A High-Pressure, Infrared Spectroscopic Study of the Solvation of Bilirubin in Lipid Bilayers†

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ABSTRACT: The location of bilirubin IXa in lipid bilayers of dimyristoylphosphatidylcholine or dioleoylphosphatidylcholine was studied by determining the effects of bilirubin on the infrared spectra of the lipids as a function of pressure. It was found for both bilayers that bilirubin intercalated into the polymethylene chain region of the bilayer, being located between the carbonyl region and the methylene group two carbons from the methyl terminus. Small amounts of bilirubin interacted with the choline region of dioleoylphosphatidylcholine. Lesser amounts interacted with the choline region of dimyristoylphosphatidylcholine. This difference between the two types of bilayers was attributed to the degradation of small amounts of bilirubin IXa to more polar isomers in the presence of dioleoylphosphatidylcholine. In dioleoyl- but not dimyristoylphosphatidylcholine, bilirubin interacted with the C=O region, probably indicating that bilirubin in the latter type of bilayer was intercalated into the polymethylene chains above and below the double bond. Bilirubin decreased the pressure required for the liquid-crystal to gel-phase transition in both bilayers at 28 °C. Bilirubin was not forced out of either bilayer at pressures as high as 20 kbar.

A general mechanism by which cells respond to changes in the environment is transport of substances across plasma membranes. The pathway for transport depends on the size and the physical chemical properties of the material being transported, as, for example, the pathway of receptor-mediated endocytosis for large proteins or transport of small polar molecules via protein channels. A relatively neglected aspect of transport across plasma membranes is that for nonpolar compounds. This is an especially interesting problem because, as compared with polar compounds, apolar compounds dissolve in membranes and can move across them passively by diffusion (Benz, 1988; Cabral et al., 1987; Daniels et al., 1985; Flewelling & Hubbell, 1986; Jordan & Stark, 1979; le Maire et al., 1987; Lieb & Stein, 1969). The entry of nonpolar compounds into cells hence could be independent of biological mechanisms, i.e., depend only on the physical–chemical properties of membranes and water-insoluble substances. This is contrary to the generally accepted view that the entry of nonpolar compounds into plasma membranes and transmembrane movement are mediated by binding proteins and transport proteins (Abumrad et al., 1981, 1984; Berk et al., 1987; Sottocasa et al., 1982; Stremmel et al., 1983; Stremmel, 1987; Wolkoff & Chung, 1980), but in the limited instances in which the idea of spontaneous entry and diffusion of nonpolar compounds across plasma membranes of cells has been tested, it has been validated (Cooper et al., 1987; Noy et al., 1986, 1990).

The factors determining the solubility of nonpolar compounds within the lipid bilayer region of a biological membrane obviously can be important for the functional integrity of the membrane and thus for the toxicity of certain compounds. In addition, however, the solubility of a nonpolar compound within the plasma membrane of a cell can determine its rate of entry into a cell, its rate of metabolism, and its concentration in the cell (Cooper et al., 1987; Noy et al., 1986). Since the concentrations of complexes between membrane carrier proteins and water-insoluble ligands will depend on the concentration of the ligand within the plane of the membrane, this concentration also can determine the rate of entry of water-insoluble compounds into cells even when transbilayer movement is facilitated.

The solubility and distribution of an apolar compound within the lipid regions of a plasma membrane will depend on its relative avidity for different regions within the membrane, e.g., the lipid–water interface, the carbonyl region, the methylene chain region, and perhaps the middle of the bilayer. The therapeutic and toxic effects of chemicals and the rates and tissue specificities of uptake of water-insoluble chemicals could be modulated in theory, therefore, by the forces determining how apolar compounds interact with these different regions in membranes and with carrier proteins in blood. Possibly, advantage could be taken too of selective interactions between water-insoluble compounds and certain types of membrane structures so as to direct compounds to specific kinds of membranes. Moreover, the carrying capacity of unilamellar bilayers used as vehicles for the delivery of agents to selected sites in the body probably can be modulated for a specific nonpolar compound by varying the lipid composition of the carrier vesicles. Describing the detailed interactions between membrane lipids and water-insoluble compounds appears therefore to be a problem with applications to many unresolved issues in pharmacology, physiology, and biochemistry. The purpose of the experiments reported in this paper was to examine the specific location of bilirubin in lipid bilayers. The method used for studying this problem was the effects of pressure on the infrared spectra of different structural regions of bilayer membranes containing bilirubin (Auger et al., 1987; Wong, 1987; Wong & Mantsch, 1985a). The results of this experimental approach are reported below.

Materials and Methods

Bilirubin IXa was purchased from Fluka and recrystallized (McDonagh & Assisi, 1971) before use. Thin-layer chro-

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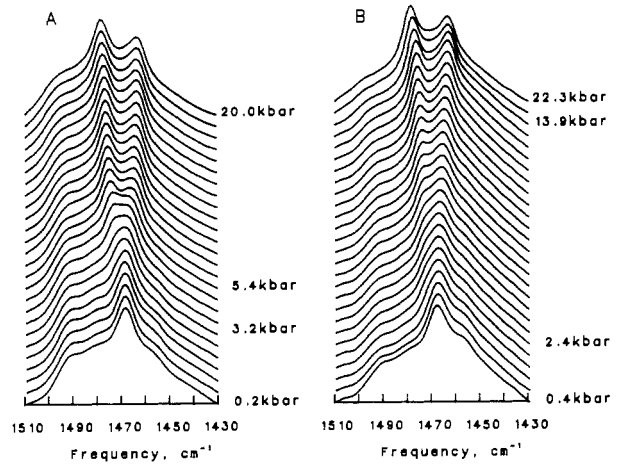

FIGURE 1: Stacked contour plots of the infrared spectra of DMPC (A) and DMPC plus bilirubin (B) in the CH_2 scissoring mode region.

matography of the recrystallized sample confirmed that it was the IXa isomer. Lipids were purchased from Avanti.

Bilirubin was added to hydrated multilamellar vesicles of lipids as follows. Dry dimyristoylphosphatidylcholine (DMPC)[1] or DOPC was added to a small polyallomer tube, and then solid bilirubin was added. D_2O was added so that the final concentration of lipid was 300 mg/mL. The concentration of bilirubin was 4 mol % in the lipids. An amount of NaOH equal to bilirubin was added, and the tubes were mixed vigorously for about 10 min on a Vortex mixer. The above procedures were carried out in dim light. Examination of the mixtures under a microscope indicated that the bilirubin was taken up completely by the lipids. Alternatively, bilirubin and pure lipids were dissolved in $CHCl_3$ and dried under a stream of N_2. After lyophilization, D_2O was added, and the mixtures were shaken vigorously. Results reported below were independent of the method used to add bilirubin to the lipid bilayers.

Small amounts of the homogeneous samples were placed at room temperature, together with powdered a-quartz, in a 0.37-mm-diameter hole in a 0.23-mm-thick stainless-steel gasket mounted on a diamond anvil cell as described previously (Wong et al., 1985). The a-quartz was used as an internal pressure calibrant, and pressures were determined from the frequency of the 695 cm^{-1} infrared phonon band of the a-quartz.

Infrared spectra were measured at 28 °C on a Digilab FTS-60 Fourier-transform spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector. A total of 512 interferograms were co-added for each spectrum. The spectral resolution was 4 cm^{-1}. Data reduction was performed by using software developed at NRCC, Ottawa Laboratory. The y scale is absorbance for all spectra in the figures.

RESULTS

Effects of Bilirubin on Pure Bilayers of DMPC. Shown in Figure 1 are stacked contour plots of the infrared spectra of DMPC, at increasing pressure, for the CH_2 scissoring region. The spectra in Figure 1A are for the pure lipid. Those in Figure 1B are for DMPC plus 4 mol % bilirubin. The spectra at atmospheric pressure were essentially the same for bilayers with and without bilirubin, with a single peak at 1467.7 cm^{-1}. This peak reflects rapid reorientational fluctuations of the relatively disordered acyl chains. As pressure is increased, the acyl chains become increasingly ordered so that a pressure-induced correlation field splitting of the CH_2 scissoring mode occurs (Wong, 1984; Wong & Mantsch, 1985b). A shoulder on the high-frequency side of the peak is the first manifestation of the pressure-induced correlation field splitting. At still higher pressure, a distinct second peak appears, which has a greater intensity than the low-frequency peak. This was the pattern displayed by bilayers of pure DMPC (Figure 1A). The spectra for DMPC plus bilirubin became different from those for DMPC at pressures that induced the correlation field splitting in pure DMPC. A correlation field splitting was observed for DMPC plus bilirubin. In fact, bilirubin decreased the pressure for the onset of the correlation field splitting for the CH_2 scissoring mode from 3.2 kbar in pure DMPC to 2.4 kbar in DMPC plus bilirubin (Figure 2A). This result means that bilirubin caused the acyl chain region of DMPC to be more orientationally ordered as compared with pure DMPC. This conclusion is predicated on the idea that the pressure needed to completely damp the reorientational fluctuations of the acyl chains depends on the initial state of orientational disorder. Higher pressures will be needed to damp the motions of more disordered chains.

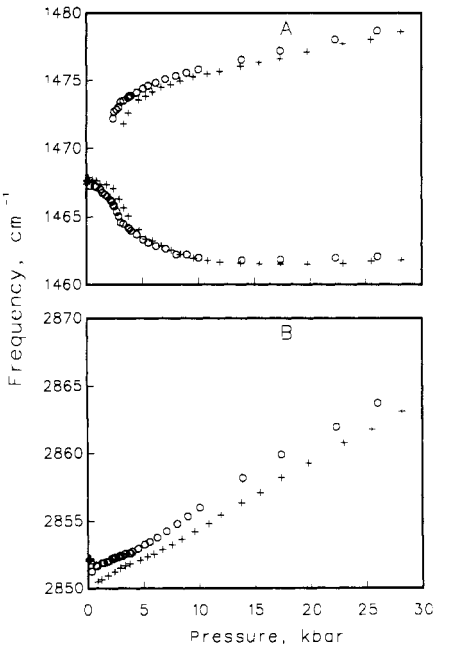

FIGURE 2: Pressure dependence of frequencies of the CH_2 scissoring mode (A) and the symmetric CH_2 stretching mode (B) of DMPC (+) and DMPC plus bilirubin (O).

Although the pressure needed to induce the correlation field splitting was lower in bilayers of DMPC plus bilirubin as compared with pure DMPC, the intensity of the low-frequency peak in DMPC plus bilirubin remained greater than that for the high-frequency peak until very high pressures were reached. The relationship between the intensities of the two peaks of the correlation field component bands is seen clearly in Figure 3. These data show for pure DMPC that the intensity of the high-frequency peak exceeded that for the low-frequency peak at about 5 kbar. This crossover of intensities did not occur for DMPC plus bilirubin until about 13 kbar. The effect of bilirubin on the distribution of intensity between the correlation field component bands for the scissoring mode of the CH_2 vibration indicates that bilirubin was located in the region of the methylene chains of the DMPC bilayers. This conclusion was reinforced by the data in Figure 2B. Inclusion of bilirubin

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine.

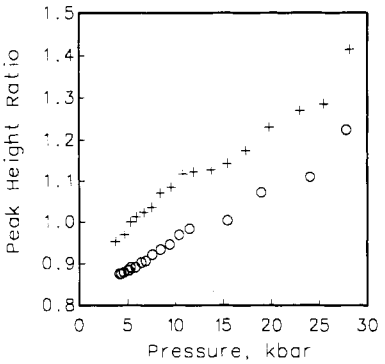

FIGURE 3: Peak height ratio between the correlation field component bands of the scissoring modes of DMPC (+) and DMPC plus bilirubin (O).

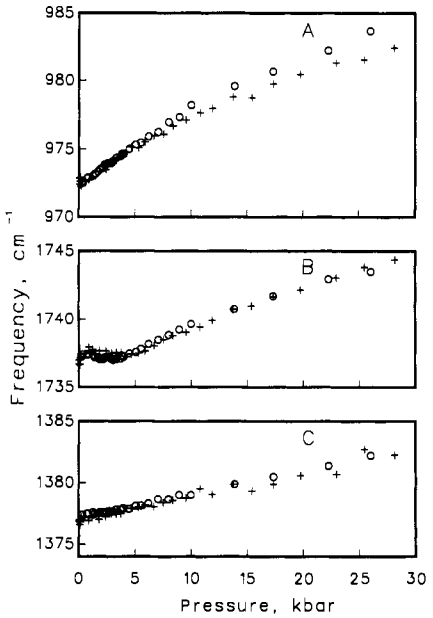

FIGURE 4: Pressure dependence of frequencies of the CN stretching mode (A), the C=O stretching mode (B), and the CH_3 bending mode (C) of DMPC (+) and DMPC plus bilirubin (O).

in DMPC increased the frequency of the symmetric CH_2 stretching mode of the methylenes at all pressures. The effect of bilirubin on the CH_2 scissoring mode was primarily on the correlation field splitting. The frequency of the CH_2 scissoring vibration (Figure 2A) was not affected by bilirubin to the same extent as the CH_2 stretching mode (Figure 2B). The reason for this difference is that the pressure-induced shift in the frequency of the scissoring vibration depends on interactions between perpendicular and parallel chains (Wong, 1987). The former interactions are intramolecular and thus will not be affected by exogenous molecules.

The data in Figures 1 and 2 indicate that bilirubin was located between the acyl chains of DMPC but they do not exclude that the bilirubin partitioned into other regions of the bilayer. The data in Figure 4 examine this possibility. The data in Figure 4A show that bilirubin influenced the frequency of the C–N stretching mode at pressures >10 kbar. The lack of an effect of bilirubin at lower pressures means that very small amounts of bilirubin were present in the polar region of the bilayers of DMPC; so perturbation of this region was seen only at relatively high pressures. The data in Figure 4B show that bilirubin had no effect on the C=O stretching mode. Interestingly, bilirubin also did not interact with the methyl terminal end of the acyl chain as evidenced by the lack of effect of bilirubin on the frequency or pressure dependence of the

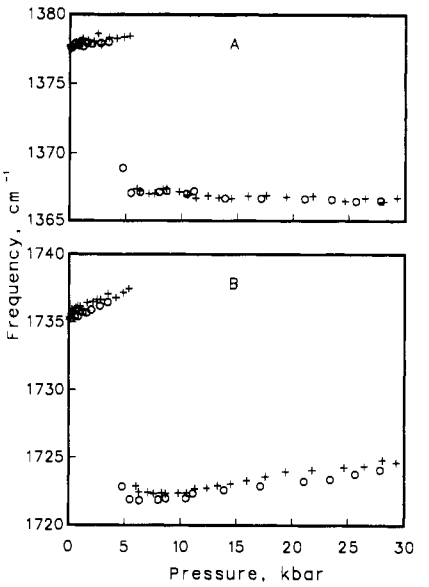

FIGURE 5: Pressure dependence of frequencies of the CH_3 bending mode (A) and the C=O stretching mode (B) of DOPC (+) and DOPC plus bilirubin (O).

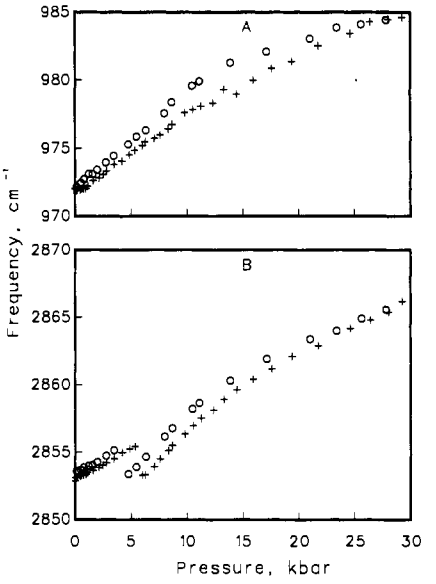

FIGURE 6: Pressure dependence of frequencies of the symmetric CH_2 stretching mode (A) and the CN stretching mode (B) of DOPC (+) and DOPC plus bilirubin (O).

CH_3 bending vibration (Figure 4C).

Effects of Bilirubin on Bilayers of DOPC. Spectra for CH_3 bending and C=O stretching modes are shown in panels A and B, respectively, of Figure 5 for bilayers of DOPC with and without bilirubin. Bilirubin had no effect on the frequency of the CH_3 bending mode nor on the pressure-dependent changes in the frequency of this band. There was a small, bilirubin-induced perturbation of the C=O stretching vibration in DOPC (Figure 5B). This result does not mean that bilirubin interacted directly with the carbonyl. Thus, the stretching vibration of this group is coupled to the adjacent methylene; so interactions between the adjacent methylene and bilirubin will perturb slightly the stretching vibration of the carbonyl. The spectra in Figure 5 show too that bilirubin displaced the pressure-induced isothermal change in the phase of the membrane to lower pressure, i.e., by 1.8 kbar.

As for bilayers of DMPC, bilirubin intercalated between the methylene chain segments in DOPC. This effect was reflected by changes in the CH_2 symmetric stretching band

of the methylene chain segments in the presence of bilirubin (Figure 6A). The data in this figure also show that bilirubin decreased the pressure needed to effect the transition from liquid-crystal to gel phase of DOPC by 1.8 kbar at 28 °C. Thus, as for the bilayers of DMPC, bilirubin caused more ordered packing of the acyl chains of DOPC than was present in pure bilayers of this lipid. On the other hand, there was no correlation field splitting of the CH_2 bending mode either in the pure lipid or in DOPC plus bilirubin. Therefore, the presence of bilirubin did not change the parallel packing of the acyl chains of DOPC (Wong & Mantsch, 1988).

The data in Figure 6B indicate that bilirubin changed the frequency of the C–N stretching mode of the choline group at low pressures. This result indicates that bilirubin interacted with the polar region of the DOPC multilayers.

Discussion

Bilirubin Interacts with the Apolar Region of Bilayers. Bilirubin contains several groups that can be solvated by water, but the physiologic isomer (bilirubin IXa) is not soluble in water (Brodersen, 1982) because the polar groups are internally hydrogen bonded (Bonnet et al., 1978). Interestingly, bilirubin IXa also has limited solubility in organic solvents (Brodersen, 1982). These observations have led to uncertainty about the nature of the interactions between bilirubin IXa with the polar or apolar regions of membranes (Brodersen, 1979; Mustafa & King, 1980). Recent experiments from this laboratory suggested that bilirubin, at concentrations as high as 20-fold of normal physiologic levels, interacted predominantly with the apolar interior of model systems and natural membranes (Leonard et al., 1989). For example, there were no detectable enthalpy changes associated with the differential partitioning of bilirubin between different membranes (Leonard et al., 1989). The data presented above confirm the general results from this work. The infrared data do show, however, that bilirubin interacted to some extent with the polar regions of DMPC and DOPC. For DMPC, changes in the choline region induced by bilirubin were seen only above 10 kbar, indicating that only small amounts of bilirubin interacted with this region of DMPC. The infrared data for DOPC do not provide quantitative information on the distribution of bilirubin between the apolar and polar regions of DOPC, but it is likely that the amount interacting with the choline of DOPC was small. Thus, selective partitioning of bilirubin into DOPC as compared with DMPC (Leonard et al., 1989) could not be attributed to selected polar interactions between bilirubin and DOPC but not DMPC. The amounts of bilirubin interacting with choline of DOPC versus DMPC were too small to detect by determining the temperature dependence of partitioning (Leonard et al., 1989).

We cannot be certain of the basis for the observed differential interactions between bilirubin and the choline moieties of DOPC and DMPC. Preexisting contamination of bilirubin with isomers other than IXa was excluded, however, in that the same preparation of bilirubin was used for studies with DOPC and DMPC, and data with DMPC were obtained for mixtures of lipid and bilirubin made before and after preparing mixtures of DOPC and bilirubin. Possibly, isomerization of a small amount of bilirubin IXa to more polar isomers was facilitated by mixing with DOPC but not DMPC.

Orientation of Bilirubin in Bilayers. The crystalline structure of bilirubin IXa consists of two planes, each comprising two pyrrole rings. The planes are at an approximate angle of 97° (Bonnet et al., 1978). The dimensions of each plane are about 9 Å across and 15 Å long, as calculated from the data in Bonnet et al. (1978). The deviation of any molecule

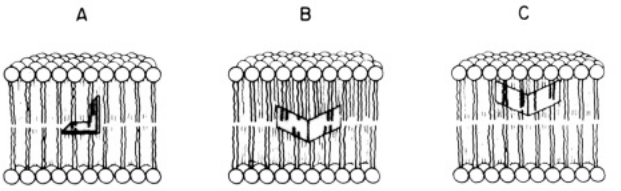

FIGURE 7: Possible orientations of bilirubin in lipid bilayers.

out of the planes of the rings is no more than 0.1 Å; so the planes are thin. Therefore, the internally hydrogen-bonded structure of bilirubin IXa can have only three possible orientations in a lipid bilayer, as depicted in Figure 7. The orientations in Figure 7A,B are excluded by the data, indicating that bilirubin did not interact with the CH_3-terminal end of the polymethylene chains. Bilirubin must insert into the bilayer, therefore, as pictured in Figure 7C. In this case, a span of about 9 Å (the distance across each plane) would have to be accommodated somewhere between the C=O of the *sn*-2 chain and the methylene group at least one carbon from the terminal CH_3.

The span of the methylene chains in DMPC is about 15 Å for half the bilayer, but about 2 Å at the CH_3 terminal cannot be occupied by bilirubin. Only one molecule of bilirubin could intercalate along the length of a polymethylene chain in DMPC, with perhaps an excess of 2 Å of length available at either side of the molecule. The distribution of bilirubin in DOPC would appear to be considerably different. Not only is the bilayer thicker, but also it has a bent configuration near the middle due to the double bond in the oleoyl chains. It is almost certain that bilirubin is present in DOPC in the region of the chain below C-8. There not only is sufficient length for bilirubin to intercalate below about C-8 in DOPC, but also this is the region of the membrane with the least dense packing of the chains (Seelig & Seelig, 1974) and thus with the largest free volume for accommodating foreign molecules. However, incorporation of bilirubin into DOPC changed slightly the stretching vibration of the carbonyl group. Some bilirubin must intercalate, therefore, on the interfacial side of the —C=C— bonds in DOPC. More bilirubin would be allowed with this sort of distribution than in DMPC, and bilirubin would avoid straddling the bent region of the oleoyl chains, which would not be allowed at high pressure (Wong & Mantsch, 1988).

Thermodynamic Basis for the Orientation of Bilirubin in Bilayers. The poor solubility of bilirubin IXa in nonpolar solvents (Brodersen, 1982) has been used to argue against its solvation by the interior of the bilayer (Brodersen, 1979, 1982). It may be that bilirubin is not solvated effectively by the methylene chain region of the bilayer but that bilirubin intercalates into this region because it fills voids. The data in Leonard et al. (1989) can be interpreted, for example, on the basis of the idea that membranes with different avidities for bilirubin contain a variable amount of void volumes dependent on the packing of the lipids. Bilirubin IXa fits into these voids, and equilibrium for the distribution of bilirubin between different kinds of membranes then occurs when the concentration of bilirubin in the void volumes is equal in different membranes. This interpretation avoids the apparent difficulty of the poor solubility of bilirubin in both polar and nonpolar solvents in that intercalation of bilirubin into the membrane, as compared with a nonpolar solvent, may not require the creation of a hole in the solvent for which the enthalpy of bilirubin–solvent interactions cannot compensate.

The largest potential void volumes in a bilayer should be in the middle of the bilayer, between the CH_3 terminals of the

acyl chains from each half of the bilayer. We have to account, therefore, for the exclusion of bilirubin from this region of the membrane, i.e., in the orientations in Figure 7A,B. One possible explanation is that the orientation in Figure 7C is favored by van der Waals interactions between bilirubin and the methylene groups. We think this is unlikely because k_1 and k_{-1} in reaction 1 are independent of temperature between

$$\text{bilirubin-}(H_2O)_n + \text{bilayer} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{bilayer-bilirubin} + H_2O \quad (1)$$

0 and 37 °C (Noy et al., 1990). This would not be so if van der Waals interactions between bilirubin and acyl chains were important determinants of the localization of bilirubin in lipid bilayers. We believe, therefore, that the orientation in Figure 7C is favored entropically. Molecules of bilirubin oriented as in Figure 7A will compete for space at the middle of the bilayer, which is smaller than the space available parallel to the polymethylene chains. Also, a molecule of bilirubin in one half of the bilayer, oriented as in Figure 7A, would exclude bilirubin from occupying a corresponding site in the other half of the bilayer. The orientation in Figure 7B will lead to coupling of the motions of each half of the bilayer; so it too should have an unfavorable entropy versus Figure 7C.

Mechanism for the Toxicity of Bilirubin. The current studies suggest a basis for the known toxicity of high concentrations of bilirubin IXa (Blanckaert & Schmid, 1982). By intercalating into the apolar region of membranes, bilirubin could alter the protein–lipid interface of selected regions of membranes thereby altering the function of membrane-bound enzymes, which in some cases are modulated by the properties of this interface. It is interesting in the context of this idea, in fact, that proteins increase the partitioning of bilirubin into bilayers as compared with bilayers of identical lipid composition but lacking proteins (Leonard et al., 1989) even though the proteins do not bind bilirubin. We have suggested that this nonspecific effect of proteins on the solvation of bilirubin by bilayers is due to void volumes created by the mismatch between the surfaces of acyl chains and integral membrane proteins (Van Hoogevest et al., 1984). If this were true, then bilirubin at greater than normal levels is likely to intercalate between the hydrophobic surfaces of intergral membrane proteins and the acyl chains of lipids that form the natural environment for these proteins.

Registry No. DMPC, 13699-48-4; DOPC, 10015-85-7; bilirubin IXa, 635-65-4.

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