The Effects of Bilirubin on the Thermal Properties of Phosphatidylcholine Bilayers

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ABSTRACT The thermotropic properties of multilamellar vesicles of dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC), as a function of the concentration of bilirubin in the range of 0.1 to 1 mol%, were measured. The exact effects of bilirubin depended on the chain length of the polymethylene chains. But the general effects of bilirubin were the same in all systems. At the lowest concentrations tested (0.1 mol bilirubin/100 mol phospholipid (0.1 mol%)), bilirubin broadened and shifted to higher temperatures the main phase transitions of all bilayers. For DPPC and DSPC, but not DMPC, this concentration of bilirubin was associated with a new transition at 25°C (DPPC) or 34°C (DSPC). Bilirubin at 0.2 mol% was required for the detection of a similar transition (at 13.7°C) in DMPC. Higher concentrations of bilirubin (>0.2 mol%) suppressed completely the main phase transitions in all bilayers but increased the enthalpy of the new transition. Maximal values of ΔH for these transitions were reached at 0.5, 0.25, and 0.2 mol% bilirubin in DMPC, DPPC, and DSPC, respectively. Values of ΔH and ΔS for these transitions were far larger than for the corresponding gel-to-liquid crystal transitions in pure lipid bilayers but were equal to those expected for a transition between crystalline and liquid crystalline phases.

INTRODUCTION

Free rotation about the central CH_2 bridge of the IXα isomer of bilirubin (Fig. 1) allows for internal hydrogen bonding between all of the polar groups in this molecule (1–3). Since the extended conformation of bilirubin IXα is sterically unfavorable as compared with the hydrogen-bonded conformation (4), the most stable structure of bilirubin IXα is one in which the pyrroles are arranged as two thin planes with an angle of about 100° between them. This structure is essentially insoluble in water except at high pH (5, 6). Surprisingly, bilirubin IXα is not highly soluble in apolar solvents either (6).

Not only is the physical chemistry of bilirubin inherently interesting, but the IXα isomer, the physiological isomer produced during catabolism of heme (7), is a neurotoxin (8, 9). The toxicity of bilirubin appears to depend on the passage of bilirubin across the plasma membranes of cells; so there is considerable interest in defining the interactions between bilirubin and biological membranes (10–14). We have shown recently that bilirubin partitions differentially into unilamellar bilayers of phosphatidylcholines (PCs)[1] as the polymethylene chains were varied (13). Since this selective partitioning was driven entropically (13), the data suggested that bilirubin did not interact with the polar region of PCs. Pressure-tuning infrared spectroscopy of multilamellar bilayers of dimyristoylphosphatidylcholine (DMPC) or dioleoylphosphatidylcholine plus bilirubin supported this conclusion by showing that bilirubin intercalated between polymethylene chains (14). As a method complementary to the pressure experiments, and as a first step in defining the effects of bilirubin on the properties of bilayers, we have examined the effect of bilirubin on the thermotropic properties of multilamellar vesicles of DMPC, dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC). These data show complex effects of bilirubin on the thermal properties of PCs; they also show that the effects of bilirubin occur at extremely small concentrations, e.g., in the range of 1 part/1000 lipid molecules.

MATERIALS AND METHODS

DMPC, DPPC, and DSPC, purity >99%, were purchased either from Avanti Polar Lipids (Alabaster, AL) or Sigma Chemical Company (St. Louis, MO). Bilirubin IXα, with purity ~99%, was from Fluka and was used without further purification. All other reagents were of reagent grade.

Sample preparations

The lipid samples were dissolved in ethanol. Bilirubin was dissolved in 0.1 N NaOH, in the dark, and was used immediately. The amount of alkaline-bilirubin solution (0–20 μl) needed to give the desired final concentration of bilirubin in phospholipids (mol bilirubin/mol phospholipid) was added directly to the solution of phospholipid in ethanol. Solvent was then evaporated under a stream of argon. The resulting films were dried under vacuum for 2 h. The dried films then were suspended in 1.0 ml of 100 mM NaCl or 100 mM HEPES (pH 7.4) and vortexed several times at temperatures below and above the phase transition temperatures of the lipids. The final pH of the system (in the range 7.4 to about 10.5) had no effect on the thermal properties of pure phospholipids or mixtures of phospholipids and bilirubin. All preparative work with bilirubin was carried out in dim light.

Differential scanning calorimetry

The thermograms were recorded on a computer-controlled (Dell 386), high-sensitivity differential scanning calorimeter (model 707; Hart Scientific, Provo, UT). Gallium was used as an internal standard to calibrate the heat capacity and transition temperature. The multilamellar vesicles (0.6 ml of 6 mg/ml of preparation) and weighed amounts of solution containing 100

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[1] *Abbreviations used:* DSC, differential scanning calorimetry; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PC, phosphatidylcholine.

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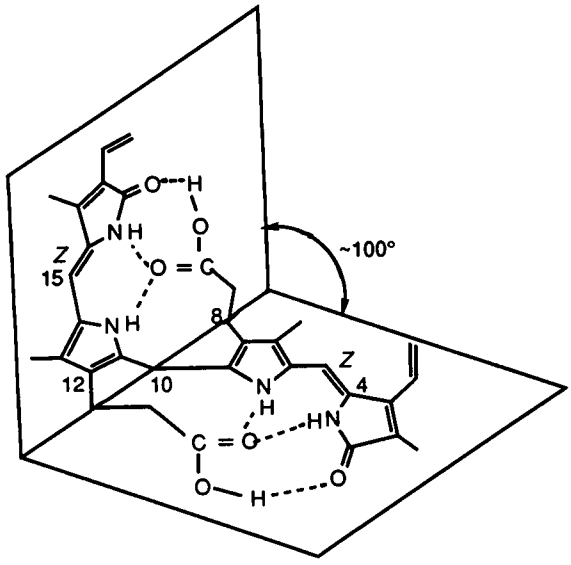

FIGURE 1 Planar representation of the intramolecular hydrogen-bonded structure of bilirubin IXα. An angle of 100° indicates the separation between the two planes.

mM NaCl or HEPES were run separately. The data file of the sample was subtracted from the buffer file to give the thermograms. The heating and cooling scans were recorded at 15°C/h. The enthalpies of transitions were computed using the software provided with the Hart calorimeter. The data were graphed on a McIntosh computer (SE/30) using Kaliedograph (version 2.1.1).

RESULTS

Differential scanning calorimetry studies of binary mixtures of bilirubin and multilamellar bilayers of PCs

The differential scanning calorimetry (DSC) data for bilayers of DMPC, DPPC, and DSPC are shown, respectively, in Figs. 2, 3, and 4. Figs. 2 *A*, 3 *A*, and 4 *A* display endothermic scans; Figs. 2 *B*, 3 *B*, and 4 *B* display exothermic scans. The concentrations of bilirubin for each scan are indicated in the corresponding figure. The scans shown were obtained in 100 mM NaCl at pH 10.5. As mentioned in Materials and Methods, we found that buffering the system with 50 mM HEPES (pH 7.4) did not change the details of the thermal properties of the systems.

The general features of the thermograms, as a function of the concentration of bilirubin, were the same for all bilayers. There were significant differences, however, in the detailed effects of bilirubin on different PCs. At the lowest concentration of bilirubin studied (0.1 mol%), there were no pretransitions and the temperatures for the main phase transitions were higher than in the pure PC. The main phase transitions were broadened at 0.1 mol% bilirubin; and the peaks were asymmetric (most notable for DSPC). On the other hand, ΔH for the main transitions, in the presence of 0.1 mol% bilirubin, were about 60% of the values in pure PCs. As the concentration of bilirubin increased above 0.1 mol%, in all bilayers, the temperature of the main phase transition decreased, and then this transition was no longer detected.

For all bilayers studied, the addition of bilirubin gave rise to a highly enthalpic transition that was not present in pure lipids. This transition took place at a lower temperature than the main phase transition in pure lipids; and in DPPC (at 25°C, Fig. 3) and DSPC (at 34°C Fig. 4), this new, low-temperature transition occurred at 0.1 mol% bilirubin. An analogous low-temperature transition appeared in DMPC but only when the concentration of bilirubin was increased to 0.2 mol% (13.7°C, Fig. 2). We note that the low-temperature transition appearing in bilayers of DMPC plus bilirubin > 0.1 mol% coincided with the pretransition in pure DMPC. We conclude, however, that this is coincidence. Thus, ΔH for the transition at 13.7°C in DMPC plus 0.5 mol% bilirubin was too large to reflect the events of the pretransition in pure DMPC. Moreover, the new low-temperature transitions in

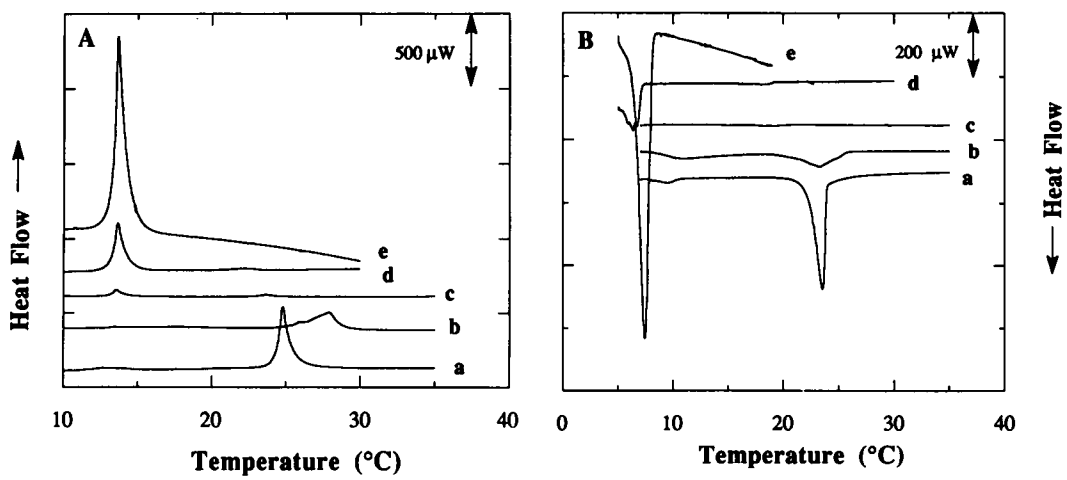

FIGURE 2 Heating (*A*) and cooling (*B*) scans of binary mixtures containing 0 (scan *a*), 0.1 (scan *b*), 0.2 (scan *c*), 0.3 (scan *d*), and 0.5 (scan *e*) mol% bilirubin in multilamellar bilayers of DMPC. The excess heat capacity is given in μW.

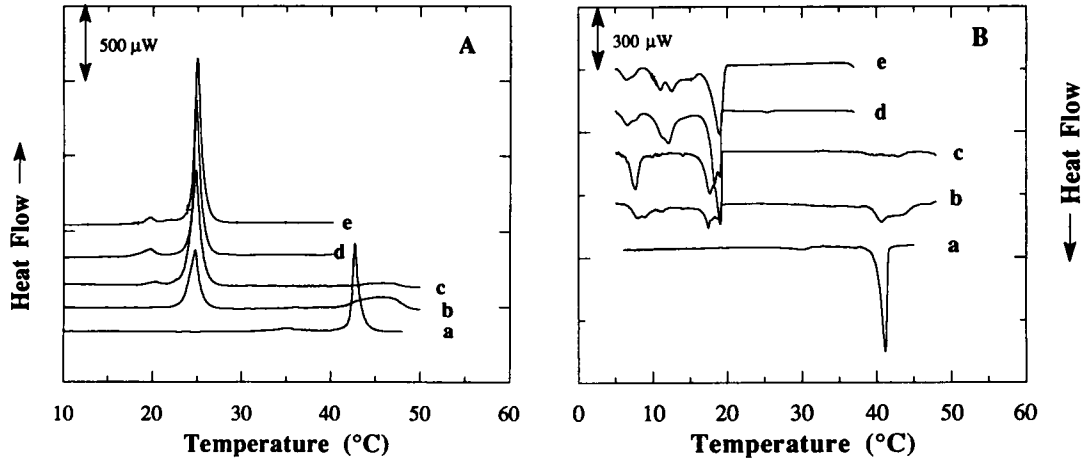

FIGURE 3 Heating (*A*) and cooling (*B*) scans of binary mixtures containing 0 (scan *a*), 0.1 (scan *b*), 0.2 (scan *c*), 0.25 (scan *d*), and 0.5 (scan *e*) mol% bilirubin in multilamellar bilayers of DPPC. The excess heat capacity is given in μW.

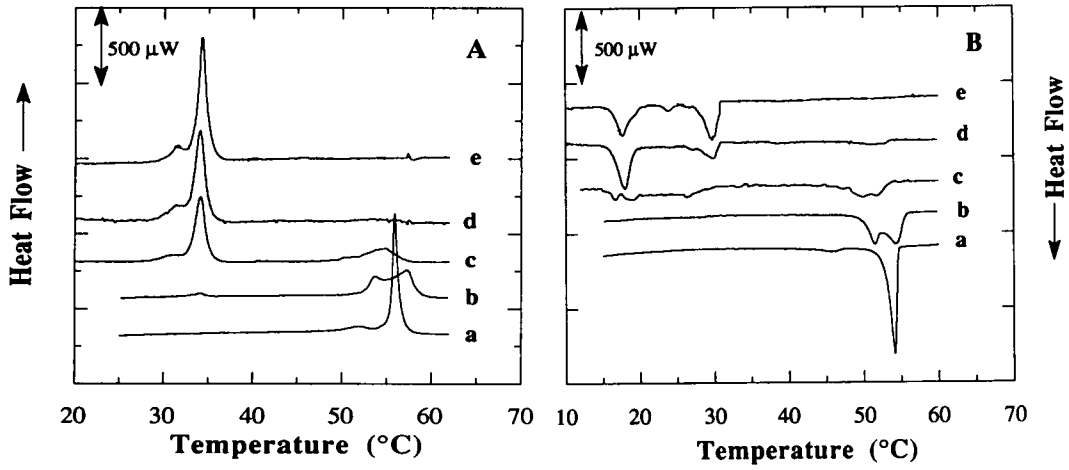

FIGURE 4 Heating and cooling scans of binary mixtures of 0.1 (*A*) or 0.2 (*B*) mol% bilirubin in bilayers of DPPC. Forward and backward arrows indicate heating and cooling scans, respectively. Heating scans were carried out immediately after completing a cooling scan. Units of excess heat capacity are the same as in Fig. 2.

DPPC and DSPC plus bilirubin did not correspond to the pretransitions in these PCs.

The data in Fig. 5 show the relationship between the structure of the PCs and the concentration of bilirubin required for achieving a maximal value of ΔH for the new, low-temperature transitions. Smaller amounts of bilirubin were needed for this effect as the length of the polymethylene chains increased. In Table 1 are listed the maximal values of ΔH and ΔS for transitions occurring at the indicated temperatures. These data also show a dependence on chain length, which is not unexpected. More interesting, however, is the observation that values of ΔH and ΔS for the transitions in Table 1 are larger than those for the main phase transitions in pure bilayers of the lipids studied. This result is compatible with the idea that the new, low-temperature transitions observed in the presence of bilirubin were associated with a greater order → disorder transition than the main transition in pure bilayers. In fact, the sum of the enthalpy changes for the subtransition, pretransition, plus main transition in bilayers of pure DPPC and DSPC equaled the maximal value of ΔH for the low-temperature transitions (Table 1) in the presence of bilirubin. The thermodynamic parameters for the low-temperature transitions in mixtures of bilirubin and DPPC or DSPC are compatible, therefore, with the conclusions that bilirubin, at concentrations above 0.1 mol%, stabilized a crystalline phase relative to a gel phase and that the crystalline phase melted directly to a liquid crystalline phase. Although there are no comparable data for ΔH for a transition from a crystal to a liquid crystal in bilayers of DMPC, ΔH for this type of transition in bilayers of di-C12:0 phosphatidylcholine is 13.4 kcal/mol, for example (15); and ΔH in Table 1 for DMPC is in the same range for crystal-to-liquid crystal transitions in several other types of PCs (16, 17). Obviously, however, the exact physical state of the lipids in mixtures with bilirubin cannot be determined directly from the DSC data alone.

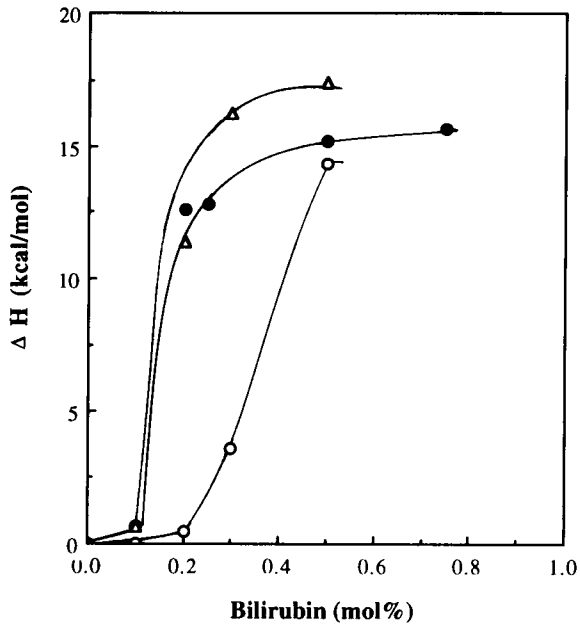

FIGURE 5 Plot of ΔH as a function of the concentration of bilirubin in bilayers of DMPC (○), DPPC (●), and DSPC (△).

TABLE 1 Thermodynamic parameters associated with the low-temperature phase transitions seen in DMPC, DPPC, and DSPC in the presence of bilirubin

Thermal parameter	DMPC	DPPC	DSPC
T_c (°C)*	13.7	25.0	34.0
ΔH‡	14.3	15.2	17.4
ΔS§	49.9	51.0	56.7

Values are given for conditions under which the enthalpies of the transitions in the heating scans were maximal.

* T_c denotes the midpoint of the transition of the new, putative crystalline phase.

‡ The values for ΔH are given in kcal/mol.

§ The values for ΔS are given in cal/mol °K.

The exothermic transition profiles for all bilayers (Figs. 2 *B*, 3 *B*, and 4 *B*) show significant hysteresis in the presence of bilirubin. But the exothermic scans confirm the findings of the endothermic scans.

DISCUSSION

It was concluded in the only previous relevant study that bilirubin had no effect on the thermal properties of PCs (18). The data in (18) are not directly comparable with ours, however, because Cestaro et al. (18) studied small unilamellar vesicles, whereas we studied multilamellar vesicles. In addition, the data in (18) were collected at rapid scan rates and over a more limited range of temperatures than were studied by us; and samples were buffered with Tris, for which there is a steep temperature dependence of pH. It is difficult, therefore, to give clear interpretations to the data of Cestaro et al. (18). Our data make it clear, however, that bilirubin has complex effects on the thermal properties of bilayers of PC. There appear to be two important features of the data. First, the qualitative effects of bilirubin on bilayers of PC depend on the concentration of bilirubin interacting with the bilayers. Second, only very low concentrations of bilirubin are required to perturb the thermal properties of bilayers.

Bilirubin interacts with bilayers in more than one mode

It appears that low concentrations of bilirubin, e.g., about 0.1 mol% in DMPC, stabilized the gel phase of PCs relative to the liquid crystalline phase. But, as the concentration of bilirubin was increased, a second type of bilirubin-bilayer interaction became manifest and the gel-stabilizing effect disappeared. Comparison of the details of scans *b*, *c*, and *d* of Fig. 2 *A* suggest, for example, that bilirubin interacted with DMPC in several different modes, which had variable effects on the thermal properties. Bilirubin, at the lowest concentration tested (scan *b*), stabilized the gel phase relative to the liquid crystalline phase; but further increases in the concentration of bilirubin (compare scans *b* and *c*) appeared to stabilize the liquid crystal phase in that this phase was present above 13.7°C. On the other hand, a new transition, not apparently associated with a gel-to-liquid crystal transition, appeared below 13.7°C. These results suggest cooperative effects on the bilayer between molecules of bilirubin. Thus, if different modes of packing between bilirubin and phospholipids were independent of each other, one would expect to find concurrent stabilization by bilirubin of the gel phase (relative to the liquid crystal phase) and of the apparently new, low-temperature phase, i.e., the thermograms should suggest the coexistence of phase-separated domains with different modes of interactions between bilirubin and lipids and hence different thermal properties. But this result was not observed.

Small concentrations of bilirubin alter the thermal properties of PCs

The effects of bilirubin on bilayers of PCs occurred at concentrations of bilirubin as low as 1 part/1000 parts lipid. Clearly, the number of polymethylene chains stabilized in the gel phase had to be far greater than the number interacting directly with bilirubin. We do not understand the mechanism for this effect, but it seems that small amounts of bilirubin must lead to an overall change in the packing of polymethylene chains independently of direct interactions between bilirubin and each chain. This is in contrast to the effects of a molecule like cholesterol (19–21). The effects of cholesterol on bilayers occur at concentrations on the order of 10–100-fold greater than the concentrations of bilirubin used in the experiments presented above. Also, whereas the condensing effect of cholesterol occurs for unsaturated chains, the effects of bilirubin were seen in bilayers comprising fully saturated chains.

We are unaware of any molecule studied to date that has been shown to affect the properties of membrane lipids at concentrations as low as those seen in the data in Figs. 2–4.

It seems reasonable to speculate about why the effects of bilirubin have not been discovered previously through studies of other molecules. A review of the literature for interactions between small apolar and/or amphipathic molecules and model membranes shows that most of these studies have utilized molecules that require space at the membrane-water interface. This usually is so because the molecule of interest has a group that forms hydrogen bonds with water or with part of the polar region of membrane phospholipids. Another reason why a molecule may require space at the membrane-water interface is that it is too large to be accommodated within the bilayer. Hexadecane, for example, will not form hydrogen bonds with water; but in bilayers of DMPC it extends into the aqueous phase (22). Bilirubin may be different from all molecules studied by DSC in that it appears to be accommodated completely within the lipid bilayer (see Fig. 7 in Zakim and Wong (14). This last result suggests a mechanism by which small amounts of bilirubin, e.g., on the order of 1 part/1000, could alter the properties of nearly all of the polymethylene chains in a bilayer. In the absence of extension to the membrane-water interface, accommodation of bilirubin within the bilayer depends on the formation of holes for it. Such holes could preexist. Alternatively, they could be formed by expansion of the bilayer or by a decrease in the free volume available to the polymethylene chains. We would not expect bilirubin to have an easily measurable effect at 0.1 mol% in the case that it simply filled a small number of preexisting void volumes. Also, the data showing that selective partitioning of bilirubin between different PCs is independent of temperature (13) do not support the idea that bilirubin expands the bilayer. Possibly, then, the effects of bilirubin on the thermal properties of PCs reflect the creation of holes by the "donation" from each polymethylene chain of a portion of the free volume available to it in pure bilayers. This would condense the bilayer and stabilize the gel phase. But this arrangement might be perturbed and no longer stable at high concentrations of bilirubin, which would account for the change in packing at relatively low and high concentrations of bilirubin.

An interesting problem suggested by the DSC data in Figs. 2–4 is that the observed changes in the thermal properties of the polymethylene chains were saturated at low concentrations of bilirubin (0.5 mol% for DMPC, Fig. 5). There is evidence that bilayers of DMPC can dissolve greater amounts of bilirubin than this (13, 14, 23). Bilirubin at concentrations greater than 0.5 mol% might enter bilayers of DMPC in a way that does not further disturb the thermal properties of the apolar interior. It is possible that there is phase separation of bilirubin at concentrations greater than 0.5 mol% in DMPC. This mechanism was not looked for in the data in the study by Zakim and Wong (14), but it is currently being explored.

Physiological implications of the DSC data

The effects of bilirubin on the thermal properties of PCs occurred at concentrations that probably occur in cell membranes in vivo at physiological to slightly elevated concentrations of unconjugated bilirubin in serum (13, 23). This implies that small elevations of the concentration of bilirubin in serum might perturb the packing of the polymethylene chains of membrane phospholipids.

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REFERENCES

1. Bonnet, R., J. E. Davies, M. B. Hursthouse, and G. M. Sheldrick. 1978. *Proc. R. Soc. Lond. B.* 202:249–268.
2. Kaplan, D., and G. Navon. 1982. *Biochem. J.* 201:605–613.
3. Trull, F. R., R. W. Franklin, and D. A. Lightner. 1987. *J. Heterocycl. Chem.* 24:1573–1579.
4. Puzicha, G., Y-M. Pu, and D. A. Lightner. 1991. *J. Am. Chem. Soc.* 113:3583–3592.
5. Hahm, J-S., J. D. Ostrow, P. Mukerjee, and L. Celic. 1992. *J. Lipid. Res.* 33:1123–1137.
6. Brodersen, R. 1982. *In* Bilirubin. Vol. 1. K. P. Heirwegh and S. B. Brown, editors. CRC Press, Boca Raton, FL. 75–124.
7. Blanckaert, N., and J. Fevery. 1990. *In* Hepatology: A Textbook of Liver Disease. 2nd Ed. D. Zakim and T. D. Boyer, editors. W. B. Saunders, Philadelphia. 254–302.
8. Diamond, I., and R. Schmid. 1966. *J. Clin. Invest.* 45:678–689.
9. Karp, W. B. 1979. *Pediatrics* 64:361–368.
10. Brodersen, R. 1979. *J. Biol. Chem.* 254:2364–2369.
11. Mustafa, M. G., and T. E. King. 1970. *J. Biol. Chem.* 245:1084–1089.
12. Suzuki, N., T. Yamaguchi, and H. Nakajima. 1988. *J. Biol. Chem.* 263: 5037–5043.
13. Leonard, M., N. Noy, and D. Zakim. 1989. *J. Biol. Chem.* 264:5648–5652.
14. Zakim, D., and P. T. T. Wong. 1990. *Biochemistry* 29:2003–2007.
15. Marsh, D. 1990. CRC Handbook of Lipid Bilayers. CRC Press, Boca Raton, FL.
16. Lewis, R. N. A. H., N. Mak, and R. N. McElhaney. 1987. *Biochemistry* 26:5570–5577.
17. Lin, H., Z. Wang, and C. Huang. 1990. *Biochemistry* 29:7063–7072.
18. Cestaro, B., G. Cervato, G. Ferrari, G. DiSilverstro, D. Monti, and P. Monitto. 1983. *Ital. J. Biochem.* 32:318–329.
19. Huang, C-H. 1977. *Lipids* 12:348–356.
20. Singer, M. A., and L. Finegold. 1990. *Biophys. J.* 57:153–156.
21. Yeagle, P. L. 1988. Biology of Cholesterol. CRC Press, Boca Raton, FL. 122–145.
22. Wong, P. T. T., and D. Zakim. 1991. *J. Phys. Chem.* 94:5052–5056.
23. Noy, N., M. Leonard, and D. Zakim. 1992. *Biophys. Chem.* 42:177–188.