Biochimica et Biophysica Acta 905 (1987) 30–38
Elsevier

BBA 73771

The mechanism of liposomal damage by taurocholate

Peter Walde [a], Junzo Sunamoto [a] and Charmian J. O'Connor [b]

[a] *Laboratory of Artificial Cell Technology, Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki (Japan) and* [b] *Department of Chemistry, University of Auckland, Private Bag, Auckland (New Zealand)*

(Received 28 July 1987)

Key words: Phosphatidylcholine; Liposome; Membrane damage; Bile salt; Taurocholate

The stability of small unilamellar vesicles formed by egg-yolk phosphatidylcholine (PC) has been examined in the presence of sodium taurocholate. The permeability of the vesicular membrane changes as the total taurocholate concentration increases, until a transformation from mixed bile salt/PC vesicles to mixed micelles occurs. Based on experiments in which the bile salt-induced release of either hydrophilic (carboxyfluorescein) or hydrophobic (Bromothymol blue) probes was studied, and on fluorescence polarization of the probe 1,6-diphenyl-1,3,5-hexatriene and turbidity measurements, a two-step process for the initial stage of liposomal damage by taurocholate is postulated.

Introduction

The effect of bile salts on the stability of liposomes has been the subject of several recent investigations [1–5]. On the one hand, it has been shown that liposomes are destroyed in the presence of relatively high bile salt concentrations under conditions which mimic the gastrointestinal situation and the significance of this destruction on orally administered drug-bearing liposomes has been discussed [1]. On the other hand, relatively small amounts of bile salts can significantly change the permeability properties of liposomes [4,6] without solubilization of the bilayers. Lysis of the liposomal membranes starts at a molar ratio of bile salt to phosphatidylcholine (PC) higher than, or close to, 3 : 1, which corresponds to the mean physiological ratio for gallbladder bile [4]. Investigation of the interactions between bile salts and liposomes may possibly also be useful in elucidation of the mechanism of bile-salt transport in the liver cell and in the interactions of bile salts with the canalicular membrane [5]. For example, it has been suggested that a possible mechanism for bile salt excretion could be the transport of bile salts in Golgi vesicles and exocytosis across the canalicular membrane.

In this work, the effect of one bile salt, sodium taurocholate (TC), on the physicochemical lysis of egg-yolk PC liposomes has been investigated at different $[TC]_{total}:[PC]$ ($[TC]_{bilayer}:[PC]$) ratios. This study provides insight into the mechanism of bile-salt damage of liposomes.

Abbreviations: BTB, Bromothymol blue; CF, carboxyfluorescein; cmc, critical micelle concentration; DPH, 1,6-diphenyl-1,3,5-hexatriene; MLV, multilamellar vesicles; PC, phosphatidylcholine; *P* value, steady-state fluorescence polarization value; SUV, small unilamellar vesicle; SUV-CF, SUV – DPH, SUV – BTB, small unilamellar vesicles with incorporated CF, DPH or BTB, respectively; TC, taurocholate; Tris, tris(hydroxymethyl)aminomethane; $R_e = [TC]_{bilayer}:[PC]$, effective molar ratio of taurocholate to PC in the vesicles.

Correspondence: J. Sunamoto, Laboratory of Artificial Cell Technology, Department of Industrial Chemistry, Faculty of Engineering, University of Nagasaki, Nagasaki, 852 Japan; C.J. O'Connor, Department of Chemistry, University of Auckland, Private Bag, Auckland, New Zealand.

Materials and Methods

Materials. Sodium taurocholate, TC, (98%) and sodium cholate (guaranteed reagent) were obtained from Nakarai Chemicals Ltd., Kyoto, tris(hydroxymethyl)aminomethane (Tris), Triton X-100 and Bromothymol blue (BTB) were from Wako Pure Chemical Industries, Tokyo, 5,6-carboxyfluorescein (CF) was from Eastman Kodak, Rochester, NY, and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Aldrich Chemical Co., Milwaukee. Egg-yolk PC was isolated and purified from egg yolk as described in the literature [7] using a column of aluminium Woelm N, akt. I from Woelm Pharma. The purity of the PC was checked by thin-layer chromatography (Silica-gel Spotfilm from Tokyo Kasei, Tokyo) with chloroform/methanol/water (65 : 25 : 4, by vol.) as eluant and detected with iodine. One single spot could be seen. The purity was also checked against the NMR and infrared spectra of an authentic sample. The purified PC was stored under nitrogen at $-20\,^{\circ}C$ in the dark. The concentration of PC in the vesicles was determined with a phospholipid test kit (Wako Pure Chemical Industries, Tokyo) using colorimetric determination of Molybdenum blue [8].

Preparation of liposomes. The preparation of small unilamellar vesicles, SUV, of egg-yolk phosphatidylcholine, egg PC, has been described [9]. The SUV which were loaded with a probe were prepared by a modification of this method. Egg PC SUV loaded with carboxyfluorescein were prepared by dispersing the dried phospholipid (30.0 mg) in 4.0 ml Tris-HCl (20 mM, pH 8.6) containing 200 mM carboxyfluorescein. Egg PC SUV encapsulating DPH in the hydrophobic domain of the bilayer [10] were prepared from 30.0 mg egg PC dissolved in 4.0 ml chloroform containing 400 μl of 1 mM DPH in tetrahydrofuran. Incorporation of DPH into the very hydrophobic domain of the phospholipid bilayer was ascertained by comparing the UV/vis spectra of SUV-DPH with those in hexane and in methanol [11]. The DPH concentration in the liposome was estimated by measuring the absorbance at λ_{max} 357 nm, ε (hexane) = 81 000 $M^{-1} \cdot cm^{-1}$ [11]. The [PC] : [DPH] ratio was 320 in the liposomes.

Egg PC SUV containing Bromothymol blue (BTB) in the lipid bilayer [12,13] were prepared in the following way: BTB was first dissolved in 0.01 M HCl under magnetic stirring at room temperature. Undissolved BTB was removed by filtration. The saturated filtrate, containing monoanionic BTB, was shaken with an equal volume of chloroform, thus extracting the dye into the organic phase. The chloroform was then evaporated and the orange BTB was dried under vacuum and stored overnight in a desiccator, over silica gel under vacuum. The phospholipid-dye mixture (33.0 mg PC and 1.4 mg BTB) was dispersed in 4.0 ml acetate buffered solution (20 mM, pH 5.0) containing 200 mM NaCl. Association of BTB with the SUV was demonstrated by comparing the UV/vis spectrum of SUV-BTB (λ_{max} 416 nm) with that of BTB (λ_{max} 433 nm) at pH 5.0 in the same buffer. The differences in the position of λ_{max} indicate different environments of the BTB chromophore in the SUV compared with the aqueous solution containing no liposomes. The exact location of nonionic BTB within the PC bilayer in vesicles has been discussed [12].

The concentration of BTB in the SUV-BTB was determined by measuring the absorbance of BTB after solubilization of the vesicle in the presence of 4.1 mM TC, or by destruction of the SUV in the presence of 0.3% Triton X-100.

Turbidity measurements. The turbidity of the vesicle suspensions was determined at $25\,^{\circ}C$ by measuring the 'apparent absorbance' at 250 nm on a Hitachi 220A spectrophotometer. The SUV (or multilamellar vesicles, MLV) suspension obtained after gel filtration with Sepharose 4B was diluted to 0.11 mM PC with 20 mM Tris-HCl buffer (pH 8.6), containing 200 mM NaCl. The turbidity measurements were carried out at 250 nm and not at a higher wavelength (where PC itself would have been optically transparent) because light scattering of the SUV at the concentration used is low. Moreover, although the absorbance of the mixed micelles (in the presence of high taurocholate concentrations) was greater than zero at this wavelength, this measurement did not affect the conclusions. (The PC concentration used was necessarily low to allow a direct comparison to be made with other aspects of this work, e.g. carboxyfluorescein release from SUV-CF.)

The effect of $[TC]_{total} \leq 8$ mM on the 'ap-

parent' absorbance of the SUV or MLV was studied. The order of addition of TC to the liposomal suspension, or vice versa, is not critical for these measurements because the turbidity of the final system is independent of its manner of preparation. This observation contrasts with that obtained in the carboxyfluorescein-release experiments (see below). However, in order to maintain a consistent experimental procedure, SUV were added to the taurocholate solution. A typical measurement was made as follows: to 2.75 ml 20 mM Tris-HCl buffer (pH 8.6) containing an appropriate amount of taurocholate, 0.25 ml of a liposomal suspension (20 mM Tris-HCl buffer (pH 8.6)) was added.

Release of carboxyfluorescein from SUV-CF. The release of carboxyfluorescein at 25 °C was followed as previously described [4,14], using a Hitachi 650-10S fluorescence spectrophotometer. The SUV-CF solution obtained after gel-filtration was diluted to 0.1 mM PC with 20 mM Tris-HCl buffer, 200 mM NaCl (pH 8.6). The carboxyfluorescein release was monitored for 20 min by measuring the increase in the relative fluorescence intensity at 520 nm after excitation at 470 nm. 100% carboxyfluorescein release was obtained by destroying the vesicles with 0.3% (v/v) Triton X-100. The order of addition of the reagents to the vesicles affected the results. In the first series of experiments, method A, a small amount of a concentrated micellar solution of taurocholate was added to the SUV-CF suspension: e.g. 20.0 μl of 123 mM taurocholate solution was added to 3.08 ml of the carboxyfluorescein-loaded SUV suspension ($[TC]_{total} = 0.8$ mM, $[PC] = 0.1$ mM). In the second series of experiments, method B, the SUV-CF solution was added to a taurocholate solution of an appropriate concentration: e.g. 200.0 μl SUV-CF was added to 2.9 ml of 0.85 mM taurocholate solution ($[TC]_{total} = 0.8$ mM, $[PC] = 0.1$ mM).

Steady-state fluorescence polarization measurements. Steady-state fluorescence polarization, P, of DPH intercalated into liposomal bilayers was measured at 25.0 °C on a Union Giken fluorescence polarization spectrophotometer FS-501S, connected to a Sord Microcomputer M-200 Mark II system. DPH was excited at 335 nm and its fluorescence was detected using a sharp cut-off filter L-39 (Hoya Glass Works, Tokyo) to shut out the light with wavelengths below 370 nm. The relationship employed to obtain the P value is given elsewhere [4].

The P values were measured after mixing the liposomal suspensions with TC solutions. The PC concentration was kept constant at 0.052 mM. At this concentration the effect of light scattering on the fluorescence polarization measurements was negligible [15].

Release of Bromothymol blue from SUV-BTB. The BTB-release measurements were carried out as follows: the SUV-BTB solution (0.2 ml) was added to 20 mM Tris-HCl (2.8 ml, 200 mM NaCl (pH 8.6)) containing an appropriate amount of taurocholate, and the absorbance at 617 nm was followed at 25.0 °C during the first 14 min after mixing. [PC] was maintained at 0.11 mM, ($[BTB]_{total} = 5.5$ μM, and $[PC]:[BTB] = 20$). [TC] < 6 mM did not show any significant effect on the absorption coefficient of BTB (6 μM, pH 8.6) at 617 nm ($\varepsilon = 3.7 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$), and the total BTB concentration in the SUV-BTB was determined after complete destruction of the liposomes in the presence of 4.1 mM taurocholate. The UV/vis spectrum of BTB at pH 8.6 was changed in the presence of 0.3% Triton X-100 solution; the absorption band at 617 nm now appeared at 622 nm and ε_{max} was decreased to 10^4 $M^{-1} \cdot cm^{-1}$. The vis spectrum of the SUV-BTB system solubilized by Triton X-100 micelles was also characterized by λ_{max} 622 nm. The two methods used for determining [BTB] gave results in good agreement with each other. The amount of BTB released at any time after mixing of taurocholate with SUV-BTB was determined from the absorbance at 617 nm. The absorbance after mixing SUV-BTB with a total concentration of 4.1 mM taurocholate was equal to 10% released BTB.

Results

Turbidity measurements

Taurocholate itself forms an aqueous micelle above its critical micelle concentration, cmc [16]. Vesicular structures of liposomes are sometimes destroyed by the coexistence of surfactant micelles [4,17]. Firstly, we investigated the destruction of the liposomal structure, upon cosolubilization of

the vesicles by aqueous taurocholate micelles, by monitoring the decrease in the turbidity of the liposomal suspension.

At taurocholate concentrations below the cmc = 3.2 mM (0.15 M NaCl, 20 °C, independent of pH) [18], no changes in turbidity were detected during the first few minutes after mixing of the SUV or MLV with taurocholate. However, at concentrations of taurocholate greater than the cmc, e.g. at 4.1 mM taurocholate, the turbidity of the solution decreased because PC was solubilized by the micelles [14]. Under our experimental conditions this solubilization process took 90 s in the case of the SUV and 5 min for the MLV. The effect of increasing total taurocholate concentrations on the turbidity decrease of a SUV suspension is shown in Fig. 1. At $[TC]_{total} > 4$ mM, the system became optically clear while at $[TC] < 3$ mM, the turbidity of the SUV suspension remained almost constant.

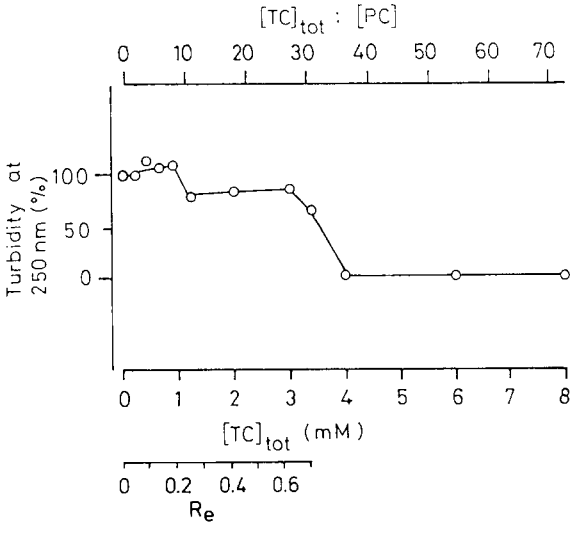

Fig. 1. Effect of taurocholate (TC) on the relative turbidity of a SUV solution, measured 1 min after mixing: 20 mM Tris-HCl, 200 mM NaCl (pH 8.6); 25 °C; 0.11 mM PC. R_e is the effective TC to PC molar ratio in the mixed TC-egg PC vesicles. $R_e = [TC]_{bilayer}/[PC] = [TC]_{total}/([PC] + K^{-1})$ [19]. The equilibrium partition coefficient, $K = 0.22$ mM^{-1}, for the TC-egg PC system has been estimated from quasi-elastic light scattering studies at 20 °C and in the presence of 0.15 M NaCl [20]. Transition from mixed vesicles to mixed micelles occurs when $R_e \approx K \cdot$ cmc [19], where cmc(TC) = 3.2 mM [18].

Carboxyfluorescein-release

Two different experimental procedures were used to study the effect of taurocholate on the carboxyfluorescein release from the interior of the SUV into the exterior bulk phase. The experimental data obtained by use of method A (not shown) revealed one major difference from those obtained by use of method B, i.e. in the former case there was an initial rapid release of carboxyfluorescein from the liposomes, caused by local destruction of the liposomal membranes immediately upon addition of the very concentrated micellar solution of taurocholate and before attainment of a homogeneous system. The relative extent of this initial carboxyfluorescein release was dependent upon both the concentration of the added taurocholate solution and the time spent on mixing.

The data obtained by use of method B are summarized in Fig. 2. Below $R_e = 0.09$ ($[TC]_{total} =$

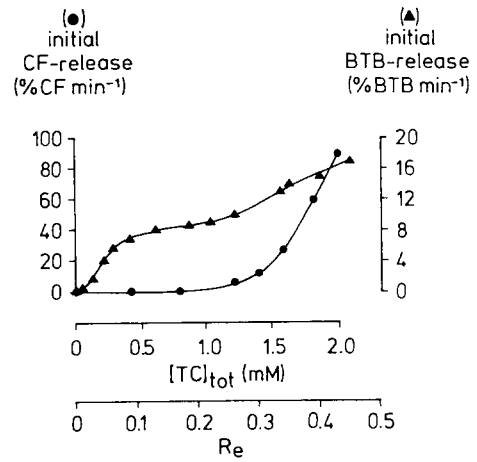

Fig. 2. Comparative diagram, showing the effect of taurocholate on the initial rates of carboxyfluorescein (CF) release (● and left hand scale) and Bromothymol blue (BTB) release (▲ and right hand scale) from SUV. Carboxyfluorescein release (calculated from percent release 1 min after mixing): 0.097 mM PC; 20 mM Tris-HCl, 200 mM NaCl (pH 8.6); 25 °C; carboxyfluorescein concentration in the interior water pool at time zero: 200 mM. Bromothymol blue release (calculated from percent release 2 min after mixing and corrected for the rapid release of weakly bound Bromothymol blue at the outer surface of the vesicles, i.e. for the Bromothymol blue released, in the absence of added taurocholate, by dilution at pH 8.6): internal pH 5.0 (20 mM NaOAc, 200 mM NaCl), external pH 8.6 (20 mM Tris-HCl, 200 mM NaCl), (see Materials and Methods); 25 °C; 0.11 mM PC, $[BTB]_{total} = 5.5$ μM. R_e values: see legend to Fig. 1.

0.4 mM, $[TC]_{total}/[PC] = 4.3$) no significant carboxyfluorescein release was observed during the first 20 min (see the legend for Fig. 1 for an explanation of the method of calculating R_e values). This result implies that any impurities which may have been present in the carboxyfluorescein sample did not lead to the preparation of unusually and highly leaky vesicles [21]. Even in the presence of 0.8 mM taurocholate ($R_e = 0.17$) the carboxyfluorescein release was small: only about 5% of the total carboxyfluorescein was released even after 20 min. However, a substantial amount of carboxyfluorescein was slowly, but obviously, leaked from the liposomes at $[TC] > 0.8$ mM. Penetration of taurocholate molecules into the phospholipid bilayer may cause a significant change in the permeability of the liposomal membranes. At $[TC] >$ cmc (e.g. $[TC]_{total} = 4$ mM, $[TC]_{total}/[PC] = 43.5$, $R_e > 0.7$), all the encapsulated carboxyfluorescein was released instantly (data not shown).

Several additional measurements were also made using sodium cholate, an unconjugated bile salt, under the controlled experimental conditions at pH 8.6. At a total cholate concentration of 1.6 mM, an effect similar to that found for 1 mM taurocholate was observed. Comparable effects were also obtained with 1.9 mM cholate and 1.3 mM taurocholate.

Bromothymol blue release

Bromothymol blue (BTB) is a very sensitive probe for determining the perturbation or the physiocochemical lysis of bilayer membranes because the dye is located very close to the surface of the membranes [12,22]. The effect of taurocholate on the initial rate of BTB-release from SUV is summarised in Fig. 2. The perturbation of the membrane with taurocholate causes the release of any BTB which is weakly bound to the membrane and then a rapid deprotonation of monoanionic BTB (yellow) to afford a very water-soluble dianionic BTB (violet) upon transfer of the BTB from the membrane to the exterior buffered system (pH 8.6). The dianionic species formed in the bulk aqueous phase is not able to return to the membrane again [12]. Even dilution of the SUV-BTB suspension with Tris-HCl buffer at pH 8.6 in the absence of added taurocholate, caused approx.

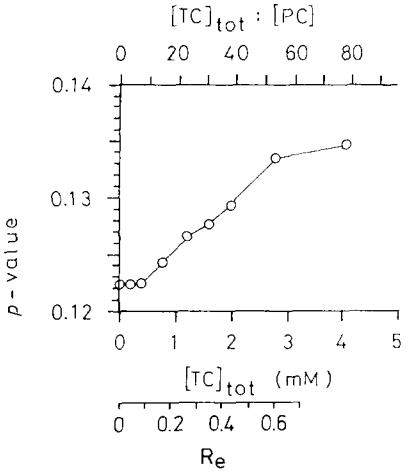

Fig. 3. Effect of taurocholate (TC) on the P value of DPH in SUV. 20 mM Tris-HCl, 200 mM NaCl (pH 8.6); 25 °C; 0.052 mM PC. [PC]:[DPH] = 320. R_e values: see legend to Fig. 1.

50% immediate BTB-release. The data in Fig. 2 have been corrected for this value. Addition of taurocholate at relatively low concentrations ($[TC]_{total} < 0.6$ mM, $[TC]_{total}/[PC] < 5.5$, R_e 0.13) induced an additional, rapid (in less than 2 min) release of the dye from the SUV. Further incubation, for at least 14 min, released no more BTB. Over the concentration range 0.8 mM $< [TC]_{total} < 3.0$ mM, there was not only an increase in the initial rate of rapid BTB-release, but also in the total amount of dye released with time. At $[TC]_{total} >$ cmc (e.g. $[TC]_{total} = 4.1$ mM, $[TC]_{total}/[PC] = 37.3$) there was immediate release of all of the BTB molecules into the external alkaline medium (data not shown).

Fluorescence polarization

The effect of taurocholate on the steady-state fluorescence polarization (P value) of DPH incorporated into egg PC-SUV is shown in Fig. 3. The P values given are mean values of seven repeated determinations made within 20 min after mixing of TC with the DPH-loaded SUV. The P value remained almost constant within this time interval in all runs. At $[TC] < 0.4$ mM ($[TC]_{total}/[PC] < 7.7$, $R_e < 0.09$), the P value of DPH remained constant and was not affected by the added bile salt. However, increasing the taurocholate concentration led to a significant increase in the P

value. Complete destruction of the DPH-loaded SUV with Triton X-100 gave a P value of 0.017.

Discussion

Although the existence of a critical micelle concentration in solutions of bile salts is currently under discussion (see, for example, Refs. 23 and 24), it is known that bile salts aggregate in an aqueous medium and that they possess a high capacity for solubilizing PC (up to 2 mol PC per mol taurocholate) through the formation of mixed bile salt-PC micelles [16]. The formation of such mixed micelles is strongly dependent on the absolute concentrations of PC and bile salt [20,25]. In this study, all measurements (except those of fluorescent polarization) were made at a constant concentration of PC, 0.1 mM.

The effect of taurocholate on liposomes formed by egg-yolk PC, as determined by different experimental methods, is discussed under three headings, according to the total taurocholate concentration used. This total bile salt concentration must not necessarily, of course, be assumed to be the concentration of surfactant associating or interacting with the phospholipid [26].

4 mM < [TC]$_{total}$ < 6 mM

Hydrophobic interactions cause bile-salt molecules to self-associate in an aqueous medium and form relatively small aggregates. In solutions of [TC] > 3.2–3.3 mM, this aggregation process occurs at 25.0 °C in 0.15–0.30 M NaCl [27]. Thus, when [TC] > 4 mM, the taurocholate micelles are able completely and rapidly to solubilize the PC molecules, which are themselves initially present in the form of SUV (Fig. 1) or MLV. This complete destruction of the liposomes results in the formation of mixed TC-PC micelles which are smaller in size [28] than egg PC-SUV or TC-egg PC mixed vesicles [20,25] and therefore the extent of light scattering is markedly decreased. The solubilization of SUV (90 s) was 3–4-times faster than that of MLV (approx. 5 min). This difference may be accounted for by the fact that, in the case of MLV, only the outermost bilayer of the multilayers is directly accessible to the surfactant molecules in the initial step of the solubilization process. The inner bilayers can be attacked only after destruction of the outer bilayer. Similar differences in the solubilization kinetics of MLV and SUV have been observed using deoxycholate as surfactant [2]. The solubilization of PC by taurocholate micelles leads to an instantaneous release of all carboxyfluorescein from the interior of SUV into the bulk phase and to a rapid release of all BTB from the membranes (Fig. 2). These observations are consistent with the postulate of a vesicle to mixed micelle transformation and demonstrate the rapid solubilziation of SUV by taurocholate micelles, under the conditions used.

[TC]$_{total}$ < 0.8 mM

At $[TC]_{total} < 0.8$ mM the turbidity of a SUV suspension is not dependent on the bile-salt concentration (Fig. 1). More specifically, the turbidity does not decrease, indicating that no mixed TC-PC micelles are formed under these conditions. Moreover, since there is no increase in turbidity under the conditions used, taurocholate does not promote vesicle aggregation or fusion, a property which has been observed in the case of many other surfactants (see, for example, Ref. 29 and 30).

In the present study, therefore, the taurocholate-induced release of carboxyfluorescein or Bromothymol blue from the liposomes is not the result of SUV-fusion and/or aggregation. At [TC] < 0.8 mM ($[TC]_{total}/[PC] < 8.7$, $R_e < 0.17$) there is no marked carboxyfluorescein release (Fig. 2). However, local disruption of the SUV (caused by addition of a concentrated bile salt solution to the liposomes) was noted, and this observation showed the importance of the experimental conditions used in this type of measurement. Even if there were a subsequent transformation of locally formed mixed micelles to vesicles after mixing of the components of the solution [20,25,31] the initial release of the fluorescent probe could not be reversed. The related BTB-release measurements (Fig. 2) were therefore carried out by adding the SUV-BTB solution to the taurocholate solution. In the presence of $[TC]_{total} < 0.8$ mM there was a substantial release of BTB from the SUV, Fig. 2. This release most probably represents diffusion of the BTB molecules which are lcoated in the outer layer of the bilayers. These perturbations of the outer layer of bilayers occur even at total taurocholate concentrations below 0.1 mM ($[TC]_{total}/[PC] < 0.9$, $R_e < 0.02$), Fig. 2.

The P values of DPH incorporated into the PC bilayer are not affected by $[TC]_{total} < 0.5$ mM ($[TC]_{total}/[PC] < 9.6$, $R_e < 0.11$), Fig. 3. In the presence of 0.8 mM taurocholate, however, the P value is significantly higher. This increase in the P value indicates monomeric penetration of taurocholate into the liposomal membranes [4,14]. In turn, there is a reduction in the rotational freedom of DPH, which is most probably localized in the very hydrophobic domain of the bilayer, and this reduction is accompanied by a considerable decrease in the membrane fluidity.

0.8 mM < [TC]$_{total}$ < 3 mM

Under the conditions used ([PC] = 0.1 mM, 25.0 °C (pH 8.6)), the presence of 0.8 mM < [TC] < 3.0 mM has only a small effect on the turbidity of SUV (Fig. 1) and of MLV (data not shown) showing that the vesicles are not transformed into smaller mixed micelles. It seems more probable that an increasing number of bile salt molecules is incorporated into the liposomes, resulting in the formation of mixed TC-PC vesicles. Because of their similar particle size, these mixed vesicles have light scattering properties similar to those of ordinary egg PC vesicles. However, these mixed TC-PC vesicles have bilayer structures with a much higher permeability for encapsulated probes such as carboxyfluorescein (Fig. 2). The carboxyfluorescein probe diffuses slowly from the interior of the vesicle into the exterior. The formation of hydrophilic pores (channels) by bile-salt molecules associated within the bilayer may provide a reasonable explanation for the increased permeability of the mixed vesicles [6]. From the results shown in Fig. 2, it can also be seen that, in the presence of [TC] < 1 mM ($R_e > 0.22$), the characteristics of BTB release from SUV are changed. This change most probably represents perturbation of the inner layer of the bilayer by the increasing concentration of taurocholate, leading to diffusion of dye molecules associated with PC molecules, in the inner layer of the membrane, into the exterior bulk phase.

Significant perturbations of the phospholipid bilayer above 1 mM TC are shown by changes in the P value of DPH, Fig. 3. When comparison is made between the results of fluorescent polarization measurements and carboxyfluorescein- or BTB-release experiments, the different concentrations of PC used should be considered. Significant changes in the P-value occur at $R_e \approx 0.11$ ($[TC]_{total} = 0.5$ mM) (Fig. 3). This value agrees with that observed for the change in the membrane permeability with added BTB (Fig. 2).

Comparison of the carboxyfluorescein-release measurements, in the presence of taurocholate or of unconjugated cholate, showed that under identical total bile salt concentrations taurocholate was the more effective agent in perturbation of the membrane. This difference may be a consequence of a different distribution of the bile-salt molecules between the water phase and the hydrophobic layers of the SUV [26,31,32]; namely, to a difference in the hydrophobicity-hydrophilicity balance between the two bile salts.

Mechanism of SUV lysis by taurocholate

Solubilization of phospholipid suspensions by surfactants has been discussed [33] and it has been suggested that the following sequence of events occurs when increasing amounts of surfactant are added. Stage I: the surfactant is incorporated into the bilayer, causing changes in its physical properties. Stage II: the phospholipid bilayers become saturated with surfactant and mixed micelles are formed, resulting eventually in a complete lamellar-micellar phase transition. (It has been suggested that dissolution of phospholipid bilayers which are saturated with surfactant occurs only after the concentration of free surfactant rises above the critical micelle concentration [34].) Stage III: the sizes of the mixed micelles decrease while the surfactant : phospholipid ratio in the mixed micelles increases. A detailed analysis of published data recently led to good characteristics and an understanding of the overall solubilization process [19].

This study provides some insight into stage I of the solubilization process. The novel finding is the initial 'two-step process' in the liposome solubilization by taurocholate. Some surfactant molecules first associate with the outer layer of the bilayer, and then (in the presence of an increased surfactant concentration) the surfactant molecules are also associated, in a second step, with the inner layer. Evidence for such a movement of surfactant molecules from one layer to the other in a bilayer

during solubilization has previously been indicated in the presence of the nonionic surfactant *n*-dodecyl octaethyleneglycol monoether [35].

At a total taurocholate concentration above the critical concentration for self-aggregation ([TC] > 4 mM) the formation of mixed TC-PC micelles occurs (Fig. 1). A current and widely accepted schematic model of mixed bile salt-PC micelles has been developed from quasielastic light-scattering studies of aqueous biliary lipid systems [28].

The main findings of this work can be summarized as follows: Before R_e reaches a value of 0.70, a transition from vesicles to mixed TC-PC micelles occurs and one can observe that taurocholate is incorporated in a stepwise manner into the phospholipid bilayer. At $R_e < 0.1$, taurocholate disturbs the outer layer of the vesicles (BTB starts to be released), without having an effect on the permeability for the water soluble carboxyfluorescein in the interior of the liposomes. Incorporation of taurocholate molecules into the PC bilayer is also shown by the changes in the P value, whereas the turbidity of the sample remains almost constant, indicating that the transformation into smaller mixed micelles does not yet occur.

The ability of bile salts to change the physicochemical properties of vesicles and to solubilize these vesicles into mixed micelles may have important consequences for the use of liposomes as drug carriers in oral administration [36].

Acknowledgements

P. Walde was a Guest Research Fellow at Nagasaki University and thanks the Matsumae International Foundation for a fellowship. J. Sunamoto acknowledges the financial support of a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (Nos. 61126001 and 61126005). C.J. O'Connor acknowledges financial assistance from the Research Committees of the New Zealand University Grants' Committee and the University of Auckland, and from the New Zealand Foundation for the Newborn.

References

1 Richards, M.H. and Gardner, C.R. (1978) Biochim. Biophys. Acta 543, 508–522
2 Lichtenburg, D., Zilberman, Y., Greenzaid, P. and Zamir, S. (1979) Biochemistry 18, 3517–3525
3 Rowland, R.N. and Woodley, J.F. (1980) Biochim. Biophys. Acta 620, 400–409
4 O'Connor, C.J., Wallace, R.G., Iwamoto, K., Taguchi, T. and Sunamoto, J. (1985) Biochim. Biophys. Acta 817, 95–102
5 Schubert, R., Beyer, K., Wolburg, H. and Schmidt, K.-H. (1986) Biochemistry 25, 5263–5269
6 Castellino, F.J. and Violand, B.N. (1979) Arch. Biochem. Biophys. 193, 543–550
7 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53–56
8 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
9 Huang, C. (1969) Biochemistry 8, 344–352
10 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521–4528
11 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
12 Sunamoto, J., Kondo, H. and Yoshimatsu, J.A. (1978) Biochim. Biophys. Acta 510, 52–62
13 Kano, K., Tanaka, Y., Ogawa, T., Shimomura, M., Okahata, Y. and Kunitake, T. (1980) Chem. Lett. 421–424
14 Sunamoto, J., Iwamoto, K., Ikeda, H. and Furuse, K. (1983) Chem. Pharm. Bull. 31, 4230–4235
15 Lentz, B.R., Moore, B.M. and Barrow, D.A. (1979) Biophys. J. 25, 489–494
16 Carey, M.C. and Small, D.M. (1970) Am. J. Med. 49, 590–608
17 Sunamoto, J., Iwamoto, K., Uesugi, T., Kojima, K. and Furuse, K. (1984) Chem. Pharm. Bull. 32, 2891–2897
18 Menger, F.M., McCreery, M.J. (1974) J. Am. Chem. Soc. 96, 121–126
19 Lichtenberg, D. (1985) Biochim. Biophys. Acta 821, 470–478
20 Schurtenberger, P., Mazer, N. and Kanzig, W. (1985) J. Phys. Chem. 89, 1042–1049
21 Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.M. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137
22 Sunamoto, J., Hamada, T. and Murase, H. (1980) Bull. Chem. Soc. Jpn. 53, 2773–2777
23 Kratohvil, J.P., Hsu, W.P., Jacobs, M.A., Aminabhavi, T.M. and Mukunoki, Y. (1983) Colloid Polymer Sci. 261, 781–785
24 O'Connor, C.J. and Wallace, R.G. (1985) Adv. Colloid Interface Sci. 22, 1–111
25 Schurtenberger, P., Mazer, N., Waldvogel, S. and Kanzig, W. (1984) Biochim. Biophys. Acta 775, 111–114
26 Lichtenberg, D., Robson, R.J. and Dennis, E.A. (1983) Biochim. Biophys. Acta 737, 285–304
27 Carey, M.C. and Small, D.M. (1972) Arch. Intern. Med. 130, 506–527
28 Mazer, N.A., Benedek, G.B. and Carey, M.C. (1980) Biochemistry 19, 601–615
29 Saez, R., Goni, F.M. and Alonso, A. (1985) FEBS Lett. 179, 311–315
30 Jackson, M.L., Schmidt, C.F., Lichtenberg, D., Litman, B.J. and Albert, A.D. (1982) Biochemistry 21, 4576–4582

31 Stark, R.E., Gosselin, G.J., Donovan, J.M., Carey, M.C. and Roberts, M.F. (1985) Biochemistry 24, 5599–5605
32 Almog, S., Kushnir, T., Nir, S. and Lichtenberg, D. (1986) Biochemistry 25, 2597–2605
33 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
34 Dennis, E.A. and Owens, J.M. (1973) J. Supramol. Struct. 1, 165–176
35 Ueno, M., Tanford, C. and Reynolds, J.A. (1984) Biochemistry 23, 3070–3076
36 Rowland, R.N. and Woodley, J.F. (1980) Biochim. Biophys. Acta 620, 400–409