Interaction of Bile Salt and Phospholipids with Bovine Submaxillary Mucin

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Purpose. The purpose of this study was to determine the distribution and diffusion of sodium taurocholate-phospholipid micelles with mucin in order to provide the foundation for understanding the transport of ingested fat and poorly water-soluble drugs through the intestinal mucous layer.

Methods. Sodium taurocholate (NaTC) was dispersed with egg phosphatidylcholines (PC) to yield mixed micelles of a specific size and concentration. A preliminary study was conducted to determine the time required for equilibration of PC/TC micellar solutions with mucin. PC/TC micellar solutions were dialyzed against fixed and variable concentrations of bovine submaxillary mucin after which the concentration of PC and NaTC was measured by an assay for total phosphorus and by HPLC, respectively. In addition, a quantitative assay of TC and PC by NMR was developed and used to estimate the mobile fraction of lipids in the samples. Finally, pulsed-field gradient spin echo NMR self-diffusion measurements were made of the water, TC, and PC in the samples obtained from dialysis.

Results. TC/PC micellar solutions achieved equilibrium with mucin in 7 days. Mucin did not affect the equilibrium concentration of PC or TC, except at high concentrations of mucin (5%), and then the effect was small. NMR quantitation was valid for PC and TC systems containing small micelles, but deviated significantly with systems containing large micelles. Mucin decreased the diffusivity of water and the phospholipids, but the effect was relatively small. Mucin dramatically affected the mobility of TC, which prevented a straightforward interpretation of the calculated diffusion coefficients.

Conclusions. Mucin has a minor effect on the equilibrium distribution of phospholipids and bile salts. However, lipids are readily accommodated by mucus, which can significantly increase the permeability of the mucous layer, particularly for poorly water-soluble drugs.

KEY WORDS: bile salt; phospholipid; diffusion; micelle; dialysis; mucin.

INTRODUCTION

The absorption of fat from the gastrointestinal tract is critically dependent on the presence of bile (1). This secretion from the gall bladder results in the formation of small aggregates, referred to as bile salt micelles, which are postulated to diffuse readily from the gut lumen to the epithelial surface. As such, the micelles serve as carriers for ingested fat through the mucous layer that lies interposed between the gut lumen and the epithelial cells (2,3). To date, a number of studies that have examined the transport of drugs by bile salts and bile salt micelles through the mucous layer have been summarized (4).

Much work has been devoted to the characterization of the mucin glycoproteins isolated from the intestine (5). Moreover, the physicochemical properties of the mucin have served as the foundation for characterizing the permeability of the mucous layer. The highly hydrated, negatively charged, entangled glycoprotein network is believed to give rise to an exclusionary effect on the diffusivity of solutes through mucin (6), binding of solutes to hydrophobic domains presumably on the polypeptide chain (7–11), and charge interaction with ions (3). Recent transport studies of mucus-containing lipid have identified a correlation of the mucous permeability with the hydrophobicity of the solute (11–13). This may be highly significant for consideration of the transport of fat and poorly water-soluble solutes. Finally, the movement of bile salt micelles through the mucous layer has been addressed within the context of mucus-producing cell cultures (14–16). With these studies and those with intestinal sections, it is often difficult to separate the contributions to the transport that arise from the membrane and those that arise from the diffusional boundary layer (unstirred water layer) in the lumen.

Although many of the features of the interaction of lipids with the mucous layer have been addressed (3,4), a quantitative study of the association of dietary constituents with mucus has not been conducted. In this study, the hypothesis that mucus affects the distribution and diffusion of bile salt-phospholipid micelles was tested. The approach involved determining the association of bile salt/phospholipid aggregates by quantifying the distribution of each constituent following equilibrium dialysis (17–19). The choice of the bile salt and phospholipids was made based on the available phase diagrams from which aggregates could be prepared of known, consistent size but variable concentration (1). In this way, the classical binding study was completed where the concentration of the ligand, consisting of the bile salt/phospholipid mixed micelle, was varied for a given mucus concentration. This approach also allowed for a fixed ligand concentration to be prepared while the mucus concentration was varied. With these samples, NMR spectroscopy was used to measure the self-diffusion coefficient and the phospholipid and sodium taurocholate (NaTC) concentrations. These measurements provided an assessment of the mobility of lipids within the mucous gel.

EXPERIMENTAL

Materials

TC, sodium taurodeoxycholate, and bovine submaxillary mucin (BSM, Type IS) were purchased from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, AL). Water was deionized and then distilled. Methanol and cyclohexane were HPLC grade. All other chemicals were reagent/analytical grade or better.

Methods

Sample Preparation

Samples containing PC and TC were prepared by first lyophilizing an ethanol/cyclohexane solution of egg PC overnight under high vacuum (Edwards 5 two-stage vacuum

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pump, model E2M5, VWR Scientific) (19). The dried PC was then reconstituted with a concentrated sodium taurocholate solution prepared in saline with 0.1% sodium azide. This dispersion was then diluted with a bile salt solution with a concentration near the chosen intermicellar concentration (IMC). Typically, BSM was dissolved and also diluted with the PC/TC dispersion or the same stock IMC solution of bile salt.

Time Dependence

The time required for bile salt/phospholipid micelles to equilibrate with BSM was determined as follows. An outer solution consisting of 15 mM PC and 21 mM TC was prepared. Two 0.5% BSM solutions were prepared; one contained 30 mM PC and 30 mM TC, and one did not contain any TC or PC. These two solutions were placed in separate, washed Spectra/Por dialysis membranes (12 mm flat width) with a molecular weight cut off (MWCO) of 300,000 Da (Spectrum Laboratories, Inc., Gardena, CA). The bags were tied with surgical thread and then placed into the outer solution held by a tissue culture flask. In addition, a solution containing NaTC equal to the IMC was added to a dialysis tube (MWCO of 1000 Da, Spectra/Por) and placed in the outer solution held by the tissue culture flask. The flask was oscillated on a shaking water bath at room temperature (Precision GCA Corp. Chicago, IL). Aliquots were taken initially and periodically thereafter for 7 days. The aliquots were then assayed for PC by a spectrophotometric assay for elemental phosphorus (20), and thin-layer chromatography (TLC) was performed on an aliquot that was extracted with chloroform/methanol (2:1) using a mobile phase of 65:35:5 chloroform/methanol/water.

Equilibration Study

Equilibrium dialysis studies were conducted as follows. Outer PC/TC solutions and IMC solutions were prepared in saline with 0.1% sodium azide based on the data reported by Duane (17). To the outer solution held by a tissue culture flask, five dialysis tubes (300,000 MWCO) containing different mucin concentrations but the same concentration of PC/TC as the outer solution were added. In addition, a dialysis tube (1000 or 500 MWCO) that contained NaTC at the IMC was added to the flask. The flask was shaken for 7 days, after which samples were taken and assayed for elemental phosphorus spectrophotometrically (20) and for TC by HPLC and NMR spectroscopy. The HPLC procedure has been previously described (19). The diffusion coefficients of water, TC, and PC were also determined for each sample using pulsed-field gradient-spin echo (PFG-SE) NMR spectroscopy (vis. infra).

In a separate dialysis experiment, four different PC concentrations were dialyzed against a fixed mucin concentration. This experiment was set up as follows. Two small width dialysis tubes (300,000 MWCO), one containing saline and the other a 0.5% BSM/TC/PC solution, were placed within a large width dialysis tube (1000 MWCO and 30 mm flat width) along with a TC/PC solution. The large width dialysis tube containing the two smaller tubes was then placed into the IMC solution. Four such large width tubes were prepared with PC concentrations of 10, 15, 20, and 30 mM PC and TC concentrations corresponding to the expected PC/TC ratio given the IMC. After equilibrating for 7 days at 25°C, the samples were assayed for the concentration of PC and TC, and the diffusion coefficients of water, TC, and PC were measured as described below.

Quantitative NMR

The procedure for quantifying TC and PC by NMR was an adaptation of a previously reported method (21). Briefly, 30 different samples containing known amounts of TC (5–80 mM) and PC (0–25 mM) were placed into 5-mm NMR tubes with coaxial insert tubes (WG234, Wilmad Glass Co. Buena, NJ) containing 115 mM 3-(trimethylsilyl)tetradeuterosodium-propionate (TSP) prepared with D_2O. The measurements were made on a Varian 500 MHz spectrometer (Palo Alto, CA) with the temperature controlled at 25 ± 0.2°C. Spectra were obtained using a sequence of a single 90° pulse preceded by a water suppression pulse. The area under a given resonance was determined after transforming the spectra, phasing, and correcting the baseline. The area of the peak for either TC or PC, AUC_{sample}, was normalized by the area of the peak of the standard, AUC_{TSP}, where the area ratio is given by:

$$(AUC_{sample}/AUC_{TSP}) = ([Sample]/[TSP])(^1H_{sample}/^1H_{TSP})(V_{sample}/V_{TSP})$$

where ^1H is the number of protons, V is the volume and sample may refer to either PC or TC.

Diffusion Measurements

The Fourier-transform PFG-SE ^1H NMR diffusion coefficients of water were measured using the standard stimulated spin-echo pulse sequence (22). The diffusion coefficients of bile salt and phospholipid were determined with a similar sequence that was preceded by a water suppression pulse. The diffusion experiments were performed at constant Δ and δ values, and a series of ten G values were used ranging from 10 to 70 G/cm. The gradient pulse length, δ, was 1 ms, and the diffusion time, Δ, was 80, 120, and 480 ms for water, bile salt, and phospholipids, respectively. The resulting transformed area under the peak was analyzed on line by the following linearized equation (23,24):

$$\mathrm{Ln}[A(\tau_1 + \tau_2)] = \text{constant} + \{-(\gamma G\delta)^2 D(\Delta - \delta/3)\}$$

where $\ln[A(\tau_1 + \tau_2)]$ is the natural logarithm of the peak area at the time of the echo, $\tau_1 + \tau_2$, γ is the gyromagnetic ratio, G is the strength of the magnetic field gradient, δ is the field gradient pulse length, D is the diffusion coefficient, and Δ is the diffusion time which is equal to the time interval between the first and second gradient pulses.

To obtain absolute values for the self-diffusion coefficients, the field gradient strength was calibrated by measurement of the diffusion coefficient of trace H_2O in D_2O in the Varian reference sample that has a diffusion coefficient of 1.88×10^{-5} cm^2/s at 25°C (25). In addition, the diffusion coefficient of water in aqueous 150 mM NaCl (2.30×10^{-5} cm^2/s at 25°C) was determined in which the 5 mm tube also contained the TSP insert for tuning and shimming (23). The diffusion coefficient of a standard of sodium dodecyl sulfate (4.6×10^{-7} cm^2/s at 37°C) was measured (19). The diffusion coefficient of water was obtained from the resonance ob-

served at 4.76 ppm. For the bile salt, the resonances at 3.5 and 3.06 ppm were used, and for the phospholipid, the resonance at 3.2 ppm that arises from the protons on the methyls attached to the choline nitrogen was used.

RESULTS

In Fig. 1, spectra obtained from samples containing TC at the IMC, TC/PC dispersion, and TC/PC/BSM are given. The spectrum (Fig. 1A) obtained from the sample containing only bile salt was relatively well resolved and consistent with previously reported spectra (21). In Fig. 1B, the spectrum obtained with a 1.2 mM/8.1 mM PC/TC dispersion is given. The bile salt resonances are well resolved from the PC resonances even though they are noticeably broadened, e.g., 3.06 ppm as well as those just above and below 3.5 ppm. Note that these provide a means to quantify the TC concentration without interference from PC. The most noticeable resonance from PC occurs at 3.2 ppm, which arises from the nine magnetically equivalent protons on the methyl groups attached to the nitrogen of the choline head group.

In Fig. 1C, a spectrum of a sample containing TC, PC and 0.5% BSM is given. The resonances of TC are further broadened in the presence of mucin, however, the mucin itself is evident only as a disturbance in the baseline. It is apparent that the resonances arising from PC are also broadened, although to a lesser extent than that observed with TC. Most importantly, the interference specifically arising from peak overlap of the BSM with TC and PC is negligible.

The results from the NMR quantitation of PC and TC are given in Figs. 2A, B, and C. In these three-dimensional plots, the ratio of the observed concentration by NMR and added concentration of PC is given as a function of the added PC concentration and added TC concentration. For PC (Fig. 2A), it is noted that at high [TC] and low [PC], the ratio is unity. As the concentration of TC is lowered or as the concentration of PC is increased, the ratio decreases. At very low [TC] and high [PC], the ratio is zero as no resonances arising from the phosphatidylcholine head group methyl groups were visible. The concentrations where the ratio fell below 0.5 correspond very well to what is called the divergence boundary (1), which is the phase boundary between mixed micelles and vesicles of the NaTC/egg PC phase diagram.

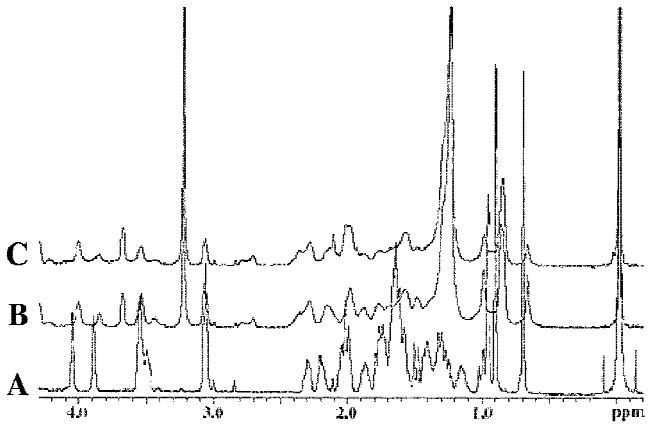

Fig. 1. Proton NMR spectra in aqueous (H_2O) saline solutions of (A) sodium taurocholate 4.34 mM, (B) 1.2 mM PC and 8.1 mM TC solution, and (C) 0.93 mM PC, 6.6 mM TC, and 0.5% BSM solution.

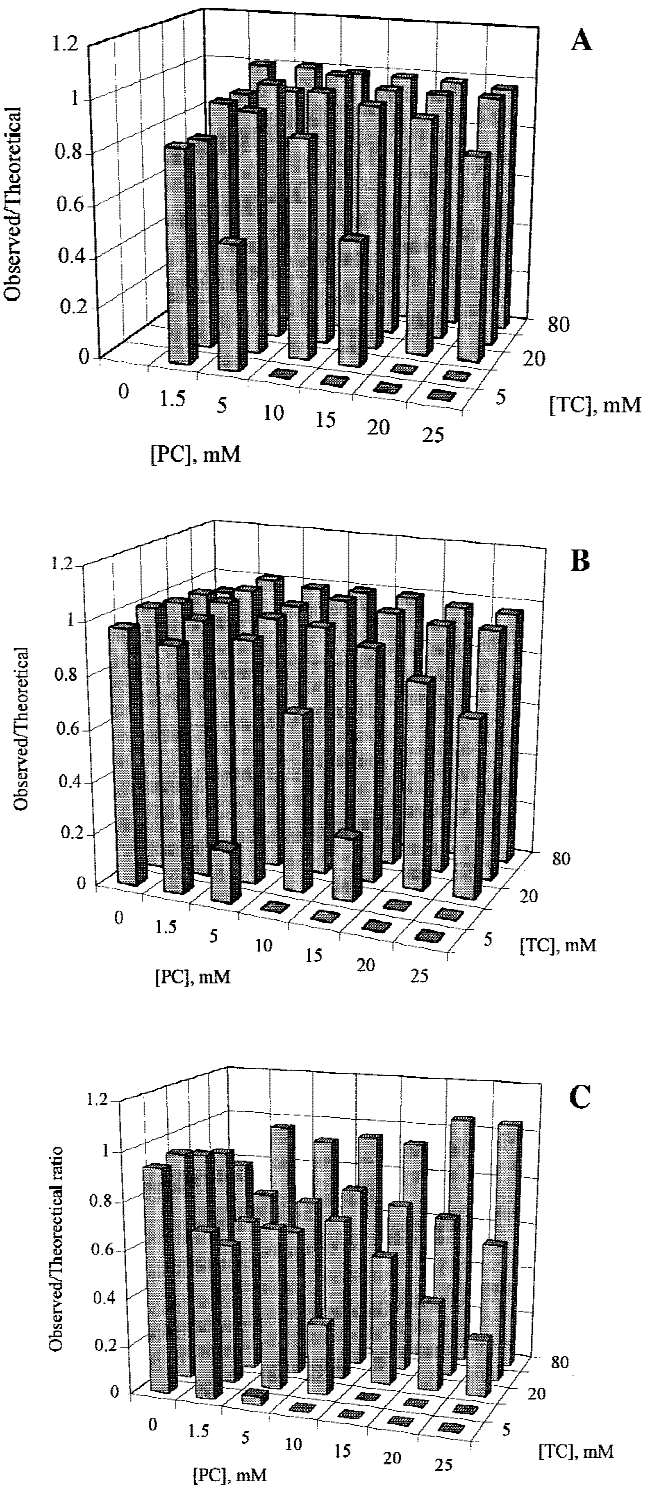

Fig. 2. The ratio of the observed concentration of (A) PC, (B) TC using resonance at 3.06 ppm, and (C) TC using resonance at 3.5 ppm by NMR quantitation and added PC concentration given as a function of added egg PC and NaTC concentration. Error bars were omitted for clarity, with observed standard deviation of less than 10% and typically 3–4%.

The results from quantifying TC with the resonance at 3.06 are given in Fig. 2B. In a similar manner to PC, the ratio was unity for samples with high [TC] and low [PC] and fell as the TC concentration was lowered or the PC concentration

was increased. The divergence limit is also characterized by samples giving rise to ratios less than 0.5, and samples with compositions that correspond to the published vesicle phase did not have visible peaks. In Fig. 2C, the results from quantifying TC with the resonance at 3.5 are shown. Although qualitatively similar to the results obtained with the resonance at 3.06, the ratios were consistently lower. It should be noted that the resonance at 3.5 ppm arises in part from protons located on the frame of the TC molecule. In contrast, the resonance at 3.06 ppm arises from protons on the taurine side chain, which, like the resonances for PC, are located in the more flexible head group region of the molecule.

The results from the time-dependent equilibration experiment are given in Fig. 3 where the concentration of PC is given as a function of time. Two samples are compared, one initially containing roughly twice the equilibrium concentration of PC and one initially containing no PC. As can be seen, the concentrations in these samples approached each other with time, and at 140 h, the assayed concentrations overlapped. The triangular data point given in the figure at 140 h represents the PC concentration of the outer solution that is somewhat higher than the concentration observed in the dialysis tubes, but the difference is not statistically significant ($p = 0.05$). From the NMR quantitation, the PC concentrations were found to be 10.6 mM for the outer solution, and the sample containing mucin had an apparent concentration of 9.82 mM.

The results from the dialysis experiment involving samples with a fixed TC/PC ratio with varying concentration

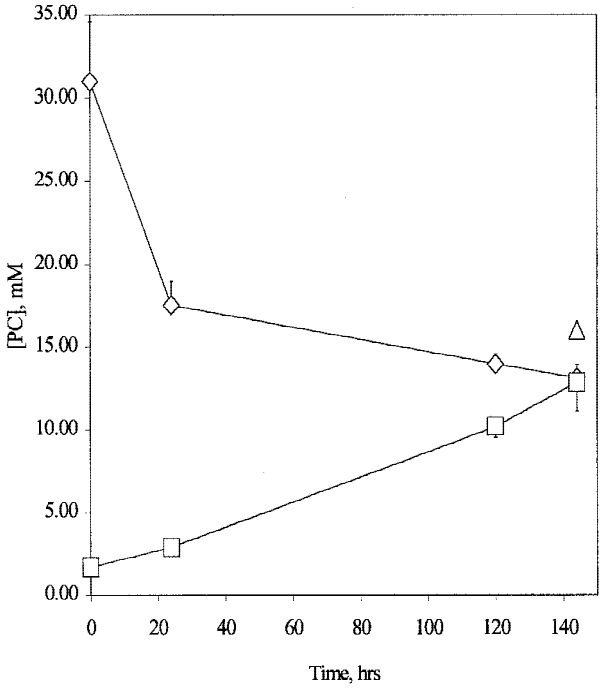

Fig. 3. Time-dependence experiment with egg PC concentration, mM, (mean ± SD, $n = 3$) given as a function of time for two samples containing 0.5% BSM, one initially loaded with PC/TC (◇) and one without PC/TC (□). The triangular data point represents the assayed concentration of the outer dialyzing solution.

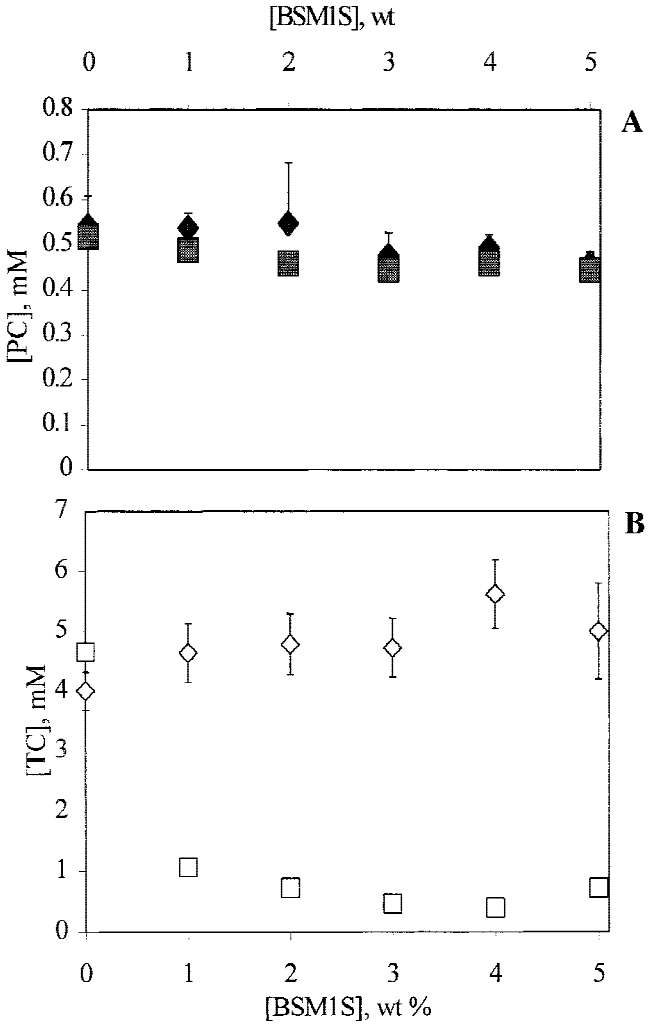

Fig. 4. (A) Egg PC concentration determined by phosphorus assay (◆, mean ± SD, $n = 3$) and by NMR (■), and (B) TC concentration determined by HPLC (◇, mean ± SD, $n = 3$) and by NMR (□) given as a function of BSM concentration.

of BSM are given in Figs. 4A and B for a low PC and high BSM concentration, and in Fig. 5 for a high PC and low BSM concentration. In Fig. 4A, the observed concentrations of PC determined by spectrophotometric measurement of elemental phosphate and by NMR are given for the samples containing BSM concentrations of 0–5%. The results for the sample with 0 wt% concentration of mucin were obtained from the outer dialyzing solution. With the phosphorus assay, the concentration of PC appears to fall with increasing BSM concentration, but the concentration of PC at 5% BSM is not statistically different ($p = 0.05$) from the sample without mucin. Similar results are observed from the NMR quantitation as well.

The results from the quantitation of TC in the same samples are given in Fig. 4B. From the HPLC analysis, it appears there is a slight increase in the concentration of TC as the concentration of BSM is increased. In contrast, the inferred concentration of TC by NMR decreases dramatically as the BSM concentration is increased from 0 to 1% and remains low at higher BSM concentrations. Although the

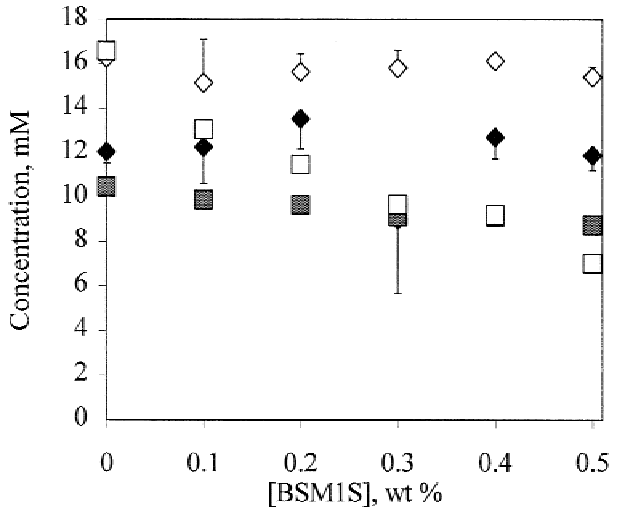

Fig. 5. Egg PC concentration determined by phosphorus assay (♦, mean ± SD, $n = 3$) and by NMR (■) and TC concentration determined by HPLC (◇, mean ± SD, $n = 3$) and by NMR (□) given as a function of BSM concentration.

NMR quantitation validation given above showed a similar discrepancy between the observed and expected concentration ratio for both PC and TC, there is only a discrepancy in the observed and expected concentration ratio with TC in samples containing mucin.

In the dialysis experiment where high PC concentrations (ca. 12 mM) are dialyzed with mucin, the concentration of PC as a function of BSM did not appear to change (Fig. 5). This was true for both the phosphorus assay and NMR method, although the values obtained with the latter method appear to decrease slightly with increasing BSM concentration. The results from the quantitation of TC are also given in Fig. 5, and no statistical effect of BSM on TC was observed with HPLC. However, there was again a decrease in the apparent concentration of TC as the BSM increased as determined by NMR.

The results from equilibrating variable concentrations of PC with a fixed concentration of BSM are given in Table 1. Four different PC concentrations were used, and the data are given under the headings of sample 1, 2, 4, and 5. The data under the heading of sample 3 are derived from the experiment of high PC concentration described above and are provided for comparison purposes. In each case, the results obtained with 0.5% BSM are paired with the control that consisted of a saline solution without mucin. For the phosphorus assay of PC and HPLC of TC, mucin had no effect on the concentration of these components. Comparison of the NMR quantitation between samples with and without mucin indicates that mucin causes a 10–20% reduction in the apparent concentration of PC and a 40–60% reduction in the apparent concentration TC.

The diffusion coefficients measured for water are given in Table 1 as well as in Fig. 6. In the table, it is noted that as the concentration of PC/TC is increased there is a reduction in the observed diffusion coefficient of water. In addition, a comparison of the samples with and without mucin indicates that mucin causes a small reduction in the diffusion coefficient of water as well. This latter point is further emphasized in Fig. 6 where the observed diffusion coefficient of water is given as a function of mucin for all of the samples in this study. It is seen from the figure that increasing the mucin concentration causes a progressive reduction in the observed diffusion coefficient of water.

Returning to Table 1, the data for the diffusion coefficients of TC and PC are given. In the control samples, the hydrodynamic radius of the micelles was 31 Å, which was found by equating the diffusion coefficient of PC to the micelle diffusivity and using the Stokes Einstein equation with the solution temperature of 298 K and viscosity of 0.01 P. The reported hydrodynamic radius for an IMC of 5 mM is 40 Å (26–28). For the variable mucin experimental control, the outer solution had a micellar PC/TC concentration ratio of 1.05 with a corresponding IMC value of 5.9 mM, which is close to the expected ratio of 0.8 for this IMC. The hydrodynamic radius was 26 Å as calculated from the observed diffusion coefficient of PC of 8.44×10^{-7} cm^2/s. Both of these values compare well with the literature, particularly because there is a large change in the micellar size with a small change in the IMC in this concentration range (24).

For PC, the presence of mucin caused a decrease in the apparent diffusion coefficient by about 15%. For TC, the intensity plotted as a function of the square of the field gradient yielded linear plots. The interesting observation is that mucin

Table 1. Concentrations and diffusion coefficients from paired samples with and without mucin. Values are reported as the mean ± SD, n = 3 when replicates were available. The diffusion coefficients are reported with the standard error of the fit.

		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
[PC], Phosphorus assay	Control	8.45 ± 0.44	10.4 ± 0.56	12.0 ± 0.5	15.0 ± 0.29	20.1 ± 0.91
	0.5% BSM1S	5.18 ± 0.007	9.9 ± 1.8	12.7 ± 1.0	15.2 ± 0.67	20.4 ± 0.63
[TC], hplc	Control	10.8 ± 0.3	14.2 ± 0.43	16.2 ± 0.6	17.9 ± 1.1	24.4 ± 0.4
	0.5% BSM1S	10.3 ± 0.3	16.2 ± 1.6	15.4 ± 0.5	17.9 ± 2.2	21.4 ± 1.2
[PC], NMR	Control	7	11	10	14	20
	0.5% BSM1S	6.2	9.3	8.7	12	18
[TC], NMR	Control	12	16	17	20	25
	0.5% BSM1S	5	7.4	7	9.9	14
Water Diffusion × 10^{-6} cm^2/s	Control	22.46 ± 0.39	22.35 ± 0.22	22.94 ± 0.31	21.65 ± 0.50	20.41 ± 0.60
	0.5% BSM1S	21.65 ± 0.52	21.47 ± 0.50	21.97 ± 0.35	20.69 ± 0.57	19.70 ± 0.67
TC Diffusion × 10^{-6} cm^2/s	Control	2.48 ± 0.052	2.09 ± 0.031	2.56 ± 0.040	1.78 ± 0.032	1.53 ± 0.020
	0.5% BSM1S	3.21 ± 0.014	2.22 ± 0.080	2.80 ± 0.060	1.95 ± 0.070	2.00 ± 0.070
PC Diffusion × 10^{-6} cm^2/s	Control	0.70 ± 0.007	0.82 ± 0.028	0.74 ± 0.023	0.76 ± 0.009	0.75 ± 0.025
	0.5% BSM1S	0.59 ± 0.058	0.68 ± 0.019	0.73 ± 0.015	0.66 ± 0.011	0.65 ± 0.013

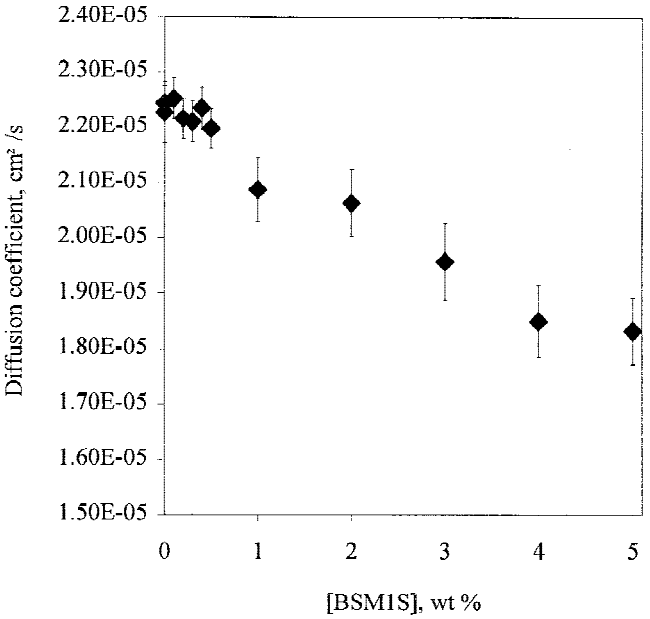

Fig. 6. Observed water diffusion coefficient given as a function of BSM concentration for all experiments. Error bars represent the fitting error from the linear regression of the transformed data (see experimental section for details).

caused an increase in the apparent diffusivity of TC from 5 to more than 50%. However, the proper interpretation of the decay curves must take into account the loss of intensity arising from exchange with sites that have different relaxation times.

DISCUSSION

The goal of this work was to begin to characterize the interaction of lipids with mucin as a prelude to obtaining a quantitative description of the transport of bile salt micelles through the intestinal mucous layer. Understanding the transport of bile salt micelles in turn is fundamental for a complete description of the absorption of ingested fat as well as poorly water-soluble drugs. The approach involved the use of dialysis experiments that provide a quantitative means to determine the effect of mucin on the thermodynamic distribution of each dialyzable component. In addition, NMR quantitation and diffusion measurements were made to provide insight into the dynamic properties of the micelle in the presence of mucin.

A relatively simple system was used to model the complex environment of the intestine. A single bile salt, TC, was chosen, which has a high critical micelle concentration (cmc) and is expected to remain ionized over the entire range of concentrations and conditions used in this study. Egg PC was chosen because the phase diagram with NaTC was reported and then used to select the appropriate concentrations to generate the desired phase to equilibrate with mucin. More importantly, the headgroup and fatty acid compositions of egg PC are very similar to that of human intestinal bile (26). As indicated in the Results section, there is good agreement between the PC/TC ratio and the IMC of this study and those reported in the literature (17,26). Finally, BSM was chosen as the model mucin because it is commercially available and has a proposed structure similar to intestinal mucin. To facilitate analysis by NMR and provide more manageable samples, most of the data were collected with a BSM concentration of 0.5%. This is about one-tenth the concentration found in vivo; however, some data were also collected at concentrations up to 5%.

Time Dependence

The time dependence study indicates that equilibration is attained after a fairly lengthy period of time. The NMR spectra and TLC did not show evidence of the presence of lyso-PC that would be a result of hydrolysis of the acyl chain of PC. The HPLC of TC also did not show any evidence of decomposition. The equilibration time is reasonable given the elongated shape of the micelle with an anhydrous 27 Å radius (26) that must pass through an estimated pore (52 Å radius) of the dialysis tubing. The results are also consistent with the work published by Higuchi and co-workers (18), who found long equilibration times with large, concentrated micellar systems. In the early experiments, a 1000 MWCO dialysis membrane was used which slowly leaked PC over time (18). In some experiments, a 500 MWCO dialysis membrane was used. Such a membrane allowed passage of TC but generally did not leak PC over 7 days of equilibration, if properly tied with multiple wrappings of surgical thread.

NMR Quantitation

The quantitation of PC and TC by NMR was found to be excellent for the PC/TC systems containing relatively small micelles. However, as expected, it tended to fail in the PC/TC systems where the micelles are larger and have enhanced transverse relaxation (27). This relaxation, also known as T_2 or spin-spin relaxation, arises from a loss of coherence among the magnetic nuclei and is observable in the spectra as a broadening of the peaks. Enhanced relaxation occurs due to more efficient exchange of magnetization among the nuclear spins that increases with a loss in the rate and/or amplitude of motion (27). Thus, as the composition of the PC/TC system is moved from the small, spherical micelles characterized by low PC and high TC concentrations to elongated cylindrical, mixed micelles characterized by high PC and low TC concentrations, there is a loss in intensity and the quantitation fails.

This failure of quantitation is particularly pronounced when the mixed micelle to vesicle phase change occurs, and the peaks arising from the headgroup protons are completely lost. It perhaps should be emphasized that this loss of quantitation is an artifact of the NMR spectrometer, and the peaks could be recovered in the spectra should the measurements be made on an instrument designed for detecting slow-moving protons. Nevertheless, it was the intent to use this change in the integrated area as a measure of a change in mobility of the protons.

The above point is further amplified by the difference in quantitation evident between the resonances from the head group protons and the resonances arising from protons attached to the frame of the bile salt molecule. The headgroup protons have sufficient freedom to undergo rotation, while the protons on the frame of the molecule are fixed. Thus, the protons attached to the cyclopentanophenanthrene ring of the bile salt molecule will only undergo rotation with rotation of the entire molecule. As such, the ratio of the observed

concentration by NMR and the expected concentration is less for the C3 protons of NaTC than that observed for the C22 protons. By a similar argument, the methyl protons of the PC tend to yield better quantitative results as well, because the axes of rotation of the methyl groups tend to lie perpendicular to the long axis of the cylindrical shaped PC molecule.

In our earlier work (28), the diffusion coefficient of PC was found to increase more than the diffusion coefficient of TC in the presence of mucin. This was interpreted as indicating that mucin dramatically affects the mobility of lipids contained within the glycoprotein network. The present study confirms this work as well as extends the findings by quantifying the extent of association. It appears that mucin has little effect on the total concentration of phospholipids and bile salts. Rather, mucin reduces the mobility of TC, and as such, a straightforward interpretation of the diffusion coefficients is not possible. The quantitative differences between the early study and the present study are readily appreciated when the effect of field strength on quantitation is considered.

Equilibrium Dialysis

The concentrations of PC that were used in the dialysis studies were chosen based on the reported composition of PC in the intestine. Dressman and co-workers (29) indicated that PC and TC concentrations of 1.5 and 5 mM would be reasonable in the fasted state. Such a low PC concentration was used and coupled with the use of a high mucin concentration to detect the presence of association. For the fed state, Dressman and co-workers suggested concentrations of 4 and 15 mM for PC and TC, respectively. These correspond well with the seminal work by Carey's laboratory (30) in which the lipid concentrations after humans were fed an oil-rich emulsion were measured. They found average concentrations of PC and TC in the aqueous subfraction of 3.2 and 13.1 mM. The sum of the averages of phospholipid, fatty acid, and monoglyceride was reported to be 15.3 mM. The fatty acids and monoglyceride components act in a similar manner to phospholipids in determining the vesicle to mixed micelle phase boundary (30). Therefore, 15 mM phospholipid was used for the variable mucin experiment. In addition, a wider range of PC concentrations was used for the fixed mucin concentration experiments to address the range of lipid concentrations that have been reported (30).

Beginning with the low PC experiment representative of the fasted state, there appeared to be a slight reduction in the PC, whereas there was a slight increase in the TC concentration as determined by the phosphorus assay and HPLC, respectively. The slight reduction of the PC may be a result of an exclusionary effect of the mucin on the distribution of PC/TC micelle. Under the conditions of additive volumes and equal densities, a 5% BSM solution should exclude 5% of the PC/TC micelles. The actual reduction may be as much as 20%, which is possible if water, bound to mucin, adds to the excluded volume effect of the anhydrous mucin.

Although there is a slight decrease in the PC concentration at high BSM concentration, there appears to be a slight increase in TC concentration in the same samples. To explore the significance, the ratio of PC/TC was examined. In the outer solution, the ratio of total phospholipid to total taurocholate was 0.135, whereas in 5% mucin, the ratio was 0.093. These values are significantly different at 95% confidence. This result implies an association of the taurocholate with mucin. The rationale is that micelles of a particular PC/TC ratio are excluded due to the presence of mucin. This will cause a proportional reduction in the micellar concentration of TC and PC. The IMC between mucin molecules would also be the same, but the contribution to the total should be reduced, again due to the excluded volume effect. The observation that the PC/TC concentration ratio is lower in the presence of mucin clearly indicates a preferential association of TC with mucin, particularly after we accounted for the reductions from the micellar and IMC portions of the TC concentration. Although an increase is demonstrated, the magnitude of the increase in the TC concentration is not overwhelming.

The NMR data reveal some interesting aspects of the mobility of the components. As the concentration of mucin was increased, the apparent concentration of PC was reduced slightly, but that of TC was dramatically reduced. From the quantitation experiment, it is expected that TC should be slightly more susceptible to motional changes of the molecule. However, the magnitude of the differences in quantitation of TC and PC cannot be explained solely by differences in the molecular-specific response. Therefore, it appears that mucin causes a significant decrease in the mobility of the TC molecule. At this time it can not be quantified because the contributions to the transverse relaxation time are many. The possible explanations for the reduction in mobility of TC by mucin include hydrogen bonding between TC and mucin as well as a specific association to the positively charged sites of mucin interacting with the negatively charged TC.

The diffusion coefficients can be used to provide additional insight into the mobility of the components. For water, the diffusivity decreased by about 18% as the mucin increased to 5%. In the absence of micelles and with an estimate of the density, the hydration layer thickness of mucin could be approximated (28). However, the binding of TC to mucin complicates the picture, and it is not possible to locate the water because the presence of the IMC TC, micelles, and mucin will all affect the diffusivity of water.

The diffusion coefficient of PC decreased by 75% in a 4% mucin solution relative to the control. However, the decay profiles used in fitting to obtain the diffusion coefficients were curved, suggesting that the micelles are present in multiple domains that are in slow exchange and give rise to different diffusivities. Nevertheless, it is clear that mucin has the power to restrict significantly the mobility of these relatively large, elongated micelles. If this occurs within the intestine, the absorption rate of these components may be reduced. There is a noteworthy consequence of the bile salt mixed micelle in the intestine. It is known that the bile salt concentration increases with time and then decreases fairly rapidly (24,30). An increasing bile salt concentration would cause the reduction in the size of the lipid aggregate, and then when the concentration decreases, micelles would grow in size. If the micelles are within the mucous layer as the concentration of bile salt falls, the large mixed micelles would become trapped within the mucus network.

The diffusion coefficients for the TC are also interesting because there was no trend with changes in the mucin concentration. It was expected that binding of the TC to the slow diffusing mucin should lead to a significant reduction in the diffusion coefficient. However, it must be recognized that the

integrated area of TC decreased by about 80%. Thus, it appears that TC exists in a number of sites, of which one or more have much shorter transverse relaxation times. This exchange affects the linewidth of the spectrum as well as the integrated area during the diffusion measurement. For a rigorous analysis, the fitted diffusivities must be corrected for simultaneous exchange, but this is not possible without specific knowledge of the exchange rate constant (27). The results for PC are not as susceptible to this problem because there was little change in the observed intensity.

For the experiments representing systems that model the fed state of the intestine, the PC concentration was varied from 10 to 30 mM while maintaining the IMC fixed near 5 mM. There was no statistically significant effect of mucin on the concentration of PC determined by the phosphorus assay or on the concentration of TC determined by HPLC. The highest mucin concentration in these studies was 0.5%.

As noted above, the [PC] and [TC] determined by NMR was low. Clearly there was an effect from the mucin on the dynamics of TC and PC that resulted in broadening of these resonances. At the lower mucin concentrations, the decays obtained from the diffusion measurements were linear. Beginning with the water, there was a clear decrease in the diffusion coefficient with an increase in mucin concentration. In addition, there was a decrease in the diffusivity of water as the micelle concentration increased as well (cf. Table 1). In the presence of mucin, the diffusion coefficient of PC was consistently lower, ranging between 82% and 99% of the control value. While significant, it is a fairly modest contribution.

As described above, the TC diffusivities observed in this experiment are also difficult to explain due to the simultaneous diffusion with exchange. However, there are a number of significant aspects. Perhaps of greatest interest is the application of these results to the absorption of fat and more importantly to the absorption of poorly water-soluble drugs. For those samples that contain only bile salt and phospholipid, the diffusivity of NaTC decreases with increasing concentration, and the observed diffusion coefficient is always higher than that observed for the phospholipid. The major contribution to the change in the diffusion coefficient is that as the concentration of phospholipid increases, a greater fraction of the bile salt is associated with the micelle and a smaller fraction is free in solution (25).

Practical Implication

There is an important practical implication of bile salt results because of the similarity with solubilized drug that would presumably also be in exchange between the aqueous and micelle phase (25). That is, the presence of phospholipids leads to a reduction in the diffusion coefficient of the bile salt, but also a dramatic increase in the concentration of bile salt due to formation of mixed micelles. For example, the bile salt concentration increases from 10.8 mM to more than 24 mM or about a factor of 2.5 when the concentration of phospholipid increases from about 8 to 20 mM. With this change in phospholipid concentration, the diffusion coefficient decreases from 2.48 to 1.53 or roughly to 60% of its original value. Because the resistance of the layer is proportional to the product of the distribution coefficient and diffusion coefficient, the concentration of the phospholipids would cause a theoretical decrease in the resistance (or equivalently increase the permeability) by the product of (2.5×0.6) or a factor of 1.5. Such effects are not directly evident in self-diffusion studies, but can be assessed by combining the results from dialysis and diffusion. Although mucus, in and of itself, does not appear capable of significantly affecting the diffusion of small molecular weight solutes, the presence of lipid within the mucous layer will significantly alter the transport of solutes. Thus, the rationale that the correlation between transport and hydrophobicity observed in an earlier study (12,13) lies in the presence of lipids in the mucous layer has been corroborated.

For this study, it can be concluded that with the stipulation that the chemical activity is adequately accounted for, the presence of phospholipids in the mucus layer causes a reduction in the resistance of transport of bile salts. This can also be true for drugs, provided they possess a similar association with the phospholipids.

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