have investigated whether the difference in the spectra of the micellar and vesicular phase can be used to quantify the amount of cholesterol and lecithin in both fractions.

MATERIALS AND METHODS

Preparation of model bile and vesicles

Model bile was prepared according to Kibe et al. (5) with slight modifications. Taurocholate (Fluka, Buchs, Switzerland) in methanol–water 85:15 was mixed with lecithin (egg yolk, Sigma Chemical Co., St. Louis, MO) and cholesterol (Sigma Chemical Co.) in chloroform. The mixture was flushed with nitrogen at 50°C to remove organic solvent and then lyophilized. The dry lipid film was solubilized in Tris buffer (20 mM pH 8.2) and incubated for 24 h at 56°C. The solutions were equilibrated for 24 h at 22°C prior to use. The final concentrations of biliary lipids were: taurocholate, 130 mM; cholesterol, 7.5, 10, 13, or 19 mM; phosphatidylcholine, 30 mM. The cholesterol saturation index (CSI) was calculated from the critical tables of Carey (16). Small unilamellar vesicles were prepared as follows. Cholesterol in chloroform and lecithin were mixed in equimolar concentrations. Chloroform was evaporated and the dried lipid film was solubilized in Tris buffer (20 mM, pH 8.2). The mixture was sonicated on ice and under nitrogen for either 5 min (mild sonication) or 45 min at 70 W in a Branson sonicator. Subsequently the vesicles were centrifuged for 1 h at either 10,000 (mild sonicated vesicles) or 100,000 *g* (extensively sonicated vesicles). The pellet was discarded. The recovery of lipid after mild sonication was 90%; after extensive sonication, 70–80%. The size and nature of the vesicles in the supernatant were studied by transmission electron microscopy after negative staining with 1% ammonium molybdate.

NMR spectroscopy

^{1}H-NMR spectra were obtained at 300 MHz and 25°C, sampling FIDs using 5-mm NMR sample tubes and a home-built spectrometer (17). The flip angle was 45°, the sampling frequency and time were 3.8 KHz and 0.8 sec, respectively. The recycle time was 4.2 sec; the number of accumulations was 40. The receiver dead time between the pulse and the beginning of the data acquisition was 80 μsec. Optimal signal-to-noise time domain windowing was applied with a time constant of 0.3 sec. Before each pulse the water signal was selectively saturated during 2 sec. Spectra were quantified by the height of the relevant peak relative to the height of the Tris reference peak (3.6 ppm).

Density gradient ultracentrifugation

The micellar and vesicular fractions in model bile were separated by KBr density gradient ultracentrifugation essentially as described previously (18). Model bile (1–5 ml) was mixed with 1.357 g KBr. The volume of this layer was adjusted to 5.5 ml with Tris buffer (50 mM, pH 8.2). On this mixture 4.8 ml of KBr solution with a density of 1.082 g/ml was layered. Finally, 2.4 ml of double-distilled water was layered and the tubes were centrifuged for 22 h at 170,000 *g* and 20°C in a Beckman SW 41 Ti rotor. The tubes were fractionated by hand into 11 fractions of 1.17 ml each.

Gel permeation column chromatography

Vesicular and micellar lipids were separated on a Sephacryl S-300 column (1.6 × 40 cm) essentially as described by Somjen et al. (13). The elution buffer contained Tris-HCl (50 mM, pH 8.2) and Na cholate (10 mM). The flow rate was 0.5 ml/min. Fractions of 1.0 ml were collected and in these fractions cholesterol and lecithin were assayed chemically. The recovery of cholesterol and lecithin was (89.3 ± 6.3%, (n = 6)).

Analytical procedures

Biliary lipids were determined using standard enzymic procedures (19–21).

Statistical analysis

The nonparametric Kruskall Wallis test was used to evaluate differences between the various sets of data.

RESULTS

The micellar and vesicular fractions were isolated from a model bile with a cholesterol saturation index (CSI) of 1.2 by density gradient ultracentrifugation. The vesicular fraction contained 10 mM cholesterol and 10 mM lecithin and less than 0.1 mM bile salt. The micellar fraction con-

tained 110 mM taurocholate, 21 mM lecithin, and 6.5 mM cholesterol. The high resolution NMR spectra of these fractions are depicted in **Fig. 1**. The micellar fraction reveals a multitude of resonances. In contrast, apart from the Tris reference peak, the vesicular fraction only gives rise to a solid state-like signal. In Fig. 1 this is not discernible because the spectrometer settings selectively aim at observing HRNMR lines such as observed in the micellar fraction.

To investigate the origin of the resonances in the spectrum of the micellar fraction, spectra were recorded from solutions containing taurocholate, lecithin, and cholesterol, alone or in combination (**Fig. 2**). It is clear that taurocholate accounts for most of the resonances in the spectrum of the micellar fraction (22). Two important resonances originate from lecithin (15): the choline head group (3.2 ppm) and the $(CH_2)_n$ (1.2 ppm) groups in the fatty acid moieties of the molecule are responsible for these resonances. These peaks can be used to quantify lecithin in the micellar fraction. No resolved signal in the spectrum could be assigned to cholesterol. Probably this is due to the fact that the concentration of micellar cholesterol is much lower than the concentration of taurocholate. Most resonances of cholesterol are overlapped by taurocholate resonances as major parts of the molecules are identical. Due to small differences in the chemical shift values of cholesterol and taurocholate in the 0.8 to 1.05 ppm region, some broadening of the lines in the spectrum in Fig. 2C is observed. Unfortunately, these facts preclude determination of micellar cholesterol. In contrast, micellar phospholipid can be quantified quite simply. **Fig. 3** depicts peak height of the choline and $(CH_2)_n$ peak as a function of the lecithin concentration. For both peaks a linear relationship between the concentration of lecithin and peak height was observed. Lecithin was solubilized in 130 mM taurocholate in the absence of cholesterol. According to the phase diagrams of Carey and Small (23), all lecithin will be present in the micellar phase at the concentrations used in these experiments. Fig. 1 shows that lecithin in the vesicular phase does not contribute significantly to the resonances in the spectrum. Hence, if a model bile contains vesicles, the amount of vesicular lecithin is given by the difference between chemically and NMR determined lecithin. Of course, the accuracy of this method depends strongly on the absence of signal from vesicular lecithin. As shown in Fig. 1, this condition is met for vesicles isolated by density gradient ultracentrifugation. However, it cannot be excluded that during this procedure salt-induced fusion of the vesicles occurs. Since vesicle size could influence the NMR signal, we have also determined spectra of vesicles prepared by mild or extensive sonication followed by high speed ultracentrifugation. Transmission electron microscopy showed that vesicles prepared by mild sonication were often multilamellar and variable in diameter. In such preparations no resonances derived from lecithin were observed (**Fig. 4B**). After extensive sonication and ultracentrifugation a monodisperse population of unilamellar vesicles with a diameter of about 50 nm was obtained (not shown). Fig. 4C shows that in these small vesicles broadening of the

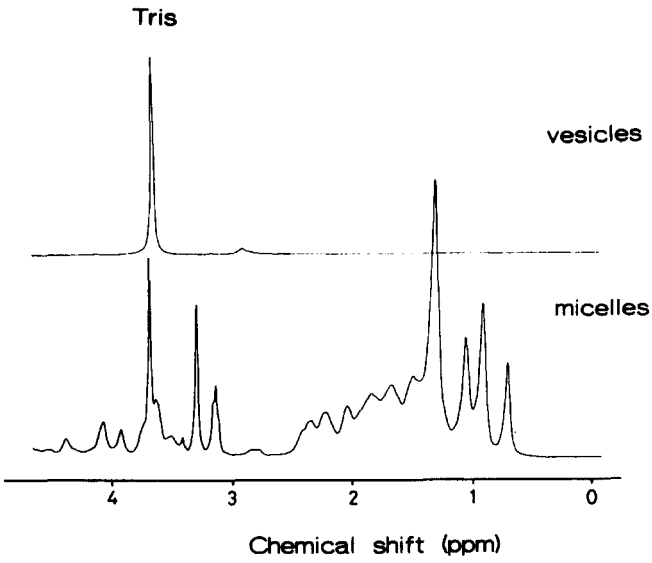

Fig. 1. NMR spectra of isolated micelles and vesicles. The vesicular and micellar phase in model bile were separated by density gradient ultracentrifugation as described in Methods. The vesicular fraction contained 10 mM cholesterol and 10 mM lecithin. The micellar fraction contained 119 mM taurocholate, 21 mM lecithin, and 6.5 mM cholesterol.

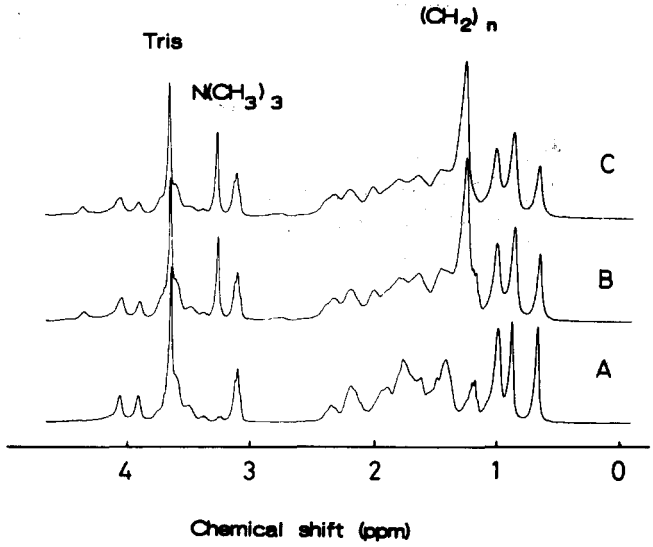

Fig. 2. NMR spectra of taurocholate/lecithin micelles, and a model bile containing taurocholate, lecithin, and cholesterol. Taurocholate (130 mM) either alone (A) or in the presence of lecithin (30 mM) (B) or lecithin (30 mM) and cholesterol (13 mM) (C) was solubilized in Tris buffer (50 mM, pH 8.2). NMR spectra were recorded as described in Methods.

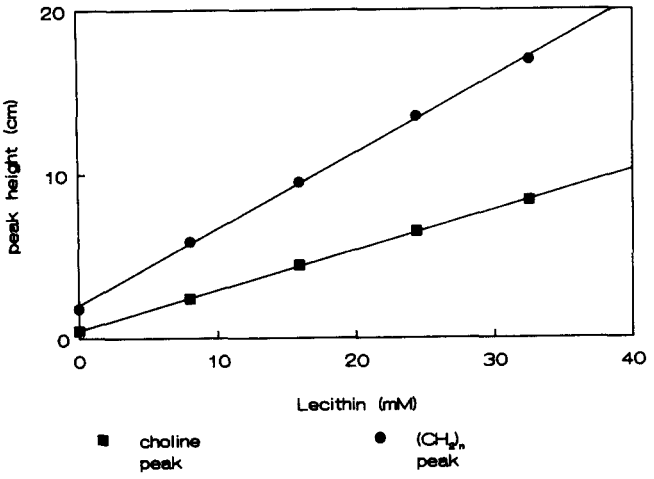

Fig. 3. Standard curve for lecithin. Different concentrations of lecithin were solubilized in 130 mM taurocholate in Tris buffer (50 mM, pH 8.2).

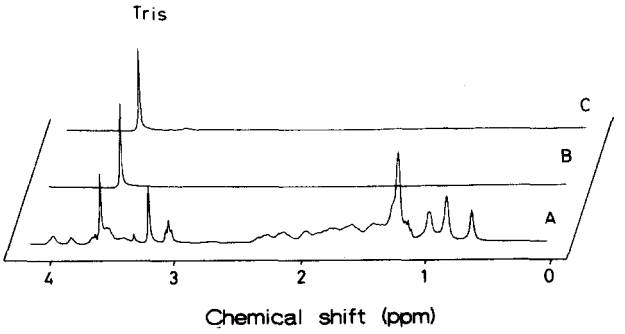

Fig. 4. The effect of sonication and ultracentrifugation on NMR resonances in isolated vesicles. The spectrum of taurocholate (130 mM) in the presence of 30 mM lecithin (A) was compared with spectra of cholesterol/lecithin vesicles prepared either by mild sonication (5 min) (B) or extensive sonication (45 min) followed by ultracentrifugation at 100,000 *g* for 1 h (C). The final concentration of cholesterol and lecithin was 10 and 10 mM after mild sonication and 7 and 8 mM after extensive sonication and ultracentrifugation.

choline-derived peak had somewhat decreased so that now a small choline resonance became visible. At 1.2 ppm, where the $(CH_2)_n$ resonance should appear, again no significant signal was observed. We have, therefore, used this resonance to quantify the amount of vesicular lecithin in model bile. Spectra were recorded from model bile with a constant concentration of taurocholate and lecithin but a varying amount of cholesterol (**Fig. 5**). Total lecithin was determined chemically and the amount of micellar lecithin was calculated from the height of the peak at 1.2 ppm in NMR spectra. In initial series of experiments, lecithin was quantified from the spectra using both peak height and peak area. An excellent correlation was obtained (r = 0.995). Since determination of peak area from these spectra is rather cumbersome, we choose to determine lecithin from peak height in subsequent experiments. At a cholesterol saturation index of 0.75, the amount of lecithin in the vesicles was negligible. As expected, when the CSI was increased, the amount of lecithin in the vesicular phase also increased. The effect of dilution on the amount of vesicular lecithin is presented in **Fig. 6**. Model bile with a CSI of 1.2 was serially diluted with Tris buffer and either analyzed directly or equilibrated for 48 h at 22°C. The amount of vesicular licithin rises as a function of model bile dilution. Clearly the equilibration is not instantaneous. After 48 h of incubation the amount of vesicular lecithin has increased at all total lipid concentrations.

Comparison of NMR, gel permeation, and density gradient ultracentrifugation

To be able to compare the NMR results with methods used in the literature, we have also separated the micellar and vesicular phases in model bile by gel permeation chromatography and density gradient ultracentrifugation. Gel permeation was carried out using a Sephacryl-S300 column and 10 mM Na cholate in the elution buffer as described by Somjen et al. (13). As shown in **Fig. 7**, with this method a good separation between the vesicular and micellar phases is obtained. By determining peak areas the amount of cholesterol and phospholipid in micelles and vesicles can be quantified. In **Fig. 8** the percentage of vesicular lecithin is plotted as a function of the CSI. The NMR data have also been plotted. Gel permeation produces slightly lower values of vesicular lecithin. We have also tested the effect of dilution on vesicular lecithin. As shown in **Fig. 9**, dilution of the

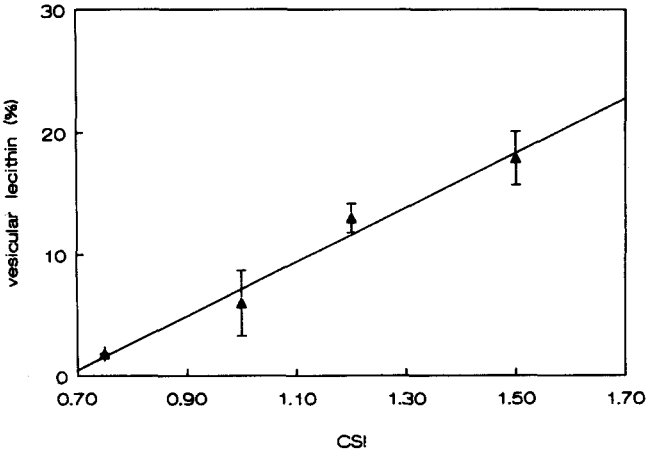

Fig. 5. The amount of vesicular lecithin in model bile as a function of the cholesterol saturation index. NMR spectra were recorded from model biles with a constant amount of taurocholate (130 mM) and lecithin (30 mM) and various concentrations of cholesterol. The amount of micellar lecithin was quantified from the spectra and the total lecithin concentration was determined chemically. Vesicular lecithin was calculated from the difference between the total and the micellar amounts of lecithin. Data are presented as mean ± SD for three or four separate experiments.

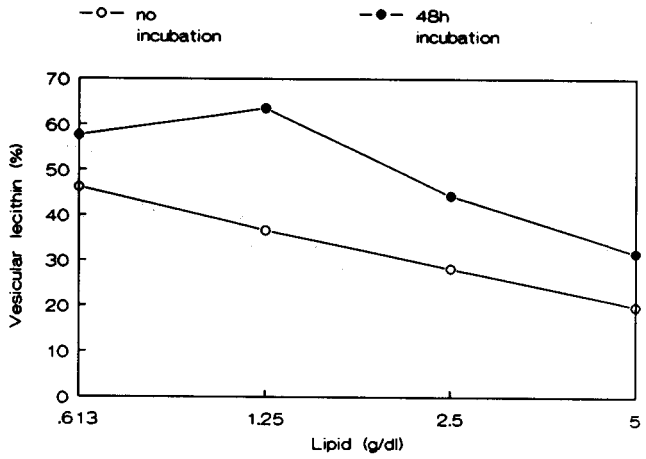

Fig. 6. The relation between the total lipid content of model bile and the amount of vesicular lecithin. Model bile containing 130 mM taurocholate, 30 mM lecithin, and 13 mM cholesterol was serially diluted as shown in the figure. NMR spectra were recorded from these dilutions either directly or after 48 h incubation at 22°C. Vesicular lecithin was calculated as described in the legend to Fig. 5.

model bile increases vesicular lecithin, as is also evident when the vesicular lipid is estimated by gel permeation. However, the percentage of vesicular lecithin remains lower than that obtained by NMR and the difference becomes significant at low total lipid concentrations.

Density gradient ultracentrifugation in the absence of added bile salt provides an excellent separation between the vesicular and micellar phases (3, 11, 18). However, as shown in Figs. 8 and 9, this technique does not give the correct value for vesicular lecithin. The underestimation of the percentage vesicular lecithin is even greater than in the case of gel permeation.

DISCUSSION

The appearance of NMR signals is determined by the spectral resolution in relation to the line width, w. In our application the spectral resolution is chosen to observe HRNMR lines with width in the order of 1 to 100 Hz; solid state-like signals are not detected. ^{1}H-NMR line width in lipid-water mixtures is determined mainly by magnetic dipolar interactions between the protons. In a frozen mixture, w is about: $w_s \approx 10^4$ Hz. In the liquid state, the dipolar interactions are averaged out if $\tau w_s \ll 1$, τ being the correlation time characterizing the reorientational motion of the vesicles or micelles. The dipolar interactions are also partly averaged out by the restricted motions of the lipid molecules within these structures. The w values for oriented lipid bilayers are in the order of 200 Hz for CH_3 groups to 3000 Hz for the chain CH_2 groups (24). Assuming that there is no cooperative interaction between micelles or vesicles, the value of τ for a sphere can be estimated by using the Stokes-Einstein equation

$$\tau = 4\pi\eta a^3/3kT$$

where k refers to the Boltzman constant, T to the absolute temperature, and a to the radius of the sphere; η is the viscosity of the fluid. Assuming that the viscosity of model bile η is equal to 1.1 centipoise (25), τ can be calculated.

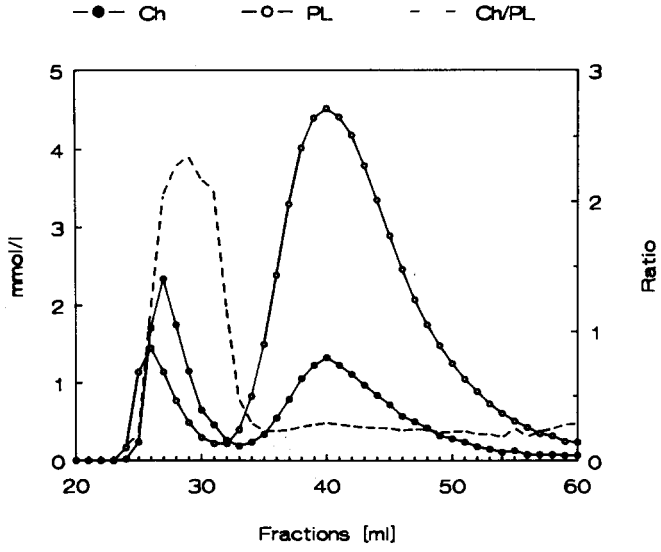

Fig. 7. Fractionation of model bile on Sephacryl S-300. Two ml of model bile containing 130 mM taurocholate, 30 mM lecithin, and 13 mM cholesterol was eluted on a Sephacryl S-300 column. The elution buffer was Tris-HCl (50 mM, pH 8.2) containing 10 mM Na cholate. The flow rate was 0.5 ml/min. Cholesterol and lecithin were assayed in the fractions by chemical means.

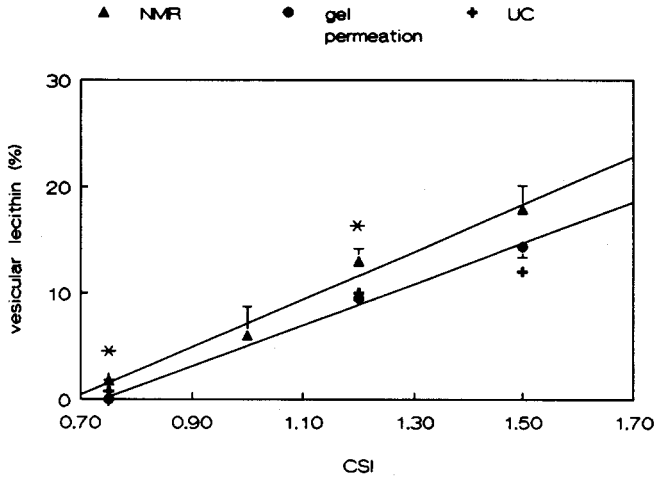

Fig. 8. Comparison of the vesicular amount of lecithin as determined by NMR, gel permeation, or density gradient ultracentrifugation. Model biles with a constant amount of taurocholate (130 mM) and lecithin (30 mM) and various concentrations of cholesterol were fractionated by gel permeation or density gradient ultracentrifugation as described in Methods. Vesicular lecithin was quantified by determining the area under the vesicular peak. Data are given as means ± SD of three separate experiments. For comparison the data from Fig. 5 have also been plotted. $^*P<0.05$ with respect to NMR.

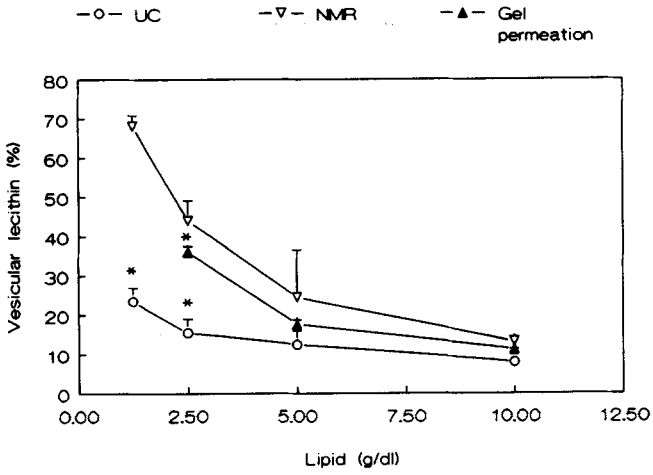

Fig. 9. The effect of dilution on the amount of vesicular lecithin as determined by NMR, gel permeation, or density gradient ultracentrifugation. Model bile containing 130 mM taurocholate, 30 mM lecithin, and 13 mM cholesterol was serially diluted as shown in the figure. The dilutions were incubated for 48 h at 22°C and subsequently subjected to fractionation by gel permeation or density gradient ultracentrifugation. The amount of vesicular lecithin was calculated as described in the legend to Fig. 8. $^{*}P<0.05$ with respect to NMR.

For micelles with a diameter of about 2.5 nm (2) τ is about 2.2×10^{-9}, whereas for the vesicles with a diameter of about 100 nm the value for τ will be around 1.4×10^{-4}. Therefore, dipolar interactions will not average out completely in the vesicular phase which induces line broadening. This effect is greater for the CH_2 groups in the fatty acids chain than for the CH_3 groups in the choline head group since the latter have more motional freedom; they reorient very fast about the N-CH_3 axis. Furthermore, these groups are located at the periphery of the vesicles. This may explain the partial visibility of the choline peak in very small vesicles (Fig. 4C) whereas the CH_2 groups remain indiscernible.

Recently, Somjen et al. (13) reported a comparison of QELS and gel permeation as methods for the determination of vesicular cholesterol and phospholipid in dilute human bile. They concluded that gel filtration on a Sephacryl-S300 column combined with bile salt-supplemented elution buffer provides reasonable lipid distribution between the micellar and vesicular phases. Our results support this conclusion with respect to model bile with a total lipid concentration of 10 g/dl. At a lower total lipid, gel permeation gives lower values for vesicular lecithin than NMR. The validity of gel permeation rests on the assumption that the concentration of bile salt in the elution buffer is equal to the intermicellar concentration (IMC) so that upon the inevitable dilution during the fractionation no micelles are converted to vesicles or vice versa. Our results suggest that a concentration of 10 mM cholate is slightly higher than the IMC under the conditions prevailing in model bile. This is not surprising in view of the findings of Somjen and Gilat (9) who showed that upon rechromatography of the vesicle fraction 70% of the cholesterol shifted to the micellar fraction.

Density gradient ultracentrifugation as carried out in this study also underestimates the amount of vesicular lipid. This was unexpected since with this technique dilution of the bile occurs in a medium without added bile salt. Theoretically this should lead to a shift from micelles to vesicles. Apparently, the concentration of KBr used to generate the density gradient affects the equilibrium between micelles and vesicles and has an opposite effect on the lipid distribution in model bile. An advantage of density gradient ultracentrifugation compared to gel permeation is the much better separation that is obtained. Halpern et al. (4) and Kibe et al. (5) used ultracentrifugation without a preformed density gradient. This results in considerable overlap between the vesicular and micellar fractions, hence the amount in both phases has to be extrapolated from control centrifugations with a sample that contains no vesicles. Theoretically, when the extrapolation is carried out correctly, the method should give reliable results. In practice this is difficult to accomplish, certainly when the amount of vesicular lipid is low. Furthermore, in contrast to gel filtration and density gradient ultracentrifugation, this technique cannot be used for human bile samples.

We have not yet thoroughly studied the applicability of ^{1}H-NMR to the determination of vesicular phospholipid in human bile. Preliminary studies have shown that the resolution of NMR spectra from human bile is similar to those from model bile. However, quantitation of the spectra is hampered by the presence of small amounts of free fatty acids in human bile. Furthermore, the composition of the fatty acid moieties in human lecithin shows individual variability (26, 27). Studies are in progress to solve these problems so that the dynamics of lecithin solubilization in bile can be studied in vitro and in vivo in both animals and humans.

Manuscript received 11 July 1989, in revised form 12 December 1989, and in revised form 26 February 1990.

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