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A ^{1}H-NMR INVESTIGATION OF THE MECHANISM FOR THE IONOPHORE ACTIVITY OF THE BILE SALTS IN PHOSPHOLIPID VESICULAR MEMBRANES AND THE EFFECT OF CHOLESTEROL

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Summary

A ^{1}H-NMR method previously applied to ionophore-mediated transport is used to investigate a similar behaviour by the bile salts. The permeability of phosphatidylcholine vesicles to Pr^{3+} is increased by several orders of magnitude over the self-diffusion rate and the kinetics indicate a transbilayer movement of inverted micelles {Pr(bile salt)$_4$}. In vesicles containing 40 mol% cholesterol the mechanism of permeability is radically altered and the di- and trihydroxy bile salts behave differently.

The need for a better understanding of the interaction of the bile salts with phospholipid bilayer membranes arises out of a number of recent developments in biomembrane research. These detergents are used in solubilisation and reconstitution of biological membranes [1], and in the formation of large vesicles [2] or vesicles without sonication [3] as model membrane systems. Knowledge of changes in permeability produced by residual bile salt in these processes is clearly important. When vesicles are used as drug-carrying systems and administered by the oral route, a principal challenge to their integrity and permeability is presented by interaction with the bile [4]. Problems concerning the stability of membranes in contact with bile remain unsolved [5] and understanding is poor of the role of the bile acids in formation of cholesterolic gall stones [6], which contain calcium [7], and in treatment of these stones by chenodeoxycholate and ursodeoxycholate [8]. A number of studies have implicated bile salts as having an effect on cation absorption or secretion, particularly of calcium [9] and copper [10], and recently the formation of complexes between calcium and glycocholate has

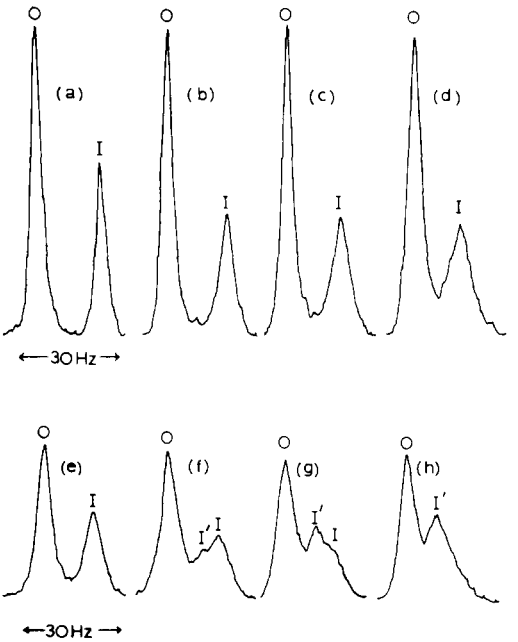

Fig. 1. (a) The head-group region ^{1}H-NMR spectrum of dipalmitoyl phosphatidylcholine vesicles (12.5 mg in 0.5 ml ^2H_2O) at 60°C in the presence of 5 mM extravesicular Pr^{3+}. Resolved signals are seen from the extravesicular (O) and the intravesicular (I) choline head-groups, $N(CH_3)_3$. Spectra b—d were recorded at intervals of 12 (b), 24 (c) and 59 min (d) after the addition of 0.8 mM chenodeoxycholate to the vesicles. (e) The head-group region ^{1}H-NMR spectrum of dipalmitoyl phosphatidylcholine vesicles containing 40 mol% cholesterol, in the presence of 5 mM extravesicular Pr^{3+} at 60°C. Spectra f—g were recorded at intervals of 1.5 (f), 5.33 (g) and 12.5 min (h) after the addition of 0.8 mM chenodeoxycholate to the vesicles. No further change in spectrum h was observed.

been demonstrated [11]. Each of these above areas implies a need to study membrane permeability produced by the bile salts and some indicate calcium permeability would be particularly interesting. In view of this, the use of the lanthanide ion, Pr^{3+}, which is a good probe ion for Ca^{2+} [12] is advantageous. Two recent reports have also indicated an increase in conductivity of planar lipid bilayers caused by bile salts [13, 14]. However, the NMR method described here has advantages of membrane stability over long periods and variable temperatures, the absence of perturbing solvents and the ability to distinguish various possible mechanisms of increased permeability. Finally, the results described below support recent proposals on the mechanism of membrane permeability based on lipid bilayer polymorphism [15] and phospholipid partition studies in the presence of cholate [16].

The vesicular membranes were prepared by sonication of the dipalmitoyl phosphatidylcholine in ^2H_2O, as described previously [17], to give a concentration of 25 mg/ml. This lipid was chosen in order to be able to compare transport rates with our previous studies [18, 19] and to investigate effects at the phase transition temperature, T_c (approx. 40°C) [17]. The ^{1}H-NMR spectra were obtained using a JEOL C60HL spectrometer fitted with a calibrated temperature control. When the extravesicular solution is adjusted to 5 mM Pr^{3+} by addition of stock solution of $PrCl_3$ in ^2H_2O, the NMR spectrum reveals separated signals from the extravesicular (O) and intravesicular

(I) choline head-groups (see Fig. 1a). Such separations of signals are now well documented [20] and result principally from a down-field shift of the extravesicular head-group signal due to dipolar interaction with the paramagnetic Pr^{3+}_{aq}. When Pr^{3+} diffuses or is carried across the lipid bilayer into the intravesicular solution, the rise in intravesicular concentration, $[Pr^{3+}]_i$, causes the signal, I, to move down-field towards signal O (see Fig. 1 b—d). By measuring the decreasing shift difference, $\Delta\nu_{O-I}$, between O and I at intervals, the rate of diffusion or transport can be obtained. This method was previously applied to follow the kinetics of Pr^{3+} transport by the calcium ionophore, A23187 [19]. Since these initial studies it has become clear that in the presence of Pr^{3+}, the stability of the vesicle is such as to allow transport or diffusion rates to be reproducibly measured over much longer time scales, even up to several weeks if required. Measured by this technique, the unfacilitated diffusion of Pr^{3+} into the intravesicular solution is slow even at 60°C, so that to give an intravesicular concentration of 2.5 mM a time of approx $5 \cdot 10^3$ min is required. Since the internal volume of the vesicles is only a few per cent of the total volume, the extravesicular concentration of Pr^{3+} remains effectively constant at 5 mM. An average value for the self-diffusion rate at 60°C measured by the movement of signal I is $8.3 \cdot 10^{-4}$ Hz·min^{-1}, which can be converted using a suitable calibration graph [19] to $1.4 \cdot 10^{-4}$ mM Pr^{3+}/min.

In order to study the effect of bile salts, stock solutions (0.1 or 0.05 M) were prepared in ^2H_2O of sodium cholate, glycocholate, deoxycholate and chenodeoxycholate. Aliquots (typically 0—15 μl) were pipetted into the NMR tubes containing 0.5 ml sonicate (12.5 mg dipalmitoyl phosphatidylcholine) and 5 mM extravesicular Pr^{3+} at 60°C. The initial extravesicular concentrations of the bile salts thus could be varied from 0.1 to approx. 5 mM, i.e., from well below and up to the critical micelle concentration, which is in the region of 2—15 mM depending on the bile salt used [1]. This range of bile salt concentrations gave half-completion times (i.e., $[Pr^{3+}]_i$ = 2.5 mM) between several days and a few minutes. The changes in $\Delta\nu_{O-I}$ for a typical run using 0.8 mM chenodeoxycholate are shown in Fig. 1b—d. For the slower experiments (and the self-diffusion, control runs), the plots of $\Delta\nu_{O-I}$ against time are linear over the initial 0—4 Hz. At faster rates, the plots are curved and the initial rates are used. Part of the curvature is due to the inability to apply the calibration plot of $[Pr^{3+}]_i$ against $\Delta\nu_{O-I}$ as used previously for ionophores [19], since at concentrations greater than about 1 mM the interaction of the negatively charged bile salt molecules induces a larger initial splitting between signals O and I. This effect is due to a decrease in ζ-potential at the vesicle outer surface, thus allowing increase of binding of the Pr^{3+} to the phosphate groups [21].

The effect of varying the concentration of the four bile salts used (between approx. 0.2 and approx. 5 mM) is shown in Fig. 2 where $\log_{10}$ (rate $\times$ 10^3 /Hz·min^{-1}) is plotted against $\log_{10}$ (molar concentration of bile salt $\times$ 10^4). The rates increase by over 2 orders of magnitude in this concentration range, i.e., from approx. $1 \cdot 10^{-3}$ to approx. $5 \cdot 10^{-1}$ Hz·min^{-1}. The slope of each of these plots is close to 4, suggesting the cooperativity of four bile salt molecules in the rate-determining step for the transport of Pr^{3+}. Now, while

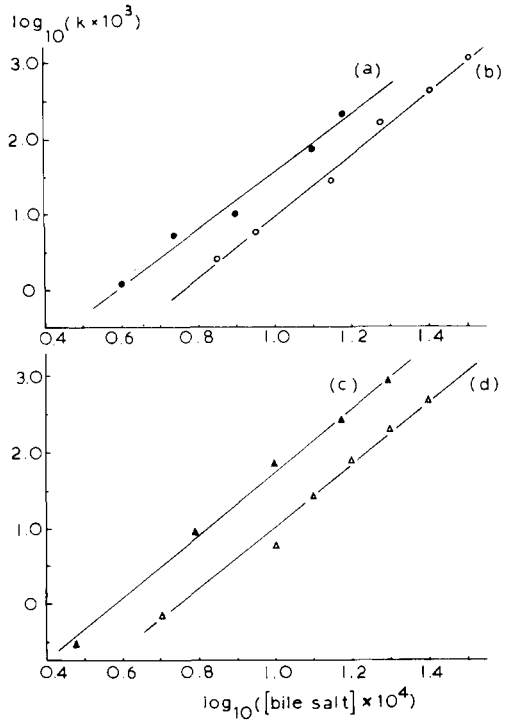

Fig. 2. The effect of concentration of the four bile salts, (a) sodium glycocholate (b) sodium cholate, (c) sodium chenodeoxycholate, (d) sodium deoxycholate, on the rate of transport of Pr^{3+} into dipalmitoyl phosphatidylcholine vesicles at 60°C. The rate, k ($Hz \cdot min^{-1}$), is determined from the changes in the spectrum as shown in Fig. 1a–d. [bile salt], initial extravesicular molar concentration of bile salt. The slope of each line, as calculated by least-squares analysis from the data points shown is (a) 3.91 (r, correlation coefficient, = 0.989); (b) 4.16 (r = 0.996); (c) 4.01 (r = 0.995) and (d) 4.20 (r = 0.990).

conductivity studies with planar bilayers give rather variable stoichiometries [13, 14], vesicle systems give more consistent results [19, 22] and we have recently demonstrated (Hunt, G.R.A., unpublished data) that the NMR method used here gives an experimental stoichiometry of 2.8 for a transported complex, $Pr(fod)_3$, where fod is a lipophilic fluorinated diketone, C_3F_7-CO-CH_2-CO-$C(CH_3)_3$, often used in NMR shift reagents [23].

Having confidence that the slope obtained in Fig. 2 does represent a true stoichiometry, we can suggest a mechanism for the increase in permeability caused by the bile salts. Small [24] has already pointed out evidence for the existence of inverted bile salt micelles in lipid bilayers, these being aggregates of two to four 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. We propose that in the presence of Pr^{3+}, such inverted micelles are stabilised by incorporating the ion in the hydrophilic cavity and the translation of the micelle across the bilayer acts as the mechanism of permeability. This translation may also include lipid molecules as part of the micelle structure.

The lowest limiting concentration of each bile salt, below which the rate is no faster than the self-diffusion rate, is approx. $3 \cdot 10^{-4}$ M, corresponding to about 50 molecules of bile salt per vesicle. Since this concentration is below the critical micellar concentration, monomers in the aqueous phase will

partition into the vesicles, where aggregates will form. It is therefore suggested that when the bile salt concentration is raised above this threshold value, the equilibrium concentration of tetramer inverted micelles in the vesicles becomes sufficient to raise the permeability above the self-diffusion rate. Recently, the dynamic formation of inverted micelles in lipid bilayers has been proposed as an explanation for lipid polymorphism and transbilayer transport in membranes [15, 16]. While other mechanisms such as flip-flop or extravesicular micelle formation [25] have been suggested to explain the self diffusion of metal ions, it seems more reasonable to propose that even in phosphatidylcholine bilayers which are not macroscopically polymorphic (as directly detectable by physical methods) the very slow rate of diffusion of Pr^{3+} takes places by means of a small equilibrium concentration of inverted micelles. The acceleration of this process by the presence of bile salts then fits neatly into one scheme. A similar suggestion has been made recently by Green et al. [16].

It should be noted that during both the self-diffusion and the facilitated transport by A23187 or the bile salts, the spectra reveal no sign of lysis of the vesicular membranes. The ratio of the signal areas, O:I, remains at 1.8 during the down-field movement of the intravesicular head-group signal I. If lytic processes occur (i.e., disruption of the membranes, or production of pores or holes which allow equilibration of the 5 mM Pr^{3+} across the membrane) then an extra shoulder to signal O appears, due to the inner head-group signal from those vesicles which have lysed, while signal I does not move steadily down-field but decreases in area (due to the decreasing population of unlysed vesicles). These changes in spectrum consequent on lysis have been reported by us previously, e.g., for dipalmitoyl phosphatidylcholine vesicles containing 15 mol% cholesterol [17].

At 38—40°C, i.e., within the phase-transition temperature range for these vesicles [17], the bile salt-mediated transport was found to cease. The same result was observed with Pr^{3+} transport by A23187. These observations also argue for a carrier-type mechanism involving bile salt micelles, rather than a channel-type mechanism.

Using transport rates obtained using a fixed concentration of bile salt at five different temperatures between 45 and 70°C, Arrhenius plots were constructred and the following activation energies obtained (in $kJ \cdot mol^{-1}$): deoxycholate, 51.7; glycocholate, 59.4; cholate, 65.1. These values are, however, considerably lower than those so far reported for carrier-type ionophore-mediated transport of Mn^{2+} [22] or Pr^{3+} [19]. There is also considerable difference in the concentration dependence of the Pr^{3+} transport by bile salts and A23187 in this system. As noted above, the limiting concentration for bile salt is approx. $3 \cdot 10^{-4}$ M, while for A23187 it is approx. $1 \cdot 10^{-6}$ M (Hunt, G.R.A., unpublished results). No doubt, part of this difference is due to the much greater partition of the bile salts into the aqueous phase.

Some exploratory investigation of the effect of incorporation of cholesterol into the vesicular membranes was also undertaken since, as mentioned in the introductory paragraphs, the interaction between bile salts, phospholipids and cholesterol is physiologically important. At 10 mol% cholesterol the bile salt-mediated rates were increased by a factor of about 4. In contrast,

the self-diffusion rate was decreased from 0.8 to 10^{-3} to $0.4 \cdot 10^{-3}$ $Hz \cdot min^{-1}$. At 40 mol% cholesterol the self-diffusion rate remained at $0.4 \cdot 10^{-3}$ $Hz \cdot min^{-1}$, but the effect of addition of bile salts was quite different. The steady down-field shift of signal I was not observed with any of the four bile salts, indicating that the presence of 40 mol% cholesterol prevents the diffusion of Pr^{3+} by $\{Pr(\text{bile salt})_4\}$ micelles. This may be due to the formation of non-transporting, bile salt/cholesterol micelles or to a decrease in fluidity of the bilayer.

We also observed that the trihydroxy and dihydroxy bile salts behave differently at this concentration of cholesterol. Thus, addition of cholate or its conjugate, glycocholate, caused little change in the head-group spectrum from the of Fig. 1e, until at approx. $0.6 \cdot 10^{-3}$ M a very rapid increase in line width was observed with increase in turbidity of sample (but without precipitation of lipid). Such a change is consistent with immobilisation of the head groups with very large structures being formed by fusion of the vesicles. These changes are currently being examined further using ^{13}C-NMR and electron microscopy. In contrast, addition of deoxycholate or chenodeoxycholate produced changes in the spectrum as shown in Fig. 1f—h. A new peak, I′, progressively appears as I disappears. The broadened nature of I′ is typical of intravesicular head-group signals when Pr^{3+} has entered the vesicles (see Fig. 1b—d and Ref. 19): in this case, approx. 2 mM Pr^{3+} has entered the vesicles, as indicated by the down-field position of I′ with respect to I. The transference of intensity from I to I′ indicates a time-dependent change from a sample in which all vesicles have $[Pr^{3+}]_i = 0$, to a sample in which all vesicles have $[Pr^{3+}]_i \sim 2$ mM, with the mechanism allowing a gated pulse of Pr^{3+} across the bilayer. Since the 40 mol% cholesterol is likely to be asymmetrically distributed between the two monolayers of the vesicle [17, 26], then equilibration of bile salt from the extravesicular aqueous phase into the outer monolayer may result in bilayer instability. If pores appear in the membrane, only while a transbilayer compositional readjustment takes place, then this will provide a mechanism for the gating process and the above spectral changes can be explained. These observations and the proposed interpretation are similar to that described above for lysis of the vesicles, except that with 40 mol% cholesterol a gated pulse of Pr^{3+} is allowed into the vesicles such that $[Pr^{3+}]_i$ rises only to approx. 2 mM.

We are currently examining the effect of bile salts on other compositions of lipid bilayer in order to clarify further the mechanisms involved in these changes. The present report demonstrates the ionophore activity of bile salts in vesicular systems and indicates that NMR spectroscopy can be useful in distinguishing different mechanisms of permeability.

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