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Interactions between biliary lipid micelles and intestinal brush border membranes investigated by ^{1}H and ^{31}P nuclear magnetic resonance

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Abstract. The effect of taurocholate and lecithin-cholesterol-taurocholate mixed micelles on the structure of isolated intestinal brush border membranes was investigated by nuclear magnetic resonance (NMR). Rabbit brush border membranes isolated by a Mg^{2+} precipitation step were chosen for this study because of their stability and integrity as revealed by ^{31}P NMR. Incubation of taurocholate with the brush border membranes does not induce significant solubilization of these membranes even when the taurocholate/phospholipid ratio reaches 3.0. ^{1}H NMR studies indicate that taurocholate is included in the membrane bilayer at low concentration (3 m*M*). However this biliary salt produces a size diminution of the vesicles when its concentration increases. Incorporation of lecithin or lecithin-cholesterol in micelles of taurocholate and subsequent incubation with brush border membranes lead simultaneously to a decrease in the ^{31}P NMR isotropic/bilayer line ratio, and to an increase in $\Delta\sigma$. These results indicate a protective effect of these compounds against lytic damage of taurocholate. Furthermore the equilibrium distribution of lecithin between mixed micelles and the membrane bilayer is strongly in favour of complete integration of micellar components in the bilayer. These data suggest that uptake of lipids from the micellar phase by isolated brush border membranes involves an interaction of the micelles with membranes followed by a fusion process.

Key words: Intestine, brush border membranes, bile salts, micelles, NMR

Introduction

Several studies have attempted to elucidate the mechanisms which regulate the functions of permeability, absorption, transport and exchange of molecules in the epithelial plasmic membrane by using in vitro preparations such as membrane vesicles (Kinne and Kinne-Saffran 1981). Among them the enterocyte membrane is a highly differentiated structure involved in the hydrolysis and absorption of dietary substances (Glickman and Brasitus 1983).

Biliary salts are known to play a crucial role in the digestion and absorption of both natural and synthetic fast soluble substances (Kuksis 1984). Of special physiological concern is micellarization in which the lipid components interact with bile salts to form micelles. The bile salts are characterized by hydrophobic and hydrophilic parts which enable the amphipathic molecules to interact with the lipid and the aqueous environment. Thus, they are natural powerful detergents frequently used in the solubilization of biological membranes (Helenius and Simmons 1975; Lichtenberg et al. 1983). Moreover, they can reversibly alter the transport of electrolytes, water and organic solutes in the gut (Wingate et al. 1973; Chadwick et al. 1979). It has been shown that such salts can produce dramatic membrane damage, suggesting that physical modifications can occur in the membrane structure.

We have recently shown that information on the structural arrangement of phospholipids and micelles in the bilayer of enterocyte brush border membranes could be obtained by ^{31}P nuclear magnetic resonance (NMR). It was demonstrated that brush border membranes exhibited high stability in contact with biliary salts, as would be expected from their biological function (Vallet-Strouve et al. 1985). Biliary salts, which naturally occur in fairly high amounts in intestinal lumen (up to 20 m*M*) produce considerable alteration in other types of membranes even at concentrations as low as 1 m*M* (Saito et al. 1983). In contrast, we have shown that taurocholate used at physiological concentrations produced a very slight brush border membrane perturbation modulated by fatty acids.

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In order to probe, at the bilayer membrane level, the structural organization of phospholipids and the alteration resulting from bile acid interaction with lipid micelles in intestinal membranes, we have used both ^{1}H and ^{31}P NMR spectroscopy.

Structural modifications of these membranes, due to the effect of different types of mixed micelles, were analysed in relation to the mechanisms of permeation, absorption and lipid transport. In this way micelles with lecithin or cholesterol which are normal components of bile, are known to protect the integrity of membranes against the disruptive action of bile salts (Coleman et al. 1979). They also modified the rate of cholesterol and fatty acid uptake from micellar suspensions into the intestine (Proulx et al. 1984 a, b, 1985).

Materials and methods

Materials

Rabbits were obtained from the slaughterhouse. L-alanine-4-nitroanilide and 4-nitrophenyl phosphate (analytical grade) were purchased from Serva. L-α-dipalmitoyl phosphatidylcholine (DPPC), cholesterol (CS), sodium taurocholate (TC), and bovine serum albumin (BSA) were obtained from Sigma. All other reagents, chemicals, solvents were of reagent grade purity or better and from either Merck or Prolabo. For thin-layer chromatography (TLC), plates of 250 µm thickness with silicagel kieselgur 60 precoating were purchased from Merck.

Phosphate buffer saline (PBS) was 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, 136 m*M* NaCl and 2.7 m*M* KCl pH 7.4. Buffer A was 20 m*M* *Tris*-HCl, 300 m*M* mannitol, 5 m*M* ethylenglycol-*bis*[β-aminoethylether) N,N′ tetraacetic acid (EGTA) pH 7.3; buffer B was 10 m*M* *Tris*-HCl, 50 m*M* mannitol, 5 m*M* EGTA pH 7.3.

Brush border membranes preparation

Brush border membrane vesicles were obtained from a process using the differential precipitation of brush border membranes by Mg^{2+} in the presence of EGTA (Hauser et al. 1980).

Intestines of several rabbits, including part of the duodenum, jejunum and ileum, were removed immediately after death of the animals, freed from most of the attached mesenteric and adipose tissue, rinsed with ice cold 0.9% NaCl solution, washed with PBS, then inverted and kept frozen at − 80 °C until needed; 50 g of this preparation were thawed and used as the starting material. Procedures for the differential centrifugation where the same as previously described (Vallet-Strouve et al. 1985). Then, brush border membrane pellets were suspended, homogenized and centrifuged in buffer B.

Purity of the membranes was ascertained by marker enzymes and phospholipid analysis.

Preparation of micellar solutions

Micelle samples for NMR were prepared as follows: the sodium salt of taurocholate (at 30 m*M* concentration) was dissolved in buffer B at pH 7.3 after thorough mixing with a magnetic stirring device (1 h at 37 °C). The micelles containing cholesterol and DPPC were prepared by dissolving the components together in organic solvent in the desired molar ratios. Samples were dried under a stream of N_2, dessicated overnight under vacuum, and hydrated with buffer B by periodic vortex mixing.

Immediately after they were obtained and just before NMR studies, brush border vesicles were incubated with micelles. One aliquot of a concentrated micellar solution was added to nine parts of brush border membrane preparation.

Lipid analysis

The entire lipid content of the brush border membrane preparations was extracted with 20 volumes of chloroform/methanol after mixing in an ultraturrax. The precipitate was re-extracted twice. After filtration of the proteins, the solvents were pooled and evaporated to dryness. Total lipid extracts were dissolved in a small volume of solvent for phosphorus analysis and for TLC to analyse the phospholipid composition of the extract. TLC plates were activated before use and were run in different solvent mixtures. Chloroform/methanol/water (65/35/6 v/v/v) and chloroform/methanol/acetic acid/water (25/15/4/2 v/v/v/v) were both used for the separation and identification of phospholipids. The areas corresponding to different phospholipids were scraped off and eluted from silica gel, as previously described (Vallet-Strouve et al. 1985), for phosphorus content analysis.

Biochemical assays

The inorganic phosphorus content was determined colorimetrically using a modification of the technique of Rouser et al. (1970) with a standard solution of potassium dihydrogenophosphate. The result was multiplied by a factor of 25 to obtain the phos-

pholipid content of the sample. Proteins were assayed with the Folin phenol reagent according to Lowry et al. (1951) using bovine serum albumin as standard.

Akaline phosphatase and aminopeptidase were assayed as brush border membrane markers. Alkaline phosphatase was determined from the increase of absorbance at 410 nm using paranitrophenylphosphate as substrate (Garen and Levinthal 1960). Aminopeptidase activity was measured at 37 °C and 410 nm according to the method of Maroux et al. (1973) using L-alanine paranitroanilide as substrate.

NMR spectroscopy

All the experiments were performed on a Bruker WM 250. Proton noise decoupling ^{31}P NMR spectra were obtained at 101.24 MHz. The sample preparation was transferred to a 10 or 15 mm tube and 15 min was allowed for temperature equilibrium. The spectra were accumulated from up to 6,000 transients using a 90° pulse (10 μs). 25 kHz sweep width and a 0.5 s interpulse delay using gated proton noise decoupling (5 W input power during the 0.0819 s acquisition time). Exponential multiplication was used to smooth the spectra with the resultant line broadening listed in the figures.

Saturation transfer ^{31}P NMR experiments were carried out using the DANTE pulse sequence (Morris and Freeman 1978) to saturate selected regions of the spectrum at discrete intervals from the excitation frequency. ^{31}P chemical shift anisotropy ($\Delta\sigma$) was calculated either from $\Delta\sigma = 3(\sigma_\perp - \sigma_i)$ with σ_i as the isotropic part of the phosphorus tensor or by simulation on an IBM A 1130 computer using a FORTRAN program containing a lineshape calculation of powder type spectra according to the equation proposed by Seelig (1978).

^1H NMR spectra were obtained at 250 MHz. Before ^1H NMR studies, brush border membranes were resuspended in a D_2O phosphate saline buffer. Spectra were obtained from up to 5,000 transients with selective irradiation for water peak suppression.

Results

^{31}P NMR characterization of the brush border membranes

As ^{31}P NMR of brush border membranes leads to the observation of the phospholipid part of the membranes, phospholipid composition was determined on brush border membrane vesicles. Lipid analysis was made within 1 hour after the preparation which is the necessary time to get a NMR spectrum. It reveals (data not shown) that the lipid extract of brush border membranes prepared by Mg^{2+} precipitation does not contain lytic compounds. So, these membranes are available for structural studies by ^{31}P NMR.

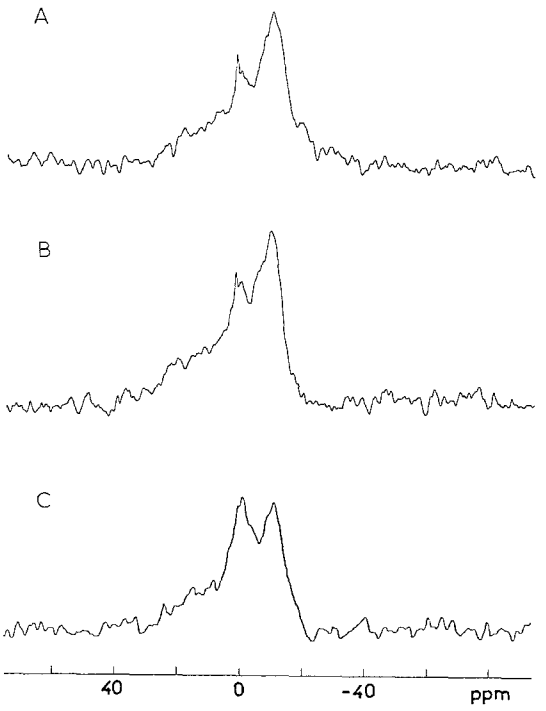

Fig. 1 A–C. 101.27 MHz ^{31}P NMR spectra of brush border membranes. 50 Hz line broadenings were applied to 6,000 free induction decays. Membrane vesicles were prepared by a Mg^{2+} precipitation and dispersed in 50 m*M* Mannitol, 5 m*M* EGTA, 10 m*M* *Tris*-HCl, pH 7.2. Protein concentration: 25 mg/ml. **A** Spectrum accumulated at 4 °C. **B** Spectrum accumulated at 37 °C. **C** ^{31}P NMR spectrum of brush border membranes incubated with micelles of taurocholate (3 m*M*) at 37 °C

Phosphorus NMR spectra of membranes were recorded at 4 °C and 37 °C (Fig. 1). At 4 °C the spectrum of brush border membranes consists of a large asymmetrical signal typical of a bilayer line shape and of a very small superimposed isotropic peak which may correspond to small phosphorated molecules entrapped in the vesicles (Fig. 1 A). After 1 h at 37 °C, a second weak isotropic peak appears but the spectrum keeps its asymmetrical shape with a chemical shift anisotropy, $\Delta\sigma = 37$ ppm, demonstrating that the phospholipids retain their bilayer configuration (Fig. 1 b).

A complete study on brush border vesicles prepared by Mg^{2+} precipitation between 4 °C and 37 °C shows a small variation in $\Delta\sigma$ (42–37 ppm). However, we observed a reversible discontinuity in the curve $\Delta\sigma = f(T)$ with a break at around 20 °C.

Taurocholate interactions with brush border membranes

Using ^{31}P NMR spectroscopy, we have recently shown that intestinal brush border membranes are not significantly perturbed by incubation with physiological concentrations of biliary salts (3, 9, 18 m*M*), thus demonstrating that the vesicles are highly stable (Vallet-Strouve et al. 1985). Incubation of brush border membranes with taurocholate (3 m*M*) leads to the emergence of a narrow peak superimposed on the broad ^{31}P NMR lineshape, indicating that a small portion of the membrane phospholipids have reached isotropic motion (Fig. 1 C).

In order to clarify the origin of the "isotropic" peak, possible exchange between bilayer and isotropic phospholipids was examined by measuring the transfer of magnetisation between their resonances. We selectively presaturated the broad resonance of the ^{31}P NMR spectra using the DANTE pulse sequence of Morris and Freeman (1978) (Fig. 2B). A control experiment was realised by positioning the carrier frequency of irradiation outside the spectrum (Fig. 2A). These two experiments were performed on the same sample of membranes containing 3 m*M* taurocholate. The spectra were accumulated simultaneously by periodic movement of the irradiation frequency in order to eliminate possible time-dependent changes in the effect of taurocholate. Figure 2C represents the difference between spectra 2A and 2B. Application of a saturation pulse train of 0.4 s results in complete disappearance of the broad phospholipid ^{31}P NMR signal (Fig. 2B). The difference spectrum, 2C, is almost entirely composed of the typical bilayer signal of the phospholipids, while the isotropic peak has completely vanished. This means that the duration of the saturation pulse train (0.4 s) is long compared to the time for a lipid molecule to experience reorientation via vesicle tumbling or lateral diffusion. Therefore, all the phospholipids responsible for the broad line undergo a loss of signal intensity (de Kruijff et al. 1980). Phospholipids responsible for the isotropic line are not exchangeable with phospholipids exhibiting a bilayer resonance during the time of saturation.

Are the taurocholate micelles incorporated in the brush border membrane bilayer? To answer this question, modifications of the taurocholate ^{1}H NMR spectrum were studied in the presence of brush border membranes. Spectrum 3B is obtained when membranes are incubated with 3 m*M* taurocholate. Compared to the spectrum of the taurocholate alone (Fig. 3C). It is interesting to see the complete disappearance of the taurocholate lines coming from protons 18 and 19. This disappearance could be due

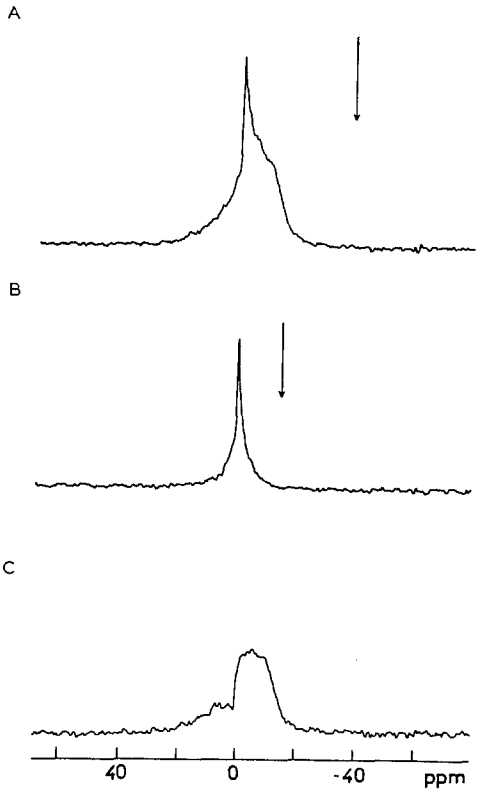

Fig. 2A–C. Saturation transfer ^{31}P NMR of brush border membranes (proteins: 15 mg/ml) incubated 15 min with 3 m*M* taurocholate. The sequence used for saturation is:

$|D_1 - (D_2 - P_1)_{NP} - D_3 - PW - AQT|_{NS}$.

In this experiment $D_1 = 0.5$ s, $D_2 = 100$ µs, $P_1 = 1$ µs, $NP = 4{,}000$, $D_3 = 600$ µs, $PW = 20$ µs, $NS = 3{,}800$. To avoid excessive heating, gated proton noise decoupling was used during the delay D_1. Carrier frequency of irradiation was alternatively changed from **A** to **B** before P_1 and the resulting free induction decay was stored on separate memory. Control ^{31}P NMR spectrum with saturating irradiation outside the broad line as indicated by the *arrow* (**A**). ^{31}P NMR spectrum after saturation at the position indicated by the *arrow* (**B**). Difference spectrum (**D**). All free induction decays were exponentially filtered resulting in a 50 Hz line broadening

to a dramatic broadening of taurocholate peaks produced by the insertion of taurocholate molecules into the membrane bilayer. Only the line of proton 25 at 3.1 ppm, which corresponds to the hydrophilic part of taurocholate, remains visible. These results indicate that taurocholate is rapidly absorbed and integrated into the lipid bilayer membrane at low bile salt concentrations.

Results illustrated in Fig. 4 show the effect of membrane incubation with increasing amounts of taurocholate on the solubilization process. When the taurocholate is added to the cloudy vesicle suspension, the turbidity shows an initial increase, passes through a maximum and then decreases. The molar ratio taurocholate/phospholipids at which the tur-

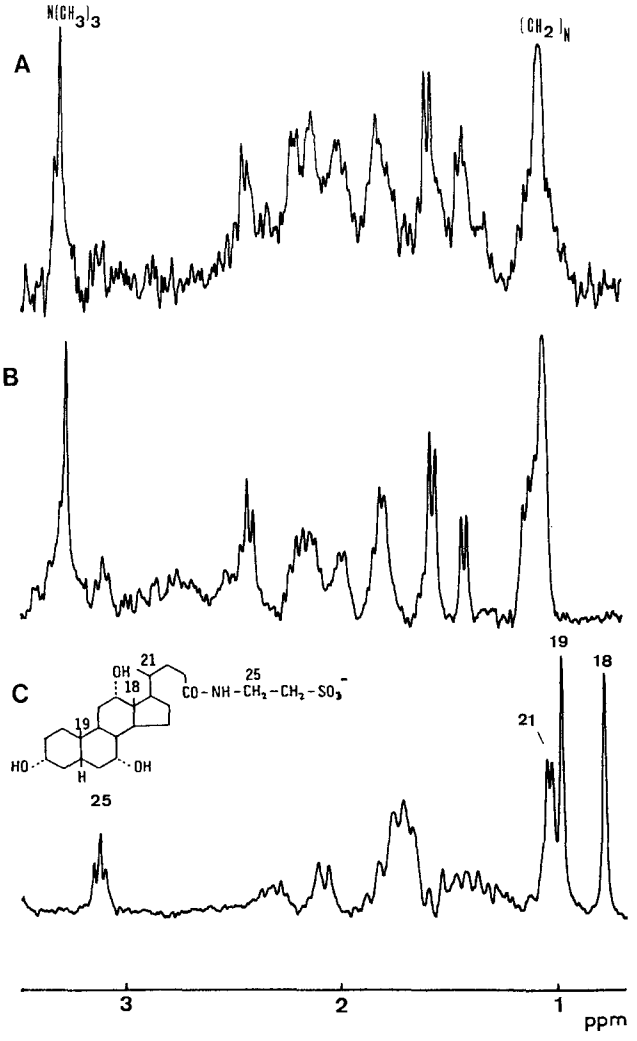

Fig. 3A–C. 250 MHz proton NMR spectra of brush border membranes at 25 °C in a D_2O phosphate buffer NaCl 136 m*M*, KCl 2.7 m*M*, pD 7.4 without taurocholate (**A**) and with 3 m*M* taurocholate (**B**). Spectrum (**C**) comes from a dispersion of 3 m*M* taurocholate in the same buffer. All spectra were obtained from up to 5,000 transients with selective irradiation for water peak suppression. Resonance assignments were made by reference to published spectra (Barnes and Geckle 1982)

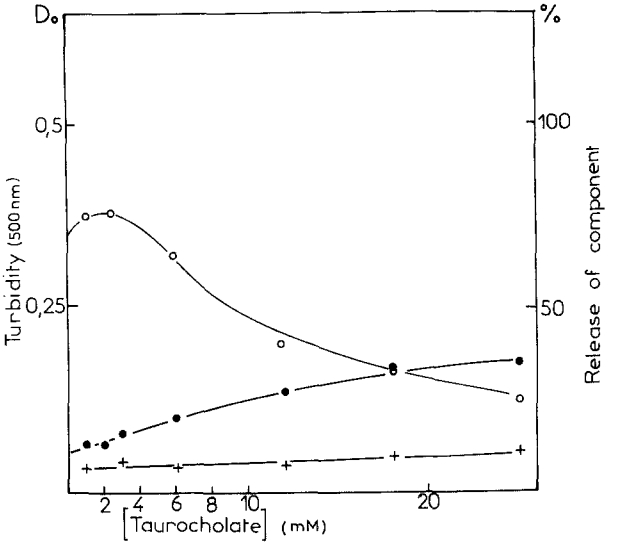

Fig. 4. Solubilizing effect of taurocholate on brush border membranes. Membranes (proteins: 20 mg/ml) were incubated 1 h at 37 °C with increasing concentration of taurocholate. Turbidity was measured at 500 nm after dilution (1/30) of the membrane vesicles in buffer B′ (○—○). Chemical determinations were realized on supernatant after centrifugation at 50,000 *g* for 30 min. Protein (●—●) and phospholipid (×—×) content in the supernatant were expressed in percent of control without taurocholate

bidity maximum occurs is about 0.3. Qualitatively, above this ratio, the results indicate that particles in suspension become smaller as the bile salt concentration increases. In order to characterize the solubilization process, the phospholipid and protein contents were determined on the supernatant after centrifugation at 50,000 *g* for 30 min. Under these centrifugation conditions, the brush border vesicles pellet, but the mixed micelles formed with TC remain in the supernatant. Figure 4 reveals that even at 25 m*M* taurocholate (which corresponds to a molar taurocholate/phospholipid ratio $\simeq 3$) most of the membrane proteins (65%) and phospholipids (90%) remain in the membrane pellet after centrifugation, demonstrating the poor solubilizing effect of taurocholate on these membranes.

Mixed micelles interactions with brush border membranes

Figure 5 shows the effect of various mixed micelle compositions on the ^{31}P NMR spectra of brush border membranes. In control spectra of taurocholate-DPPC (Fig. 5B) or taurocholate-cholesterol-DPPC (Fig. 5D) micelles, the isotropic feature of the lines demonstrated that phospholipids (DPPC) undergo rapid reorientation via tumbling and lateral diffusion around structures which do not exceed 100 nm (Burnell et al. 1980). This agrees with the dish like mixed micelles of 300 Å maximum diameter proposed by Mazer et al. (1980). Incubation of brush border membranes with taurocholate-DPPC micelles (1/1 mole/mole) leads to the complete disappearance of the isotropic peak originating from micelles (Fig. 5C). This seems to indicate that most of the phospholipids coming from micelles are integrated in the brush border membranes. Concomitantly, the chemical shift anisotropy is slightly increased ($\Delta\sigma = 33$ ppm) compared to taurocholate micelle incubation ($\Delta\sigma = 30$ ppm, Fig. 5A). Furthermore, the percentage of the isotropic peak in

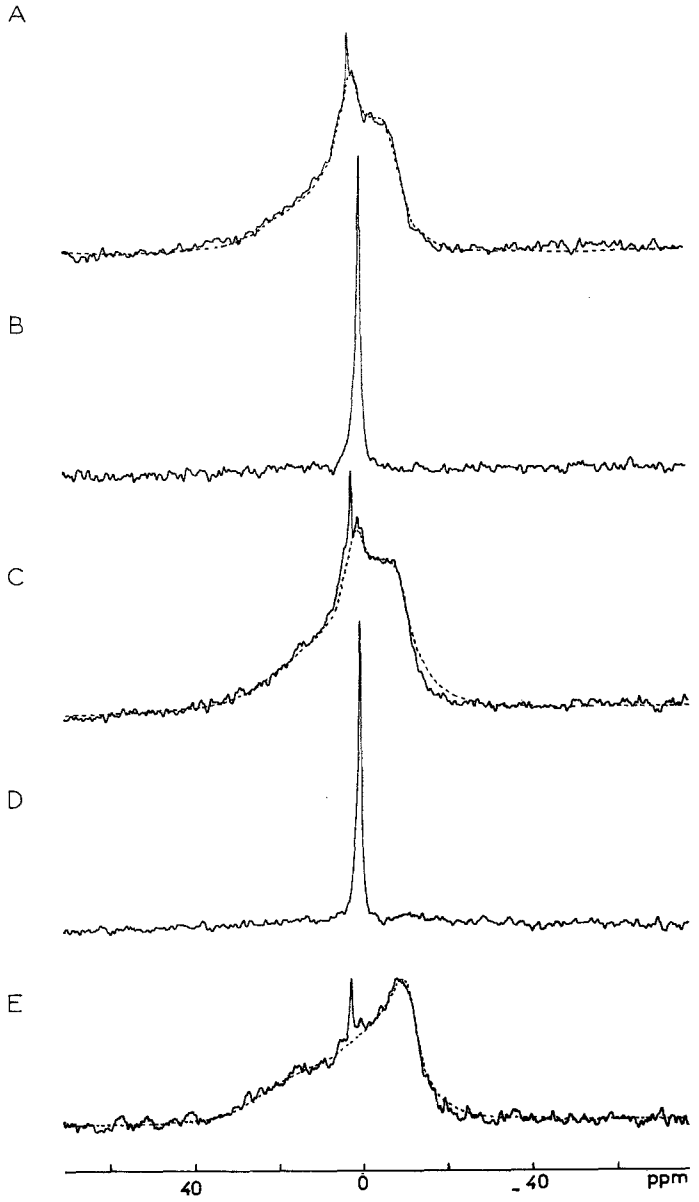

Fig. 5A–E. 101.27 MHz ^{31}P NMR spectra of brush border membranes at 25 °C incubated with micelles of TC, 3 m*M* (**A**), TC/DPPC, 3 m*M*/3 m*M* (**C**) and TC/CS/DPPC, 3 m*M*:3 m*M*:1 m*M* (**E**). ^{31}P NMR spectra of the micelles without membranes: TC/DPPC (**B**), TC/CS/DPPC (**D**). All spectra were recorded in the same conditions (2,700 scans, 50 Hz line broadening). *Dashed lines* represent computer simulated spectra with corresponding parameters ($\Delta\upsilon_{iso}$: full line width, $\Delta\upsilon_{aniso}$: anisotropic line width, $\Delta\sigma$: chemical shift anisotropy, R: isotropic/bilayer ratio) **A**: 350 Hz, 200 Hz, 30 ppm, 0.26; **C**: 350 Hz, 200 Hz, 33 ppm, 0.18; **E**: 300 Hz, 150 Hz, 37 ppm, 0

Fig. 5A (26%) is higher than in Fig. 5C (18%) demonstrating a slight protecting effect of DPPC on bilayer organization. Addition of cholesterol in the micelles of taurocholate-DPPC (TC/CS/DPPC; (3/3/1) does not prevent the incorporation of DPPC in membranes as is suggested by the absence of isotropic peak at 0 ppm in Fig. 5E. Also a slight increase in the chemical shift anisotropy of spectrum E ($\Delta\sigma = 37$ ppm) is observed.

Discussion

^{31}P NMR spectra obtained from brush border membrane vesicles show a pseudo-axially symmetric powder pattern characteristic of phospholipids in a bilayer configuration. It has been demonstrated that the reorientation time of the phospholipid molecules in a vesicle. τ_c, is an important factor for the phosphorus lineshape and that a bilayer shape is retained for $\tau_c > 1.4 \cdot 10^{-2}$ s (Burnell et al. 1980). Electron microscopy revealed that the vesicles were of fairly uniform size with a mean diameter of 1,500 Å (data not shown). The correlation time τ_c for the isotropic rotational motion of a spherical vesicle can be related to the vescile size by the following equation: $1/\tau_c = 6/r^2(D_t + D_{diff})$ where $D_t = kt/8\,\pi\, r\,\eta$ is the tumbling dependent part of the brownian rotational diffusion and D_{diff} the rate of lateral diffusion of lipid molecules in the bilayer (Cullis 1976). Using the measured value η 37 °C $= 2.5 \cdot 10^{-2}$ P for the viscosity of the membrane suspension and $r = 750$ Å, D_t was calculated to be $9 \cdot 10^{-9}$ cm$^2 \cdot$ s^{-1} and D_{diff} was estimated to be in the range of 10^{-8} to 10^{-9} cm$^2 \cdot$ s^{-1} which is in agreement with previous work on natural membranes (Bayerl et al. 1984; Scandella et al. 1972). The small variation of $\Delta\sigma$ in the temperature range 4° – 37 °C shows that the transition observed around 20 °C, revealed by electron spin resonance, differential scanning calorimetry (Mütsch et al. 1983) or Arrhenius plots of D-glucose transporter (Brasitus and Schachter 1980), only slightly influenced the orientation and the mobility of the head group region.

With regard to the incubation of brush border membranes with micelles of taurocholate, previous work by ^{31}P NMR (Vallet-Strouve et al. 1985) showed that incubation of brush border membranes with taurocholate (3–18 m*M*) produces an increase of the isotropic peak originating from phospholipids with high molecular reorientation (Fig. 1C). Present results indicate that this detergent effect is not due to membrane phospholipid solubilization (Fig. 4). but rather to a decrease in the size of membrane vesicles. This is attested by *(i)* the decrease of the turbidity measurement with increasing taurocholate concentrations and *(ii)* the absence of exchange between phospholipids from large and small vesicles (Fig. 2).

The mechanism by which the taurocholate interacts with brush border membranes seems to involve

an integration of the detergent inside the membrane bilayer. ^{1}H NMR spectra (Fig. 3) show that most taurocholate ^{1}H resonances undergo a dramatic line broadening after incubation with the membrane. This disappearance of ^{1}H resonances, excepting the one from proton 25, suggests that taurocholate is probably embedded in the membrane bilayer parallel to phospholipid acyl chains. Similar results have been obtained with ^{2}D NMR when a low concentration (1 m*M*) of deoxycholate was incubated with lamellar multi-layers of egg lecithin (Saito et al. 1983). At low levels of detergent, the turbidity of the vesicle dispersion slightly increases (Fig. 4) probably due to an expansion of the membrane bilayers after taurocholate integration (Jackson et al. 1982). Consequently equilibrium between micelle and membrane taurocholate seems to be largely in favour of membrane integration, demonstrating the high ability of intestinal brush border membranes to incorporate bile salts without noticeable solubilization (Wilson and Treanor 1977).

Addition of lecithin to micelle of taurocholate is known to protect membranes against the disruptive action of bile salts (Coleman et al. 1979) and to modify the uptake of fatty acids and cholesterol (Proulx et al. 1984a, 1985). The possibility has been considered that phosphatidylcholine causes an increase in size, as well as changes in shape and charge of the micelles, in such a manner as to retard diffusion across the unstirred water-layer (Hollander and Morgan 1980). Our results demonstrate that the incubation of DPPC-taurocholate or DPPC-cholesterol taurocholate micelles with brush border membrane leads to a complete integration of micellar DPPC in the membrane bilayer (Fig. 5). The higher ^{31}P chemical shift anisotropy observed with membranes incubated with mixed micelles could be attributed to the bilayer expansion produced by DPPC and cholesterol integration, the resulting increase in vesicle size leading to a smaller motional averaging of chemical shifts anisotropy. Furthermore, it is known that the insertion of these two components produces an acyl chain order effect (Jacobs and Oldfield 1979) into membranes. This phenomena could be responsible of their resultant low ability to absorb more cholesterol from micellar suspensions as has been observed by Proulx et al. (1984b).

However, the mechanism by which incorporation takes place remains unclear. The great ability of intestinal brush border membranes to incorporate all micelle components suggests that micelles interact with them directly by a fusion process.

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