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Bilirubin diffusion through lipid membranes

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The possibility that bilirubin can diffuse through lipid bilayers is investigated with liposomes prepared from dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (egg PC) with 22 mole percent cholesterol, and a lipid extract preparation from N115 neuroblastoma cells. Liposomes were prepared with internalized bilirubin and bovine or human serum albumin, and bilirubin efflux into an exogenous solution of human serum albumin was measured. Efflux from DPPC liposomes was significantly higher above the phase transition temperature than below it. This change was dependent on the lipid undergoing a phase transition and could not be accounted for by 6 K change in temperature. Maximum bilirubin efflux from egg PC-cholesterol liposomes was found to depend on the relative internal and external albumin pools, suggesting an equilibrium distribution of bilirubin between them. These observations demonstrate that bilirubin can diffuse freely through these lipid membranes.

Bilirubin is a bile pigment produced by the catabolism of hemoproteins. Under certain clinical conditions, bilirubin can be toxic to the central nervous system of the newborn infant [1]. While the mechanism of cytotoxicity has not yet been completely elucidated, numerous studied have demonstrated that the activities of several membrane bound enzymes are disturbed as a result of bilirubin binding to the lipid regions of cellular and intracellular membranes [2]. While much work has been done relating to the nature of bilirubin binding to lipid bilayers, the mechanism of bilirubin transport through the membrane, if in fact it enters the cell, remains unclear. In this study, we investigated the possibility that bilirubin can pass through phospholipid bilayers by simple diffusion alone.

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Our experimental model involved measuring efflux of tritiated bilirubin from liposomes into which it had been incorporated. Three types of liposomes, differing in their lipid compositions, were prepared with an internal solution of bilirubin and bovine serum albumin (BSA). An attempt was then made to extract this bilirubin from the intact liposomes into an exogenous solution of human serum albumin (HSA). HSA has a much higher affinity for bilirubin than BSA [3], and this differential affinity represents the 'driving force' for bilirubin efflux.

Tritiated bilirubin was extracted from bile collected from Sprague-Dawley rats into which the precursor δ-amino[3,5(n)-^{3}H]levulinic acid (New England Nuclear) had been injected [4]. [^{3}H] Bilirubin was purified from the bile as described by McDonagh [5], and was found to contain more than 98% bilirubin IXα by HPLC (absorption at 454 nm). Bovine serum albumin and human serum albumin were obtained from Sigma (fraction V,

essentially fatty acid free). Bilirubin-albumin solutions of varying molar ratios were prepared in 0.05 M Tris buffer to a final bilirubin concentration of 115 μM, and a pH of 8.3.

The three types of liposomes were prepared from: dipalmitoyl-DL-α-phosphatidylcholine (Sigma, Grade 1, approx. 99%, crystalline, synthetic); L-α-phosphatidylcholine from eggs (Sigma type XI-E, approx. 99%) with 22 mole percent cholesterol (Sigma, Chromatography grade); and a lipid extract preparation from N115 mouse neuroblastoma cultures. N115 lipid extracts were prepared as described by Bligh and Dyer [6], and found to contain 21.6 mole percent cholesterol relative to total lipid [7,8].

Chloroform solutions of these lipids were prepared to contain 62.5 μmol of phosphatidylcholine. In each case, 6.0 nmol cholesteryl [^{14}C]oleate (New England Nuclear) was included as a marker to determine liposomal integrity, and 32 nmol butylated hydroxytoluene was added as an antioxidant which did not appear to affect the permeability of the bilayers. These solutions were then evaporated in vacuo for 4 h. The dried lipid was then dispersed with the bilirubin-albumin solution to a phospholipid concentration of 4.2 μM by agitating for two minutes at room temperature (or 50°C for DPPC dispersions). The multilamellar liposomes were collected by centrifugation at 10000 $\times$ g and washed four times with Tris buffer (pH 8.3). All operations were performed under dim red light to prevent photoisomerization of bilirubin IXα. All aqueous solutions were saturated with N_2 gas.

Having prepared the liposomes, samples were taken for analysis of bilirubin uptake. It was observed that when the lipid was dispersed with a solution of bilirubin and BSA in a molar ratio of 1 : 1, incorporation of bilirubin by the liposomes was as high as 385 nmol bilirubin per mmol of phospholipid at pH 8.3. As consecutively greater molar equivalents of BSA were included in the dispersing solution (keeping bilirubin constant), incorporation of bilirubin into the liposomes decreased greatly. It was found, however, that at bilirubin : BSA molar ratios of 1 : 8 or less, bilirubin uptake remained constant at a minimum value of 16.6 $\pm$ 0.5 nmol per mmol phospholipid. These results were interpreted to reflect a partition of bilirubin between the aqueous albumin and the phospholipid bilayers. As the ratio of albumin to bilirubin increases, this partition shifts towards the albumin, and bilirubin dissolution into the forming bilayers is minimized. Hence, it was assumed that at bilirubin: BSA ratios of 1 : 8 or less, virtually all the bilirubin taken up by the liposomes was bound to albumin molecules in internal aqueous solution.

DPPC, egg PC-cholesterol, and N115 lipid extract liposomes were prepared with a solution of bilirubin and BSA in a molar ratio of 1 : 8 and then incubated in a Tris-buffered 20.0 μM solution of HSA. Egg PC-cholesterol, and N115 liposomes were incubated at 37°C. DPPC liposomes were incubated above (43°C) and below (37°C) the gel-liquid crystalline phase transition temperature for DPPC vesicles (41.0°C [9]). At specified time intervals, aliquots of each suspension were taken, the liposomes spun down, and the supernatant analyzed for [^{3}H]bilirubin by liquid scintillation counting. In addition, samples of the supernatant were extracted with chloroform/methanol (2 : 1, v/v) and bilirubin content was determined by HPLC (absorbance at 454 nm). This was necessary to confirm that tritium counts in the HSA extraction media represented intact bilirubin IXα, and not unknown decomposition products.

As can be seen in Fig. 1, it was possible to extract most of the bilirubin from DPPC liposomes above the gel-liquid crystalline transition temperature within 15 min. In contrast, bilirubin efflux from DPPC liposomes at 37°C, below the

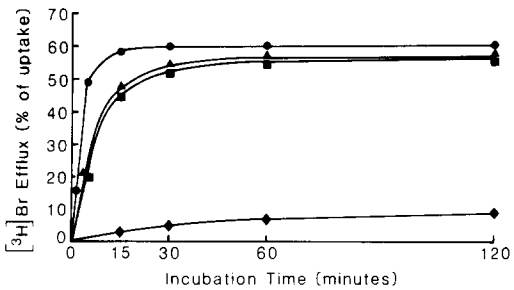

Fig. 1. [^{3}H]Bilirubin ([^{3}H]Br) efflux from DPPC liposomes at 43°C (●), egg PC-cholesterol liposomes at 37°C (▲), N115 lipid extract liposomes at 37°C (■), and DPPC liposomes at 37°C (◆). Each point represents the average of eight separate determinations, and the standard error of the mean was not significant in each case.

phase transition temperature, was comparatively very slow and reached a maximum of only 8% after 2 h. This lipid phase dependent efflux appears to be consistent with a model of simple diffusion of bilirubin through the bilayer.

It can also be been in Fig. 1, that bilirubin efflux from egg PC-cholesterol and N115 lipid extract liposomes was similar to that from the DPPC liposomes in the liquid-crystalline state, although the initial rate of efflux and the maximum bilirubin efflux were somewhat less. Furthermore, the efflux profiles from the egg PC-cholesterol liposomes and the N115 lipid extract liposomes were virtually superimposable, demonstrating the usefulness of the egg PC-cholesterol system as a model to replace expensive cellular lipid extracts.

In all cases, the fraction of cholesteryl [^{14}C]oleate detected in the extraction medium did not exceed 0.5% of that incorporated into the liposomes. This demonstrates that the liposomes were stable, and that the bilirubin efflux was not the result of liposomal breakage and the formation of smaller lipid structures. Measurements of bilirubin efflux made by HPLC were very similar to those made by liquid scintillation counting, confirming that the tritium counts in the extraction medium did in fact represent bilirubin IXα.

The model described above for investigating bilirubin movement across lipid bilayers, involves measuring efflux of bilirubin from liposomes into which it has been incorporated. Therefore it was imperative to demonstrate that the bilirubin initially 'incorporated' into the liposomes was in fact in aqueous solution within the liposome. This was especially so since various studies have shown bilirubin binding to lipids in vesicular suspension, either through relatively strong ionic interactions between anionic bilirubin and the polar head groups of various phospholipids [10–12], or as aggregates of bilirubin acid incorporated into the apolar regions of the bilayer [12,13]. Extraction of bilirubin bound to liposome membranes in such a way would not demonstrate bilirubin diffusion across lipid bilayers per se.

As mentioned above, we are confident that with the bilirubin : albumin molar ratios used to generate the liposomes, bilirubin binding to, or dissolving in the bilayers was minimized, and that the bilirubin was stabilized in internal aqueous solution. Nevertheless, we found that it was possible to examine liposomal membrane permeability to bilirubin with a method in which the initial placement of bilirubin in the liposome was not important. In this approach, we attempted to demonstrate that the maximum bilirubin efflux from the liposomes actually reflects an equilibrium distribution of bilirubin between internal and external albumin pools. Taken together, the quantity of albumin (binding capacity), as well as the type of albumin (binding affinity), determine the binding potential of either albumin solution. Providing that the liposomes are permeable to bilirubin, altering the binding potential of either the internal or external albumin pools would be expected to elicit a shift in the final distribution of the bilirubin between these two pools.

This is consistent with the finding that the maximum amount of bilirubin that could be extracted from egg PC-cholesterol liposomes was a function of the quantity of HSA in the extraction medium. When the liposomes were incubated in Tris buffer without HSA, less than 1% of the internalized bilirubin could be recovered. As the amount of external HSA was increased, the maximum bilirubin efflux also increased.

To examine the effect of internal albumin on maximum efflux, three types of egg PC-cholesterol liposomes were prepared, differing only in the internal albumin pool. Type 'A' liposomes were prepared with an internal bilirubin : BSA ratio of 1 : 8; type 'B' with a bilirubin : BSA ratio of 1 : 12; and type 'C' with a bilirubin : HSA ratio of 1 : 8. Incorporation of bilirubin into the three types of liposomes was measured and found to be the same, varying by less than 3 percent. These liposomes were then incubated at 37°C in a Tris-buffered HSA solution (2.0 μM), and bilirubin efflux measured at specific time intervals as before.

Bilirubin efflux from type 'A' and 'B' liposomes is compared in Fig. 2A. Efflux from type 'A' liposomes reaches a maximum of 35% compared to 27% for type 'B' liposomes. It can be seen that increasing the amount of internal albumin relative to a external bilirubin has the effect of reducing bilirubin efflux out of the liposomes. A similar comparison is made in Fig. 2b

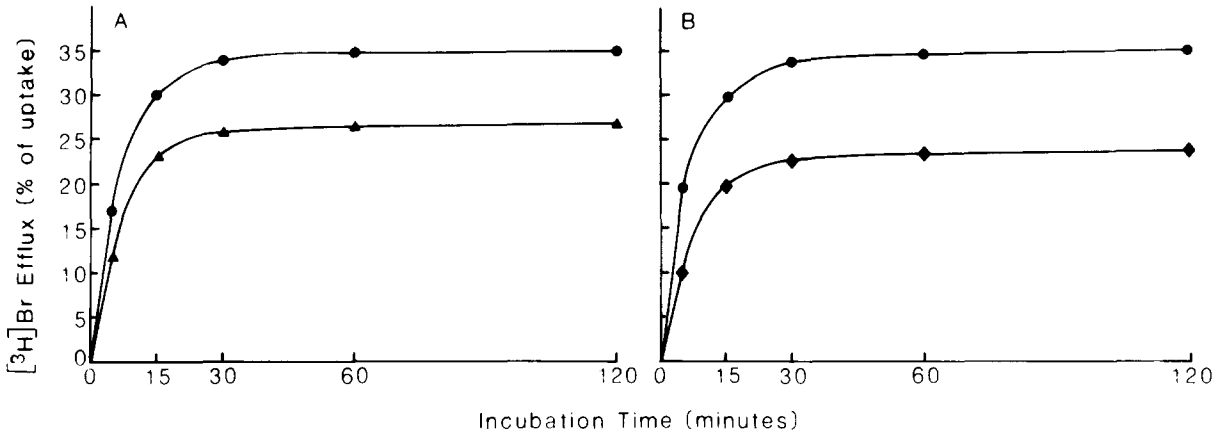

Fig. 2. Efflux of [^{3}H]bilirubin ([^{3}H]Br) from egg PC-cholesterol liposomes prepared: (A) with internal bilirubin : BSA molar ratios of 1 : 8 (●) (Type 'A') and 1 : 12 (▲) (Type 'B'), and (B) with internal bilirubin : BSA (●) (Type 'A') and bilirubin : HSA (◆) (Type 'C') in molar ratios of 1 : 8 at 37°C. Each point represents the average of six separate determinations, and the standard error of the mean was not significant in each instance.

between efflux from type 'A' liposomes and type 'C' liposomes. Bilirubin efflux from liposomes containing HSA (which has a higher affinity for bilirubin than BSA), was significantly less than from liposomes containing the same molar quantity of BSA -- efflux from type 'C' liposomes reaches only 24% compared to 35% for type 'A' liposomes.

The fact that bilirubin is in equilibrium between the internal and external albumin pools, appears to be established by the observation that altering the binding potential of either pool results in a shift in the maximum bilirubin efflux. Yet, if bilirubin were merely in simple equilibrium between internal and external albumin pools, one would expect that it should be possible to make calculations about the steady-state distribution of bilirubin given the relative sizes and types (or binding potential) of both albumin pools. In fact, no such equation was possible. As can be seen in Fig. 1, even when external HSA was in considerable excess to internal BSA (30 : 1), only 60% of the bilirubin could be extracted from egg PC-cholesterol liposomes after 2 h of incubation. Further increases in external HSA had a minimal effect on the maximum efflux. Even if the liposomes were then pelleted and resuspended in fresh HSA, only a small fraction of the remaining bilirubin could be recovered.

One possible explanation to account for this observation is that the bilirubin may have been loading up in the lipid bilayers in a way that was not completely reversible. Eriksen et al. [12] have reported the formation of bilirubin aggregates containing small amounts of phospholipid in the apolar regions of vesicular bilayers. The dissolution of these aggregates was found to be incomplete even under favourable conditions. In the present study, it was observed that maximum bilirubin efflux under any given set of conditions, varied with the composition of the liposome. It was found that maximum efflux was significantly higher from liposomes prepared from egg PC alone, than it was when increasing proportions of cholesterol were included in the liposomes. This seems consistent with the finding of Nagoaka and Cowger [10] that the inclusion of 5% cholesterol in DPPC liposomes significantly increases the association constant for bilirubin (as determined by a static fluorescence quenching method), over that of liposomes prepared from DPPC alone.

If this hypothesis of bilirubin loading in the membrane is valid, bilirubin would be expected to be distributed proportionately between internal and external albumin pools, as well as the lipid bilayer itself. Whatever complicating factors exist, however, they do not negate the observation that varying the internal and external albumin pools

effects the final distribution of bilirubin. These findings suggest that internal and external albumin pools are exerting competitive influences for bilirubin binding, and this fact necessitates the movement of bilirubin across the liposome bilayer, qualitatively substantiating the hypothesis that bilirubin can pass through these various membrane systems by simple diffusion alone.

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