Distribution and Diffusion of Sodium Taurocholate and Egg Phosphatidylcholine Aggregates in Rat Intestinal Mucin

Timothy Scott Wiedmann,[1,4] Heather Herrington[1] Cinthia Deye,[1] and Deborah Kallick[2,3]

Received April 23, 2001; accepted July 30, 2001

Purpose. The permeability of rat intestinal mucin (RIM) to sodium taurocholate/egg phosphatidylcholine (TC/PC)-mixed micelles has been investigated.

Methods. The time dependence for the equilibration of TC/PC-mixed lipid micelles with isolated RIM was determined. Thereafter the distribution of TC/PC-mixed lipid micelles was assessed at low and high PC and intermicellar concentrations (IMC) and with different RIM concentrations. The equilibrium distribution of PC and TC was determined by analysis for phosphorus and by high-performance liquid chromatography, respectively, as well as by nuclear magnetic resonance spectroscopy. In addition, the diffusion coefficients of water, PC, and TC were measured by pulsed field gradient nuclear magnetic resonance spectroscopy. Two model solutes, phenylmethyltrimethylsilane (PTMS) and tetramethylsilyl-tetradeutero-proprionic acid (TSP), were added to the high PC, low IMC samples, and the diffusion coefficients were determined.

Results. The time to reach equilibrium was 2 days for a system with a high intermicellar concentration of sodium taurocholate. At low PC concentrations, RIM had slightly higher PC concentrations relative to the control. In contrast, at high PC concentrations, RIM samples had lower PC concentrations. The concentration of TC was largely independent of mucin concentration. The water diffusivity was reduced proportionately to the concentration of RIM, and analysis indicated that about 150 g of water moved as a kinetic unit with each gram of mucin. The diffusion coefficients of PC were also reduced with increasing RIM concentration. The magnetization decay of TC did not always follow a monoexponential decay, reflecting the simultaneous diffusion and exchange among sites imparting different relaxation behavior on the TC. Magnetization decay curves were simulated and the diffusivity of TC in mucin was estimated. The diffusion coefficient of TSP was 10 times larger than that of PTMS in the presence of micelles and mucin.

Conclusions. RIM is highly hydrated, and dilute solutions have a minor exclusive effect on the high concentration of PC/TC micelles. At low concentrations of PC, there appears to be preferential association of the PC with the RIM. The permeability of mucin to solutes in the presence of bile salt mixed micelles critically depends on the degree of association of the solute with the micelle.

KEY WORDS: bile salt; phosphatidylcholine; intestinal mucin; diffusion; sodium taurocholate.

[1] Department of Pharmaceutics, University of Minnesota, 308 Harvard St., SE, Minneapolis, Minnesota 55455.

[2] Department of Medicinal Chemistry, University of Minnesota, 308 Harvard St., SE, Minneapolis, Minnesota 55455.

[3] Current Address: Incyte Genomics, 3160 Porter Drive, Palo Alto, California 94304.

[4] To whom correspondence should be addressed. (e-mail: wiedm001@tc.umn.edu

INTRODUCTION

The interaction of bile salt with phospholipids has an essential role in the absorption of fat from the gastrointestinal tract (1–3). Moreover, this function of the bile salts/phospholipids is largely understood within the context of the phospholipid/bile salt/saline ternary phase diagram (3). Specifically, various types of aggregates are formed by compositions in the aqueous rich corner of the phase diagram, including vesicles, simple micelles, mixed micelles, and hexagonal shaped rods (HI phase). These phases, as well as the transitions between these phases, are important for the absorption of fat (1,2).

The current paradigm for the bile-assisted absorption of fat is as follows (1,2). Bile secreted by the gall bladder interacts with emulsified ingested fat within the intestinal lumen. Coincident with the action of enzymes that degrade triglycerides and phospholipids, mixed micelles are formed from the bile salts and the metabolic products of fatty acids, 2-monoglycerides, and lysophosphatidylcholine. These mixed micelles are small and thereby sufficiently mobile to diffuse from the turbulent bulk region of the lumen, through the progressively quiescent mucous layer, and ultimately to the epithelial surface. Reaching the surface, there is rapid exchange of the lipid between the micelle and epithelial surface.

For absorption of poorly water-solubile drugs, the various phases of bile salt/phospholipid must be considered (4–9). Micelles composed of only bile salt molecules, known typically as simple micelles, are small and have been shown to diffuse rapidly within the mucin (10,11). Moreover, a number of studies have also demonstrated that bile salt solubilized solutes are readily absorbed (12–14). However, simple micelles are poor solubilizers and therefore provide a rapid transport mechanism of low capacity. In contrast, the combination of bile salts with phospholipids causes the formation of mixed micelles that can be considerably larger than simple micelles. In fact, the size of the mixed micelle is related to the phospholipid/bile salt ratio within the micelle in a predictable manner (3). Studies have shown that an increase in the solubilization power is obtained with an increase in the ratio of micellar phospholipid to bile salt (4–9). However, the increase in size has the negative consequence of reducing the diffusivity of the micelle. Thus, the mixed micelle provides a slow transport mechanism of high capacity (4,5). As such, in and of themselves, neither simple micelles nor the mixed micelles offer an efficient mechanism for transport.

An added complexity is the structural environment presented by the mucous layer in the intestine (11). This glycoprotein-rich network has the potential of dramatically reducing the diffusivity of large aggregates. There is also the possibility of exclusion of the negatively charged micelle by this negatively charged network. The exclusive effect will also depend on the extent of hydration of mucin. These factors can also adversely impact the transport of fat and poorly water-soluble drugs to the epithelial surface. Although many studies have been conducted that involve the transport of micelle-solubilized drugs by *in vivo, in situ,* and *in vitro* approaches, the many nuances of this problem have thwarted efforts at presenting a detailed paradigm that accounts for the experimental results.

In this study, the exclusion by/association to an intestinal

mucin of bile salt/phospholipid mixed micelles, as well as the diffusivity of aggregates and model compounds within the network, were investigated. The ultimate goal was to provide the foundation for the absorption of ingested fat and poorly water-soluble drugs by the intestine. Our specific hypothesis was that isolated rat intestinal mucin affects the distribution and diffusivity, and hence the permeation, of bile salt/phospholipid micelles. To address this question, a dialysis technique was used. In this approach, the association/exclusion of bile salt micelles, along with the diffusion coefficient of the bile salt, water, phospholipid, and added solutes, were measured (11).

THEORY

The observed diffusion coefficient of water in the presence of mucin is a result of a rapid exchange between free water and that associated with mucin (15). The observed diffusion coefficient, $D_{obs,w}$, is given as

$$D_{obs,w} = D_{w,f}(1 - F_{b,t}) + \Sigma_j D_{b,j} F_{b,j} \quad (1)$$

where $D_{w,f}$ is the diffusion coefficient of free water in the sample, and F_b is the summed weight fractions of all bound water, $F_{b,j}$ is the weight fraction of bound water at the j^{th} site, and $D_{b,j}$ is the diffusion coefficient of bound water at the j^{th} site. Because the diffusion coefficient of the bound water is much less than that of the free and the free fraction is larger than the bound fraction, the second term may be dropped. The diffusion coefficient of unbound water in the presence of obstacles may be related to the free aqueous diffusion coefficient by introducing a correction factor that is given as a function of the excluded volume, f(Φ) (16). The functional relationship was taken to be 1.5 Φ. Thus, the expression may be written as

$$D_{obs,w} = D^{\circ}_{w,f}(1 - F_{b,t})(1 - 1.5\Phi) \quad (2)$$

where the volume fraction is a function of the concentration, c_i in g/mL, and specific volume, v_i in L/g, of each component representing an obstacle and the bound water. That is,

$$\Phi = c_m v_m + c_g v_g + c_w v_w F_{b,t} \quad (3)$$

The subscripts m, g, and w refer to the micelles, glycoprotein mucin, and water, respectively. The grams of bound water/gram of mucin or micelle, N_g or N_m, is defined in terms of the fraction of bound water as

$$F_{b,t} = (N_g c_g + N_m c_m)/c_w \quad (4)$$

The expressions for the fraction bound and volume fraction of obstacles may be substituted into the expression for the observed diffusion coefficient. The rather lengthy algebraic expression may be simplified by neglecting the terms dependent on the product of c_m and c_g as being small and retaining only those terms that depend on c_g. The expression is

$$D_{obs,w}/D^{\circ}_{w,f} = 1 - [1.5v_g + 1.5v_w N_g + (N_g/c_w)]c_g + [(1.5\, v_w N_g^2 + 1.5 v_g N_g)/c_w]c_g^2 \quad (5)$$

This indicates that a plot of the relative diffusion coefficient as a function of the mucin concentration should decrease in a nonlinear fashion with the fitted coefficients proportional to the grams of bound water/gram of mucin.

EXPERIMENTAL

Materials

Sodium taurocholate and 11α-hydroxyprogesterone were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium trimethylsilyl-tetradeutero-propionate (TSP) and phenylmethyltrimethylsilane (PTMS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL). Rat intestinal mucin (RIM) was isolated from male Sprague–Dawley rats (250–275 g) and characterized as previously described (17). Water was deionized and then distilled. Methanol used in the high-pressure liquid chromatography (HPLC) was HPLC grade, and the cyclohexane was spectroscopic grade. All other chemicals were reagent/analytical grade or better.

Methods

Sample Preparation

Samples containing phosphatidylcholine (PC) and sodium taurocholate (TC) were prepared by first lyophilizing overnight under high vacuum an ethanol/cyclohexane solution of egg phosphatidylcholine (16). The dried PC was then reconstituted with a concentrated sodium taurocholate solution prepared in saline with 0.1 % sodium azide.

Time Dependence

The time required for bile salt/phospholipid micelles to equilibrate with RIM was determined with two different PC/TC concentrations as follows. Two 0.1% RIM solutions were prepared; one containing about twice the expected equilibrium concentrations of TC/PC and one not containing any TC/PC. These solutions were placed in separate, washed Spectra/Por dialysis membranes (12-mm flat width) with a molecular weight cut off of 300,000 daltons (Spectrum laboratories, Inc, Gardena, CA 90248-2904). The bags were tied with surgical thread and then placed into a tissue culture flask containing the outer solution of TC and PC without mucin. In addition, an intermicellar concentration (IMC) of bile salt was added to a dialysis membrane (Spectra/Por) with a MWCO of 500 daltons and placed in the same tissue culture flask. The flask was oscillated on a shaking water bath at room temperature. Samples were taken from the mucin solutions initially and periodically thereafter for 7 days. The samples were assayed for phosphatidylcholine by a spectrophotometric assay for elemental phosphorus (18), and thin-layer chromatography was performed to check for the presence of lyso-phosphatidylcholine using a mobile phase of 65:35:5 chloroform:methanol:water.

Equilibration Studies

Equilibrium dialysis studies were conducted as previously described (16). Briefly, an outer PC/TC solution was placed into a large tissue culture flask along with an IMC solution contained within a dialysis tube with a 500 MWCO. Five tied dialysis tubes (300,000 MWCO), which contained concentrations of PC and TC equivalent to the outer solution as well as different mucin concentrations, were placed in the

flask. The flask was shaken for 7 days, after which samples were taken and assayed for elemental phosphorus spectrophotometrically (18) and for TC by HPLC (10). Nuclear magnetic resonance (NMR) spectroscopy was used to measure the diffusion coefficients and for quantifying PC and TC (10).

Quantitative NMR

The procedure for NMR has been described previously (10). Briefly, samples were placed in NMR tubes with inserts containing TSP in D_2O. The measurements were made on a Varian 500 MHz spectrometer with the temperature controlled at 25 ± 0.2°C. Spectra were obtained using a single 90° pulse preceded by a 10-s saturation delay to suppress the water signal. The areas for the TC and PC resonances were expressed as a ratio of the area determined for the internal standard, TSP.

Diffusion Measurements

The Fourier-transform pulsed-field gradient spin-echo ^{1}H NMR diffusion coefficients of water were measured using a stimulated spin-echo pulse sequence. For the bile salt and phospholipids, the same sequence was used, but it was preceded by a water suppression pulse. The resulting transformed area under the peak was analyzed on line by the following linearized equation (15):

$$\mathrm{Ln}[A(\tau_1 + \tau_2)] = \text{constant} + \{-(\gamma G\delta)^2 D(\Delta - \delta/3)\} \qquad (6)$$

where $\mathrm{Ln}[A(\tau_1 + \tau_2)]$ is the natural logarithm peak area at the time of the echo, $\tau_1 + \tau_2$, γ is the gyromagnetic ratio, G is the strength of 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.

The diffusion experiments were performed at constant Δ and δ values, and a series of 10 or more G values were used ranging from 10 to 65 G/cm. The gradient pulse length was 1 ms, and the diffusion time was 80 ms for water and TSP, 120 ms for the bile salt, and 480 ms for PC and PTMS. Absolute values of the self-diffusion coefficients were obtained by calibrating the field gradient strength (10).

Water Diffusion Analysis

The diffusion coefficients in the presence of mucin were expressed as a ratio with respect to the solution without mucin and were plotted as a function of the weight percent of mucin. The data were fit (Kaliedograph™) by a quadratic function and the linear term was used with equation 5 to estimate the grams of water bound/gram of mucin. The specific volume of anhydrous mucin was taken to be 0.74 ml/g (16).

PC Diffusion Analysis

The hydrodynamic radius of the micelle, R_h, was calculated by assuming that all of the PC resides within the micelle and that Stokes–Einstein equation applies. That is,

$$R_h = kT/6\pi\eta D \qquad (7)$$

where D is the diffusion coefficient of PC, and η is the viscosity of saline at 25°C with a value of 0.01 poise.

TC Diffusion Analysis

The observed magnetization of TC is influenced by diffusion as well as exchange between sites with different relaxation properties (10). To analyze the data, the TC was assumed to follow a two-site model with sites A and B, which are characterized by diffusion coefficients, D_i, initial magnetization, M_o, and rate constants for exchange, k_i. The analytical expression is given as (15):

$$M = \left(\frac{Mo}{2} + \frac{\alpha}{\beta}\right)\exp[(-\varphi + \Psi)T] + \left(\frac{Mo}{2} - \frac{\alpha}{\beta}\right)\exp[(-\varphi - \beta)T] \qquad (8)$$

where

$$\alpha = \psi[M_{Bo} - M_{Ao}] + M_{Ao}k_A + M_{Bo}k_B$$
$$\psi = (1/2)[k_A - k_B + D_A q^2 - D_B q^2]$$
$$\beta = (1/2)[k_A + k_B + D_A q^2 + D_B q^2]$$
$$\varphi = \sqrt{(\psi + k_A k_B)}$$

and q is equal to the product of the gyromagnetic ratio, field gradient strength and field gradient pulse length and T is the diffusion time. It is noted that $k = k_A/M_{Bo} = k_B/M_{Ao}$. The ratio of M_{Ao} and M_{Bo} define the initial equilibrium distribution, $K_{eq} = M_B/M_A$, and k is the first-order rate constant for the exchange between the sites independent of the distribution.

Using the equation above, the change in magnetization with the gradient parameter can be simulated. In this study, the fraction of TC in site A, X_A, was calculated by dividing the measured IMC by the total TC concentration. The diffusion coefficient of TC in site B, D_B, was equated to the diffusion coefficient of PC, which is taken to be equal to the diffusion coefficient of the micelle (19). Knowledge of the pulse sequence and calibration of the field gradient strength with solutes of known diffusion coefficient allow determination of q and T. Thus, the three free parameters are k, M_o, and D_A, of these, the exchange rate and diffusion coefficient of the monomeric TC are of interest.

From the simulation, the effective diffusivity of TC that determines the permeability of the mucin may be calculated independent of the exchange process as the weighted average of the two sites, that is (16)

$$D = X_A D_A + (1 - X_A)D_B \qquad (9)$$

where X_A is the mole fraction of TC at site A.

RESULTS

For the time dependence experiment, the initial concentrations of PC in the two tubes containing mucin were different with one nearly twice the final equilibrium concentration and the other with no PC. As can be seen in Figure 1 (filled symbols), the concentrations of PC in the mucin solutions approached each other rapidly with time, and after 2 days, no statistically significant difference was observed among the samples. At 188 h, the concentration PC in the outer solution is also provided which was indistinguishable from the samples containing mucin. Similar results are seen for TC, also given in Figure 1 (open symbols).

The equilibrium dialysis experiments were planned as a two factor (PC concentration and IMC), two level statistical design for five different RIM concentrations ranging from

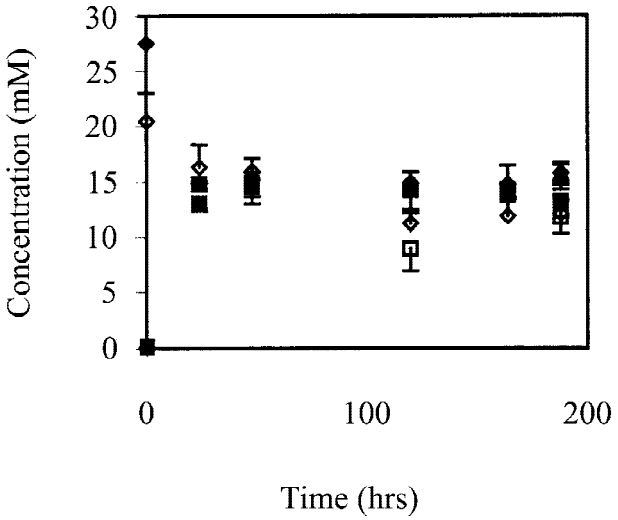

Fig. 1. Time dependence experiment with (♦,■) egg PC concentration and (◇,□) TC concentration (mean ± standard deviation; n = 3) given as a function of time for two samples containing 0.1% rat intestinal mucin, one initially loaded with phosphatidylcholine/sodium taurocholate and one without. The triangular symbols represents the assayed concentrations of (▲) PC and (△) TC of the outer dialyzing solution.

0.02 to 0.1% and a control without mucin. In addition, an experiment was conducted with a series of higher mucin concentrations. Because the samples were dialyzed, it was not possible to obtain the same IMC and concentrations of PC. The observed PC and TC concentrations for the control samples are given in Table I. The low PC concentrations were between 1.1 and 1.2 mM and the high PC concentrations were near 12.5 mM. The IMC was 4 mM or below for one level and near 5.25 for the higher level.

The PC concentration, based on the assay of total phosphorus and relative to the control, is shown in Figure 2. The low PC studies had progressively more PC with increasing RIM concentration. In contrast, the concentration of PC for the high PC samples decreased with increasing RIM concentration. Both of these effects were statistically significant at $P < 0.05$. The TC concentration in mucin samples was not appreciably different from the controls in any of the studies (data not shown). The data of samples with the highest mucin concentration of 0.1 % is shown in Table I for comparison purposes. For the quantitation by NMR, the apparent concentrations of PC and TC fell with increasing concentration of mucin (data not shown).

For the diffusion coefficients of water, the decays of magnetization plotted as a function of the field gradient squared were well-fit ($r^2 \geq 0.999$) by a monoexponential function (equation 6). The diffusion coefficients of water are expressed as a ratio of the control and plotted as a function of RIM concentration in Figure 3. The values measured in the samples from all five equilibration experiments progressively decreased as the RIM concentration increased from 0 to 0.1% for the design study and also for the experiment ranging from 0 to 0.2%. Each data set was independently fit to equation 5, from which the linear term was used to estimate the mass of water bound to mucin. The linear term ranged from 3.2 to 4.0/wt%, and the mean ± standard error of the mean was 3.72 ± 0.33/wt%. This corresponds to 148 g of water bound/gram of mucin.

The PC decays of magnetization as a function of the field gradient squared were well fit to the single exponential function. The calculated diffusion coefficients of PC are given as a function of [RIM] in Figure 4. From the control samples, the diffusion coefficient was used to estimate of the hydrodynamic sizes of the micelle, which are given in Table I. The hydrodynamic radii for the high IMC samples were similar. At an IMC of 3.84 mM, the radius was 60 Å reflecting an increase in the micelle size with decreasing IMC. With increasing RIM concentration, there was a decrease in the observed diffusion coefficient particularly with those samples with large diffusivities (Fig. 4). The slopes were found to be statistically different from zero ($P < 0.05$).

The TC diffusion coefficients measured in aliquots of the IMC solution were between 4.57 and 4.96 × 10^{-6} cm^2/s consistent with the expected diffusivity of monomeric TC (Table II). The decay curves of TC in the presence of PC and PC and mucin were often linear and characteristic of a monoexponential decay, but at times were not. The diffusion coefficients from a single exponential fit are given in Table II. The diffusion coefficients all fell within a relatively narrow range. The range was limited at the upper end by the monomeric TC diffusivity of the IMC sample and on the lower end by the diffusivity of PC.

The data were simulated by allowing the diffusivity of the free TC, the total magnetization, and the exchange rate constant to vary while using the experimentally determined dif-

Table I. Concentrations of Phosphatidylcholine (PC) and Sodium Taurocholate (TC) after the Dialysis Experiments

	PC (mM)		TC (mM)		
IMC[b] (mM)	Control	0.1% RIM	Control	0.1% RIM	Control, Rh, Å
4.01 ± 0.091	1.13 ± 0.096	1.35 ± 0.12[a]	6.71 ± 0.58	6.8 ± 1.4	18.5
5.25 ± 0.45	1.191 ± 0.021	1.349 ± 0.063[a]	7.80 ± 0.73	7.25 ± 0.38	20.6
3.84 ± 0.17	12.68 ± 0.46	12.20 ± 0.59[a]	11.50 ± 0.018	11.23 ± 0.12[a]	60.0
5.21 ± 0.39	12.11 ± 0.16	9.96 ± 0.50[a]	20.48 ± 0.39	19.38 ± 0.90[a]	26.8

[a] Values significantly different than control using paired *t* test with $P = 0.05$.
[b] IMC refers to the intermicellar concentration. The control solution was dialyzed against the solution containing 0.1 % RIM.
(mean ± SD, n = 3).

fusivity of the bound TC and the TC free fraction. With the results from the simulation, the effective diffusivities of TC were calculated by equation (9) and are given in Table III. These values are higher than the diffusivities calculated from a monoexponential fit (Table II), reflecting the fact that the exchange process introduces another pathway for the loss of magnetization giving rise to an overestimation of the diffusivity. Nevertheless, the correction is relatively small as can be seen by comparing the values in Table II and Table III. The weighted average values are sensitive to the free fraction as shown by the lower diffusivities obtained with the samples with a higher concentration of PC. The presence of mucin had no apparent effect on these diffusivities ($P = 0.05$).

In Figure 5, the diffusion coefficients of water, TC, PC, TMS, and PTMS are given for the samples with an IMC of 3.84 mM. The addition of these silyl derivatives did not significantly change the diffusion coefficients of water or PC. The diffusion coefficient of TSP was 6×10^{-6} cm^2/s and decreased with increasing mucin concentration ($P < 0.0005$). The diffusivity of PTMS was about an order of magnitude smaller at about 5×10^{-7} cm^2/s and very close to the diffusivity of PC. It also was significantly reduced with increasing mucin concentrations ($P < 0.05$).

DISCUSSION

In this study, the interaction of bile salt/phospholipid mixed micelles with an intestinal mucin, as well as the diffusivity of these lipid aggregates within the glycoprotein network, was investigated. Our specific hypothesis was that isolated rat intestinal mucin affects the distribution and diffusivity of bile salt/phospholipid micelles. This study provides the individual contributions of the diffusivity and distribution coefficient to the permeability of the mucous layer to TC/PC aggregates.

The choice of the micellar system was guided by the earlier work that involved measurement of the lipid concentrations within the intestine (1,2). In these studies, there is a distinction between the fed and fasted state as the concentration of bile salts and phospholipids are different (5). This led to the use of two levels of PC in the dialysis experiments. However, rather than performing the studies with the most physiologically relevant, complex mixture of bile salts, sodium taurocholate was the sole bile salt used in conjunction with egg phosphatidylcholine. The rationale is that the TC/PC/saline phase diagram is known and thereby micelles of known size and concentration could be prepared for a specific total bile salt/PC ratio and given IMC (3). That is,

$$R_h = \gamma/2\sigma([PC]_{tot}/([TC]_{tot} - IMC)$$

where γ and σ are constants, and the subscript, tot, indicates the total concentration in solution. Finally, this micellar system is also insensitive to pH changes over a large range.

Rat intestinal mucin was chosen as the model system and was isolated with standard procedures. The composition and properties are in excellent agreement with the literature (17). RIM is characterized by a high carbohydrate-to-protein ratio and is particularly rich in the negatively charged carbohydrate, sialic acid. The material as isolated was an inhomogeneous gel with discrete particles indicative of large aggregated or entangled molecules representative of intact intestinal mucin. It is noteworthy that this isolated mucin is distinct from samples prepared from commercially available pig gastric mucin and bovine submaxillary mucin that yield homogeneous solutions (10). Finally, time dependence studies were performed to ensure that equilibrium was attained. Although a relatively short time for equilibration was observed, longer times have been noted with systems that are more concentrated as well as those systems with large micellar aggregates (4,5).

For the most part, mucin had a minor effect on the distribution on PC and TC as determined by the phosphorus assay and by HPLC. In the absence of association of the lipid, mucin should decrease the concentration of other solutes in

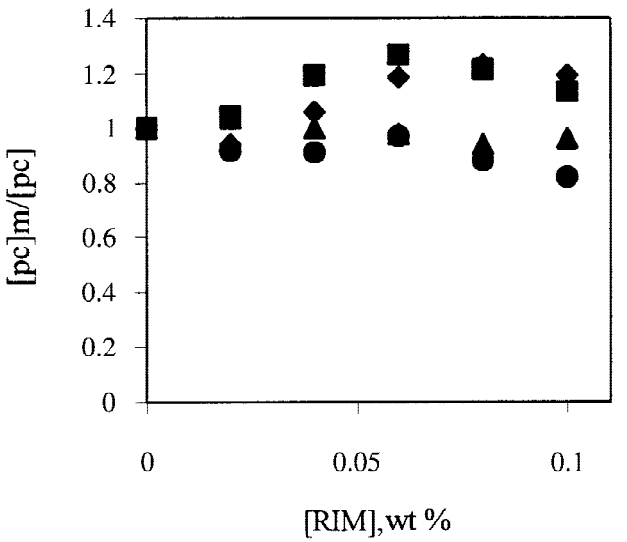

Fig. 2. Egg phosphatidylcholine concentration in samples with mucin relative to the control determined by phosphorus assay for intermicellar concentration values of (♦) 4.01, (■) 5.25, (▲) 3.84, and (●) 5.21 mM. Error bars omitted for purposes of clarity.

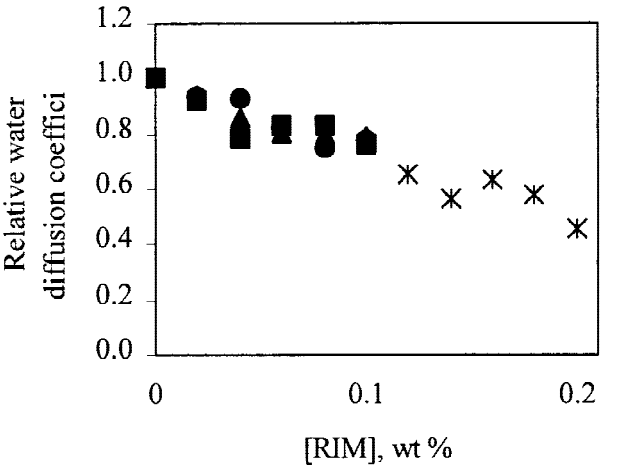

Fig. 3. Water diffusion coefficient relative to the control given as a function of rat intestinal mucin concentration for samples dialyzed against solutions with an intermicellar concentration of (♦) 4.01, (■) 5.25, (▲) 3.84, and (●) 5.21 mM and (∗) the high rat intestinal mucin concentration experiment. The error bars from the fitting error from the linear regression of the transformed data are omitted for clarity but are less than 5%.

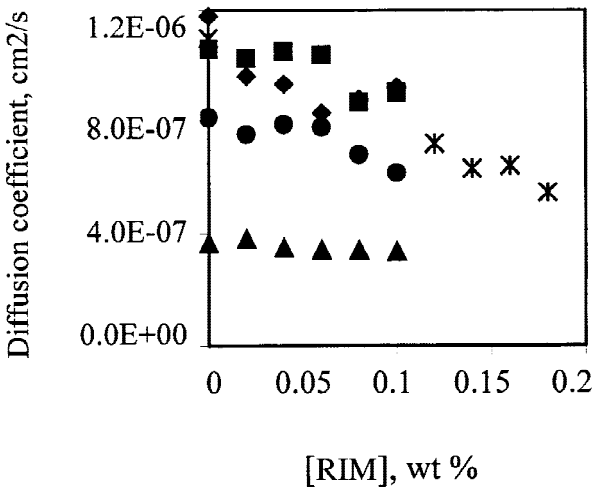

Fig. 4. Diffusion coefficients of egg phosphatidylcholine given as a function of rat intestinal mucin concentration for samples dialyzed against solutions with an intermicellar concentration of (♦) 4.01, (■) 5.25, (▲) 3.84, and (●) 5.21 mM and (∗) the high RIM experiment. The error bars from the fitting error from the linear regression of the transformed data are omitted for clarity but are less than 5%.

proportion to the excluded volume effect (20). With the maximum concentration of RIM used in these studies of 0.2%, the effect was slight but statistically significant and only observed in samples with the higher concentration of PC. With the samples containing a lower concentration of PC, there was a statistically significant preferential association of PC with mucin. The effect was modest, but noteworthy in that it complicates the analysis of the excluded volume effect. Nevertheless, extrapolation to physiological concentrations of 1–5 % indicates that a significant reduction in the concentration of PC would occur at high PC concentrations due to an excluded volume effect.

The water diffusion coefficients were significantly reduced in the presence of mucin. This is consistent with the expectation of a negatively charged, hydrated glycoprotein (11). Winne and Verheyen (21) found that the diffusion of tritium was reduced by about 50% by native rat intestinal mucin. This is comparable to the reduction observed with a

Table II. TC Diffusion Coefficients Listed by the IMC of the Sample and Concentration of Mucin as Obtained from Linear Regression of Transformed Data

Observed TC diffusion coefficients (cm^2/s)				
[RIM], wt %	4.01[a]	5.25	3.84	5.21
IMC	4.71E-06	4.85E-06	4.96E-06	4.57E-06
PC/TC control	3.63E-06	3.56E-06	1.83E-06	1.94E-06
0.02	3.71E-06	3.45E-06	2.16E-06[b]	1.92E-06
0.04	3.88E-06	3.48E-06	1.94E-06[b]	2.52E-06
0.06	3.19E-06	ND[c]	1.99E-06	2.52E-06
0.08	3.57E-06	5.88E-06	1.85E-06	2.73E-06
0.1	3.31E-06	6.27E-06	1.70E-06	2.69E-06

[a] IMC of solution.
[b] Decay curves non-monoexponential.
[c] Not determined.

Table III. TC Diffusion Coefficients Listed by the IMC of the Sample and Concentration of Mucin as Obtained from Calculating the Weighted Average Diffusion Coefficient of the Free and Bound TC as Obtained from Simulated Data

Simulated TC diffusion coefficients (cm^2/s)				
[RIM], wt %	4.01[a]	5.25	3.84	5.21
PC/TC control	4.74E-06	4.25E-06	2.58E-06	2.56E-06
0.02	4.55E-06	4.22E-06	2.97E-06	2.36E-06
0.04	3.88E-06	4.23E-06	3.06E-06	3.17E-06
0.06	3.58E-06	ND[b]	2.88E-06	2.88E-06
0.08	4.2E-06	5.85E-06	2.69E-06	3.05E-06
0.1	3.64E-06	5.9E-06	2.61E-06	2.95E-06

[a] IMC of solution.
[b] Not determined.

0.15% purified mucin in this study. A similar NMR diffusion study of water with bovine cervical mucin failed to find any significant reduction in the diffusion coefficient of water (22). Analysis of the data in terms of association of water with mucin suggests that as much as 150 g of water/g of mucin move at the same kinetic rate. The reported degree of hydration of mucin varies with respect to the type of mucin. Earlier work in our laboratory with bovine submaxillary mucin showed that at 0.25%, there were 30 g of water bound/gram of mucin (11). This decreased to 13 g/g at 1%. Because the protein and carbohydrate compositions are similar among these mucins, these results may indicate that the nature of the association is distinct leading to differences in the binding of water. Alternatively, because water is bound to these mucin, there may be differences in the relaxation behavior, specifically a much shorter traverse relaxation time. Under this condition, there may be simultaneous diffusion and exchange that may complicate the interpretation of the diffusion coefficient.

Along with a reduction in the diffusion coefficients of water, there also was a reduction in the micelle diffusivities in the presence of mucin. As shown in Figure 4, the diffusion coefficients are seen to decrease by over 50% as the mucin

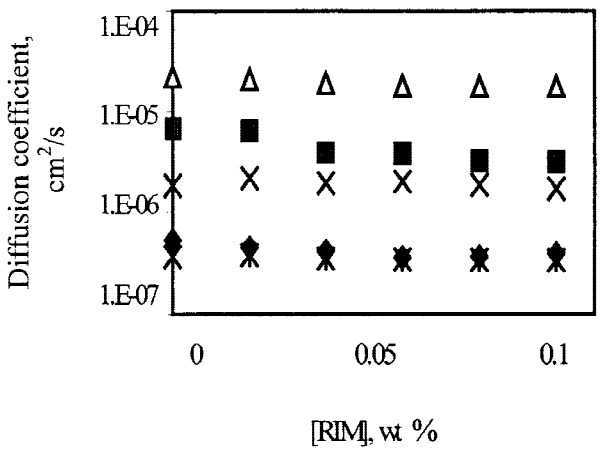

Fig. 5. Diffusion coefficients of (Δ) water, (X) taurocholate, (∗) phosphatidylcholine, (■) sodium trimethylsilyl-tetradeutero-propionate, and (♦) phenylmethyltrimethylsilane given as a function of rat intestinal mucin concentration for samples dialyzed against solutions with an intermicellar concentration.

concentration is increased from 0 to 0.2%. Although there is some scatter, the values tend to decrease in proportional to mucin concentration. This decrease in the diffusion coefficients with an increase in mucin concentration is not significantly greater than that observed with water. Theoretically, larger micelles should have a greater reduction in their diffusivity relative to water due to the excluded volume effect, which is a function of solute size (20). In the present experiment, the exclusive effect of the worm-like coils of mucin on the elongated rod shaped micelles must be considered. Because the experimental results indicate a relatively minor effect, it appears that the relatively large openings expected of the mucin glycoprotein network readily allow the diffusion of the micelles resulting in a small excluded volume effect (15).

The diffusivity of TC is complicated by changes in the relaxation behavior. As previously reported (10), the quantitation of TC fails with larger aggregates as a result of the short transverse relaxation time of these solutes. Although the contributions to this relaxation time are numerous in these systems, they are dependent on the rate and/or amplitude of the motions of the protons being examined. For these samples, it would appear that a reduction in the rotational motion is responsible for the significant decrease in the transverse relaxation time.

One approach to separate the effects of diffusion from the relaxation is to consider that the TC molecule resides at different sites and define an exchange rate constant for the movement of the TC molecule between each class of sites (15). In general, for a diffusion measurement made on a molecule undergoing exchange, the magnetization decay of the solute is a function of the relaxation time and diffusion coefficient at each site as well as the exchange rate constants between the sites (15). An analytical solution for the magnetization decay has been obtained in the case of exchange between two sites, which was shown to be a biexponential function (15). In the case of an exchange rate much faster than the diffusion rate, a single exponential function is obtained and the slope of the magnetization decay is related to the weighted average diffusion coefficient of the two sites.

The decay of the magnetization of TC was simulated with Equation 8. The preferred method would have been to fit the decay of the magnetization to Equation 8 and obtain parameters with the appropriate confidence intervals. However, the magnetization was obtained as a function of the gradient strength. Because the gradient strength appears both in the exponential and preexponential factor, fitting the data was not possible. Alternatively, the data could have been collected as a function of diffusion time rather gradient strength. However, with this process, differences in the spin-lattice relaxation would cause a problem in the interpretation in the magnetization decay. Thus, data were collected by incrementing the gradient strength and the subsequent matching of the simulations to the observed experimental data. By comparing the data with the simulated diffusion coefficients of TC, it is very clear that the correction is relatively small despite the less than optimal method of analysis.

The magnetization decay of phosphatidylcholine was distinct from taurocholate for a number of reasons. First, the protons used in the diffusion analysis are located on rapidly rotating methyls on the head group quaternary amine. Second, most if not all of the PC is restricted to the micelles as the aqueous solubility is vanishingly small. Thus, the decay of the magnetization of PC was described by a monoexponential function.

The addition of the model compounds complements the results obtained with TC. TSP is a water-soluble compound that exists almost exclusively in the free state. In contrast, PTMS is a hydrophobic compound that resides almost exclusively in the micelle (19). These represent the extremes, whereas TC is the intermediate case of being distributed between the micelle and free in solution. Although TSP and PTMS are expected to have similar diffusion coefficients in solution, the diffusivities ranged from that of the micelle to values characteristic a small molecule in aqueous solution. Thus, it is clear that the observed diffusivity is essentially determined by the degree of association with the bile salt/phospholipid micelle. This provides confirmation for the observed correlation between the permeability of mucin and drug hydrophobicity (23,24).

Moreover, in the present study, the contributions to the distribution and diffusion are clearly delineated. In the case of taurocholate, the monomer concentration is on the order of 4 to 5 mM. With the addition of PC, the concentration of TC rises to about 7 mM to over 20 mM depending on the concentration of PC. Thus, if mucin contains lipids, hydrophobic solutes will be preferentially taken into the layer. The presence of lipids also affects the diffusion coefficient of TC. Monomeric TC has a diffusion coefficient of about 5×10^{-6} cm^2/s, but in the presence of micelles and mucin, it was reduced to a low value of 2.56×10^{-6} cm^2/s. The expected permeability may be deduced from the product of the diffusion coefficient and distribution coefficient. In all cases, there would be an increase in the permeability of mucin to TC in the presence of phospholipids, since the increase in the distribution coefficient is more than sufficient to offset the decline in the effective diffusivity. Therefore, the presence of lipids and their aggregation state becomes of paramount importance in determining the permeability of mucin.

In summary, rat intestinal mucin was shown to be highly hydrated, and a quantitative value of the extent of hydration was obtained. There were also some important implications of this study for the intestinal absorption of fat and poorly water-soluble drugs. Evidence of binding of phospholipids when present at low concentrations has been obtained, whereas exclusion dominates at high phospholipid concentration. This indicates that mucin acts to modulate the concentration of lipid that is seen by the epithelial cell surface. With hydrophobic drugs, the effective diffusivity of solutes through the mucin layer will depend on the presence of bile salt micelles. In contrast, the diffusion coefficient of hydrophilic compounds will not be appreciably affected by mucin or the presence of bile salt micelles. Finally, in determining the permeability of the mucous layer, consideration must be given to the distribution when the concentration of lipid in the mucin is distinct from that in the lumen.

ACKNOWLEDGMENTS

We acknowledge the excellent technical support provided by Drs. David Live and Beverly Gaul Ostrowski in the NMR facility of the University of Minnesota. We recognize the support of NIH DK-53419. A portion of this work was presented at the 74th Colloid and Surface Science Symposium, Bethlehem, PA.

REFERENCES

1. J. E. Staggers, O. Hernell, R. J. Stafford, and M. C. Carey. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase analysis and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry* **29**:2028–2040 (1990).
2. O. Hernell, J. E. Staggers, and M. C. Carey. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry* **29**:2041–2056 (1990).
3. D. J. Cabral and D. M. Small. Physical chemistry of bile. In S. G. Schultz, J. G. Forte, and B. B. Rauner (eds.), *Handbook of Physiology-The gastrointestinal system III*, American Physiology Society, Waverly Press, 1989 pp. 621–662.
4. M. Rosoff and A. T. M. Serajuddin. Solubilization of diazepam in bile salts and in sodium cholate-lecithin-water phases. *Int. J. Pharm.* **6**:137–146 (1980).
5. L. J. Naylor, V. Bakatselou, and J. B. Dressman. Comparison of the mechanism of dissolution of hydrocortisone in simple and mixed micelle systems. *Pharm. Res.* **10**:865–870 (1993).
6. W. I. Higuchi, C. C. Su, N. Daabis, A. Wanichsiriroj, and A. F. Hofmann. Mechanism of cholesterol monohydrate dissolution in taurocholate-lecithin media-correlation between equilibrium dialysis results and dissolution rates. *J. Colloid Interface Sci.* **98**:9-19 (1984).
7. W. I. Higuchi, M. Arakawa, P. H. Lee, and S. Noro. Simple micelle-mixed micelle coexistence equilibria for the taurocholate-, taurochenodeoxycholate-lecithin systems. *J. Colloid Interface Sci.* **119**:30–37 (1987).
8. C-Y. Li, C. L. Zimmerman, and T. S. Wiedmann. Solubilization of retinoids by bile salt-phospholipid aggregates. *Pharm. Res.* **13**: 907–913 (1996).
9. X. Cai, D. J. W. Grant, and T. S. Wiedmann. Analysis of the solubilization of steroids by bile salt micelles. *J. Pharm. Sci.* **86**: 372–377 (1997).
10. T. S. Wiedmann, C. Deye, and D. Kallick. The interaction of sodium taurocholate and phospholipids with bovine submaxillary mucin. *Pharm. Res.* **18**:45–53 (2001).
11. C-Y. Li, C. L. Zimmerman, and T. S. Wiedmann. Diffusivity of bile salt/phospholipid aggregates in mucin. *Pharm. Res.* **13**:535–541 (1996).
12. V. L. Sallee, F. A. Wilson, and J. M. Dietschy. Determination of unidirectional uptake rates for lipids across the intestinal brush border. *J. Lipid Res.* **13**:184–192 (1973).
13. H. Westergaard and J. M. Dietschy. The mechanism whereby bile acid micelles increase the rate of fatty acid and cholesterol uptake into the intestinal mucosal cell. *J. Clin. Invest.* **58**:97–108 (1976).
14. F. G. J. Poelma, R. Breas, J. J. Tukker, and D. J. A. Crommelin. Intestinal absorption of drugs. The influence of mixed micelles on the disappearance kinetics of drugs from the small intestine of the rat. *J. Pharm. Pharmcol.* **43**:317–324 (1991).
15. C. S. Johnson Jr. Diffusion ordered nuclear magnetic resonance spectroscopy: principles and applications. *Prog. NMR Spect.* **34**: 203–256 (1999).
16. C.-Y. Li and T. S. Wiedmann. Concentration-dependent diffusion of bile salt/phospholipid aggregates. *J. Phys. Chem.* **100**:18464–18473 (1996).
17. T. S. Wiedmann, H. Herrington, C. Deye, and D. Kallick. Analysis of the interaction of bile salt/phospholipid micelles with mucin. *Chem. Phys. Lipids*, in press.
18. P. S. Chen, Jr., T. Y. Toribara, and H. Warner. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756–1758 (1956).
19. P. Schurtenberger and B. Lindman. Coexistence of simple and mixed bile salt-lecithin micelles: An NMR self-diffusion study. *Biochemistry* **24**:7161–7165 (1985).
20. W. M. Saltzman, M. L. Radomsky, K. J. Whaley, and R. A. Cone. Antibody diffusion in human cervical mucus. *Biophys. J.* **66**:508–518 (1994).
21. D. Winne and W. Verheyen. Diffusion coefficient in native mucus gel of rat small intestine. *J. Pharm. Pharmacol.* **42**:517–519 (1990).
22. D. F. Katz and J. R. Singer. Water mobility within bovine cervical mucus. *Biol. Reprod.* **17**:843–849 (1978).
23. A. Wikman-Larhed, P. Artursson J. Grasjo, and E. Bjork. Diffusion of drugs in native and purified gastrointestinal mucus. *J. Pharm. Sci.* **86**:660–665 (1997).
24. A. Wikman Larhed, P. Artursson, and E. Bjork. The influence of intestinal mucus components on the diffusion of drugs. *Pharm. Res.* **15**:66–71 (1998).