A COMPARISON OF TRITON X-100 AND THE BILE SALT TAUROCHOLATE AS MICELLAR IONOPHORES OR FUSOGENS IN PHOSPHOLIPID VESICULAR MEMBRANES

A ^{1}H NMR method using the lanthanide probe ion Pr^{3+}

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1. Introduction

Studies on the interaction of detergents such as the bile salts and Triton X-100 with membranes have largely concerned solubilisation methods [1]. They increase the permeability of liposomes [2,3] or planar lipid bilayers [4,5] at concentrations which do not disrupt the membranes. This report describes experiments in which single-bilayer vesicular membranes are used to investigate the mechanism of the ionophore-like action of these surfactants. The results indicate the most likely transport mechanism to be the formation of inverted micelle structures within the lipid bilayer, and that Triton X-100 can also act as a fusogen and lytic agent depending on the physical state of the membrane.

These results are also significant in that they lend support to evidence recently introduced for the existence of inverted micelle and hexagonal phases in dynamic equilibrium with the bilayer phase [6]. The proposed functional role of these non-bilayer phases in transport and fusion processes [6,7] has not yet been effectively demonstrated in membranes, but these experiments suggest that such phases, when induced and stabilised by surfactants, do promote both transbilayer transport and membrane fusion. The usefulness of NMR and lanthanide probes in distinguishing mechanisms of transport, fusion and lysis is also demonstrated.

2. Materials and methods

DL-α-Dipalmitoyl phosphatidylcholine (DPPC) and cholesterol (puris grade) were obtained from Koch Light and sodium taurocholate from Calbiochem. Triton X-100 (scintillation grade) was purchased from BDH and av. M_r 624 (9.5 oxyethylene units) was used in calculating molarities. Praseodymium and dysprosium chloride were obtained from Lancaster Synthesis and D_2O (99.8%) from Prochem. The vesicular membranes (25 mg DPPC/ml) were prepared by sonication in D_2O as in [8]. This lipid was chosen to compare transport rates with our studies in [9] and to investigate effects at the phase transition temperature, T_c, which for these vesicles occurs over the range 37–43°C [8].

The ^{1}H NMR spectra were obtained using a JEOL C60HL spectrometer at 60 MHz, fitted with a calibrated temperature control. Transport of Pr^{3+} was followed using the method described for investigation of facilitated transport by the calcium ionophore A23187 [9]. This involves adjusting the extravesicular solution to 5 mM Pr^{3+} by addition of stock solution of $PrCl_3$ in D_2O. The ^{1}H NMR spectrum of the vesicles then shows separated signals from the extravesicular and intravesicular choline head-groups as seen in fig.1(a) where the outer head-group signal O has been shifted down-field by the extravesicular Pr^{3+}_{aq} [10]. Assuming a bilayer width of 4 nm, the ratio of signal areas O : I = 1.8 can be used to calculate the average vesicular diameter as 26 nm. When the Pr^{3+} diffuse or are transported into the intravesicular solution the rise in intravesicular concentration of Pr^{3+} causes signal I to move down-field towards signal O (fig.1(b–e)). By measuring the decreasing shift difference, $\Delta\nu_{(O-I)}$, between O and I at time intervals, the rate of diffusion or transport can be obtained. During these changes the extravesicular concentration of Pr^{3+} remains effectively constant at 5 mM since the total internal volume of vesicles is only a few % of the volume of sonicate. This method also allows a distinction to be made between diffusion or carrier-mediated transport and mechanisms which involve lysis. The effect of vesicle lysis on the NMR spectrum has been illustrated in [8] and is further discussed below with the results obtained at T_c.

To study the effect of Triton X-100 and taurocholate, stock solutions were prepared in D_2O and aliquots (typically 0–15 μl) were pipetted into the NMR tubes containing 0.5 ml sonicate (12.5 mg DPPC) and 5 mM extravesicular Pr^{3+} at 60°C. The initial extravesicular concentration of detergents was thus varied from ~0.1–5 mM.

3. Results and discussion

3.1. *Transbilayer transport by inverted micelles*

Since the initial studies with ionophores X537A and A23187 [9,11] it has become clear that the stability of the DPPC vesicles in the presence of Pr^{3+} allows transport rates to be measured reproducibly over long periods (>1 week). Using the above technique the unmediated self-diffusion of Pr^{3+} into the intravesicular space of the DPPC vesicles at 60°C (i.e., well above the phase transition) is slow. The rate as measured by the movement of signal I is 8.3×10^{-4} Hz/min. (This can be converted using a suitable calibration graph [9] into 0.14 μM Pr^{3+}/min.)

Addition of Triton X-100 or taurocholate up to ~0.25 mM causes no increase in rate of transmembrane movement of Pr^{3+}. This lower concentration limit represents ~40 detergent molecules vesicle and is slightly above the critical micelle concentration of Triton (0.24 mM) but well below that for taurocholate (10–15 mM). At >0.25 mM the rates increase with concentration and can be measured up to $\sim 5 \times 10^{-1}$ Hz/min, i.e., >2 orders of magnitude greater than the self-diffusion rate. Typical changes in the spectrum observed, illustrating the down-field movement of signal I with time, are shown in fig.1(b–e). Plots of $\Delta\nu_{(O-I)}$ against time are linear over the first 0–4 Hz of shift and the slopes of this initial portion of the plots are measured to determine the rates in Hz/min.

The effect of increasing concentration of Triton and taurocholate on the transport rate is shown in fig.1(f,g), where the slopes of the logarithmic plots are 3.0 and 3.95, respectively, suggesting the cooperativity of 3 molecules of Triton and 4 molecules of taurocholate in the rate-determining step for the transport of Pr^{3+}. That these figures represent true stoichiometries is supported by a number of other determinations using NMR of vesicular membranes. Thus the expected 2 : 1 ratio was found for X537A : Mn^{2+} [12] and we have obtained [13] an experimental

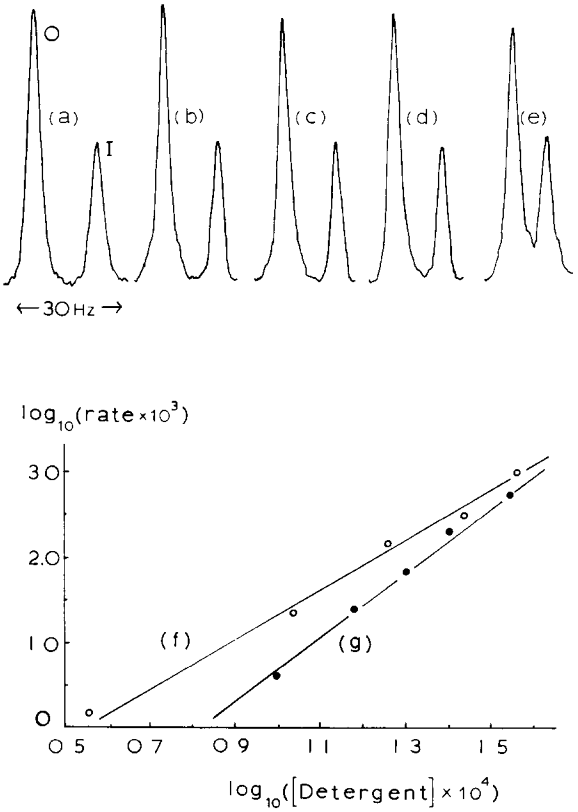

Fig.1. (a) The ^1H NMR spectrum of the extravesicular (O) and intravesicular (I) choline head-group signals $N(CH_3)_3$, from dipalmitoyl phosphatidylcholine vesicles at 60°C in the presence of 5 mM extravesicular Pr^{3+}. Spectra (b–e) show the result of transport of Pr^{3+} into the intravesicular region by Triton X-100 after. (b) 6.75, (c) 11.83, (d) 24.25 and (e) 93.0 min. The initial extravesicular concentration of Triton X-100 was 2.7 mM. Lipid vesicles were 34 mM. (f, g) The effect of increasing concentration of detergents Triton X-100 (f) and sodium taurocholate (g) on the rate of transport of Pr^{3+} into dipalmitoyl phosphatidylcholine vesicles at 60°C. The rates are expressed Hz/min as measured by the movement of peak I as shown in fig.1(a–e). [Detergent] = initial extravesicular molar concentration of detergent. Extravesicular concentration of Pr^{3+} was 5 mM.

stoichiometry of 2.8 for a transported complex $Pr(fod)_3$, where Hfod is the lipophilic fluorinated diketone $C_3F_7-CO-CH_2-CO-C(CH_3)_3$, often used in NMR shift reagents [14]. Finally, we have examined transport rates for 4 other dihydroxy and trihydroxy bile salts and in each case the stoichiometry is close to 4 [15].

Evidence for the existence of inverted bile salt micelles in lipid bilayers already exists [16], these being aggregates of 2–4 bile salt molecules with their hydrophilic sides bound inwards by hydrogen bonds between the hydroxyl groups, leaving their hydrophobic sides facing outwards to interact with the lipid acyl chains. A similar arrangement of Triton X-100 can occur by the folding of the molecule into two halves, one hydrophilic and one hydrophobic. A mechanism for the detergent-facilitated transport described above can then be suggested as follows: When the concentration is raised to $> \sim 0.25$ mM sufficient detergent partitions from the aqueous phase into the vesicular membranes to produce a significant concentration of oligomer inverted micelles in the bilayer. These inverted micelles are stabilised by incorporating the Pr^{3+} and translation of the micelle across the bilayer acts as the mechanism of increased permeability. This translation may also include lipid molecules as part of the micelle structure.

It also seems more reasonable to propose that the slow self-diffusion of Pr^{3+} observed in pure DPPC vesicles at 60°C also occurs by means of a small equilibrium concentration of inverted micelles, rather than by flip–flop or extravesicular micelle formation [17]. The acceleration of the transport by Triton and taurocholate then fits in with a more general scheme in which the dynamic formation of inverted micelles provides an explanation for lipid polymorphism and transbilayer transport in membranes [6,7].

3.2. *Effects at the phase transition*

The above rates of transport decrease with temperature until, with taurocholate, transport ceases at T_c. A similar behaviour is observed with A23187. This similarity is further evidence for a carrier-type mechanism by bile salt-inverted micelles.

The complete ^1H NMR spectrum of DPPC vesicles (60°C, 5 mM extravesicular Pr^{3+}) is shown in fig.2(a). Signals H and M are due to the acyl chain methylenes $(CH_2)_{14}$ and terminal methyls, respectively. On reducing the temperature to 38–40°C and returning it to 60°C little change in spectrum is seen. However, on repeated cycling through T_c (~10 min at 38–40°C, each cycle) the spectrum reveals that a small fraction of vesicles lyse completely (i.e., allow equilibration of the 5 mM Pr^{3+} across the bilayer) on each cycle. The spectrum after a number of such cycles is shown in fig.2(b), where partial transference is seen of signal I

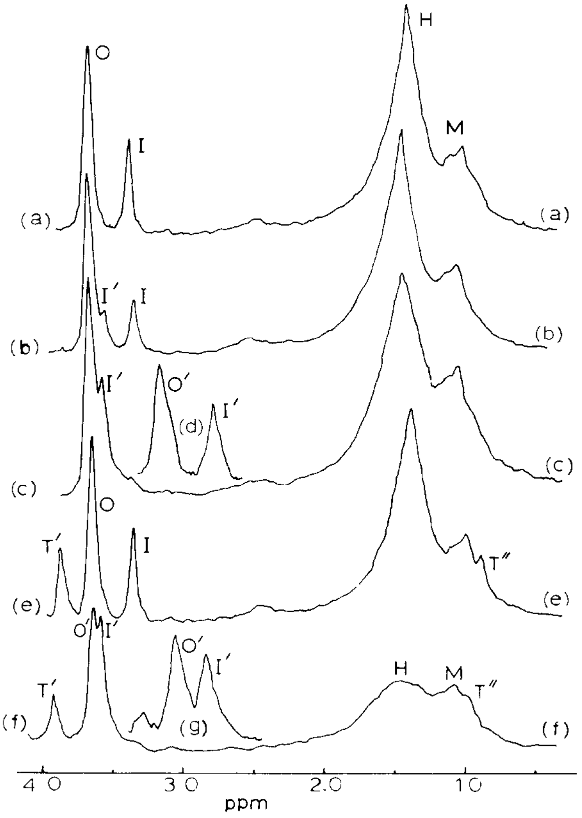

Fig.2. (a) ^1H NMR spectrum of dipalmitoyl phosphatidylcholine vesicles at 60°C in the presence of 5 mM extravesicular Pr^{3+}. Signals shown are: (O) extravesicular, (I) intravesicular head-groups, (H) acyl chain methylenes; (M) acyl chain terminal methyls. (b) The spectrum after continuing cycling of the vesicles through the phase transition temperature (38–40°C). (c) The spectrum after further cycling through the phase transition temperature over several days. Insert (d) shows the head-group region spectrum as in (c) following further addition of 0.2 mM Dy^{3+} to the vesicles. (e) The spectrum of vesicles as in (a) shortly after the addition of 1.8 mM extravesicular Triton X-100. Peaks T' and T'' are due to Triton X-100. (f) The spectrum of the vesicles plus Triton X-100 after lowering the temperature to the phase transition region (38–40°C) and then increasing it to 60°C. Insert (g) shows the head-group region spectrum as in (f) following the addition of 0.1 mM extravesicular Dy^{3+} to the vesicles. All vesicle samples are 34 mM (25 mg lipid/0.5 ml D_2O). Chemical shifts are shown with respect to external TMS.

(inside head-groups of unlysed vesicles) to signal I′ (inside head-groups of lysed vesicles). Eventually, after repeated cycles over several days, all the vesicles lyse (fig.2(c)). The insert, fig.2(d), shows the result of adding 0.2 mM Dy^{3+} to the lysed vesicles. Separation of signals O′ and I′ with area ratio O′ : I′ = 1.8 indicates that vesicular integrity and size are maintained during this lysis.

Fig.2(e) is the ^1H NMR spectrum at 60°C shortly after adding Triton X-100 to give an extravesicular concentration of 1.8 mM. (Signals T′ and T″ are from Triton.) On cooling to 38–40°C for 10 min, and reheating to 60°C, the spectrum appears as in fig.2(f). Complete transference of signal I to the shoulder I′ on O′ indicates complete lysis. Further addition of 0.1 mM Dy^{3+} (insert (g)) demonstrates vesicle integrity but indicates a fall in ratio of O′ : I′ to 1.2, corresponding to an increase in vesicle size to 85 nm. This, together with the partial collapse of signals H and M due to slower tumbling of the larger vesicles [18], indicates that limited fusion of the original 26 nm vesicles has occurred. Transmission electron microscopy using negatively stained vesicles also confirmed the presence of larger structures. Similar experiments using lower concentrations of Triton showed that at <1.6 mM (~250 Triton molecules/vesicle) only a fraction of the vesicles lysed on lowering to T_c.

The mechanism of increased permeability of phospholipid bilayers at T_c is still under discussion [19]. It is significant that direct evidence for transient pore formation at T_c has been obtained using planar lipid membranes [20]. The lysis described above at T_c is consistent with pore formation, but with a sufficiently high activation energy for opening the channels so that only some vesicles lyse during each passage through T_c.

The results using Triton then suggest that the pores are stabilised by Triton since only one cycle (at 1.8 mM) is sufficient to lyse all the vesicles. This is again consistent with the planar membrane studies where transient pore formation by Triton has been demonstrated [21]. Comparison of the results in [20,21] shows that the Triton-induced channels occur at lower voltages, and that larger currents over longer time periods are obtained, compared to those formed at T_c alone. It can be suggested that the pores stabilised by Triton are similar to those proposed for the polyene ionophore amphotericin [22] with folded Triton molecules lining the channels.

Also compatible with the two different mechanisms discussed above (carrier- and channel-mediated permeability) is the observation that in planar membranes both bile salts and Triton X-100 increase the permeability [4,5] but only Triton produces transient channels [21].

The observation that Triton X-100 also causes fusion of the vesicles is consistent with the behaviour of other amphipathic channel-forming ionophores such as alamethicin, which is also fusogenic [23,24]. The mechanism of membrane fusion most often proposed involves the formation of inverted micelles between the two fusing membranes [6,24] so that the carrier-type transport and fusion caused by Triton can have a common origin in inverted micelle formation. These mechanisms are exactly those recently put forward in connection with the occurrence of non-bilayer lipidic phases in membranes [6,7] and we are attempting to use the above NMR method to demonstrate transport and fusion with vesicles of appropriate mixed-lipid compositions [6]. The lytic process discussed above is best explained in terms of stabilised channels, but further kinetic studies with Triton involving the simultaneous measurement (from the ^1H NMR spectra) of the rates of lysis and fusion will be required to establish whether or not these two processes have a common mechanism.

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