Influence of Cholesterol on the Polar Region of Phosphatidylcholine and Phosphatidylethanolamine Bilayers†

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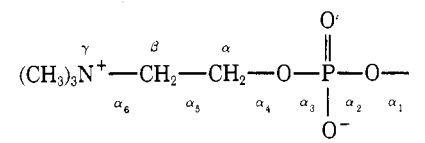
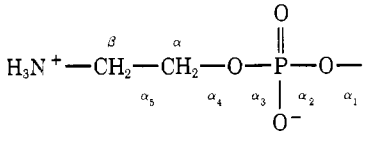
ABSTRACT: The structural changes in the polar head group region of unsonicated bilayer membranes of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine produced by addition of cholesterol have been determined using deuterium and phosphorus-31 NMR. Incorporation of up to 50 mol % cholesterol produces little change in the phosphorus-31 chemical shielding anisotropies, compared with the values in pure bilayers above the phase transition temperatures, while some of the deuterium quadrupole splittings are reduced by almost a factor of two. Adjustment of the head group torsion angles by only a few degrees accounts for the observed spectral changes. Addition of cholesterol therefore has opposite effects on the hydrocarbon and polar regions of membranes: although cholesterol makes the hydrocarbon region gel-like, with an increased probability of trans conformations, the conformation of the polar head groups is very similar to that found in the liquid crystalline phase of pure phospholipid bilayers.

Deuterium magnetic resonance has proven to be a sensitive tool to detect even small conformational changes in lipid molecules (Seelig and Seelig, 1977). In combination with phosphorus-31 NMR we have employed this method to study the orientation and flexibility of the choline and ethanolamine head groups in bilayers composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Gally et al., 1975; Seelig et al., 1977) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Seelig and Gally, 1976). In both bilayers the polar head groups are found to be bent at the position of the phosphate group, probably due to a gauche-gauche conformation of the phosphodiester linkage, so that both the phosphocholine and phosphoethanolamine dipoles are aligned parallel to the membrane surface. In the case of DPPC[1] this result is further strengthened by recent neutron diffraction studies of selectively deuterated phospholipids (Büldt et al., 1978). Addition of trivalent ions (shift reagents) induces dramatic changes in the deuterium quadrupole splittings and, depending on the ion added, also in the phosphorus-31 chemical shielding anisotropy, which must be attributed to conformational changes in the head group structure (Brown and Seelig, 1977). In this paper we address ourselves to the question of whether cholesterol induces conformational changes in the polar head group region. The bulk of previous work has been directed toward elucidating the effects of the rigid sterol nucleus on the segmental motions of the lipid hydrocarbon chains and the precise nature of the phase separations induced by cholesterol in lipid bilayers (for reviews, see Chapman, 1975; Demel and de Kruyff, 1976). However, many of the physical and functional properties of membranes also depend strongly on the nature of electrostatic or dipolar interactions involving the lipid head groups, so that it is important to know to what extent, if any, the orientation and mobility of the head groups are influenced by the high cholesterol contents found in many biological membranes. We report here a study of the effects of cholesterol on the phosphorus-31 chemical shielding anisotropies and deuterium quadrupole couplings of unsonicated multibilayers of DPPC and DPPE selectively deuterated in the polar head groups. Preliminary reports of the effect of cholesterol on the choline methyl rotors have been published (Stockton et al., 1974; Gally et al., 1976). The influence of cholesterol on the phosphorus-31 chemical shielding anisotropy of various 3-*sn*-phosphatidylcholines has also been previously investigated (Cullis et al., 1976).

Materials and Methods

Cholesterol was purchased from Fluka, Switzerland. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine was deuterated at three carbon segments ($(CD_3)_3N^+$-DPPC, $^+NCD_2CH_2$-DPPC, $^+NCH_2CD_2$-DPPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine at two carbon segments ($^+NCD_2CH_2$-DPPE, $^+NCH_2CD_2$-DPPE) as described previously (Gally et al., 1975; Seelig and Gally, 1976). For the purpose of discussion, we refer to the various deuterated segments as the α, β, and γ positions and the corresponding deuterium quadrupole splittings as $\Delta\nu_\alpha$, $\Delta\nu_\beta$, etc., as indicated below





The nomenclature for the various head group torsion angles (Sundaralingam, 1972) is also indicated.

Samples were prepared by dissolving the appropriate amounts of cholesterol and phospholipid in chloroform/methanol. The solutions were transferred to glass ampoules and the solvent was removed by blowing a stream of nitrogen over the samples followed by at least 24 h under high vacuum. Buffer (0.2 M sodium acetate-acetic acid, pH 5.7, 10^{-4} M EDTA) was added to yield a final lipid:buffer ratio of 50:50

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[1] Abbreviations used are: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine.

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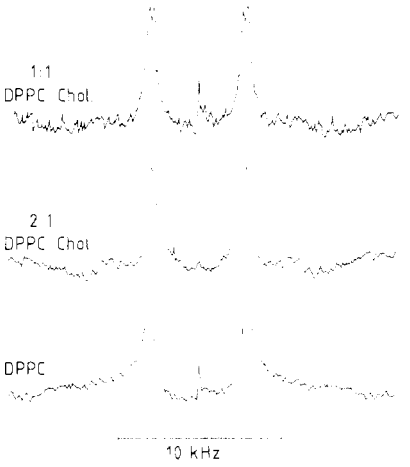

FIGURE 1: Effect of cholesterol on the deuterium NMR spectra of $^{+}NCH_2CD_2$-DPPC. Deuterium resonance frequency 13.8 MHz; unsonicated samples; temperature 58 °C. The numbers refer to the molar ratios of phospholipid:cholesterol. Lower trace: $^{+}NCH_2CD_2$-DPPC without cholesterol.

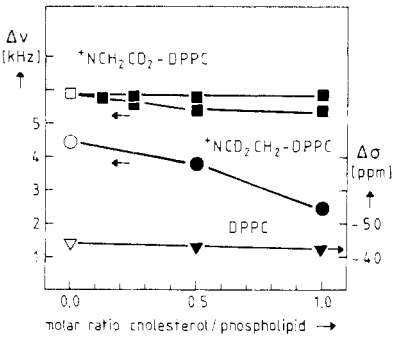

FIGURE 2: Dependence of the deuterium quadrupole splittings and phosphorus chemical shielding anisotropy of DPPC on cholesterol concentration. Temperature 58 °C.

wt %. The ampoules were sealed and the contents always thoroughly mixed above the phase transition temperature of the pure phospholipids immediately before use.

The deuterium and phosphorus-31 NMR measurements were made with a Bruker-Spectrospin HX-90-FT spectrometer (deuterium frequency 13.8 MHz, phosphorus frequency 36.4 MHz) equipped with a home-built quadrature detection unit. The experimental details are the same as described previously (Gally et al., 1975; Seelig and Gally, 1976). The deuterium quadrupole splittings and the phosphorus-31 chemical shielding anisotropy were evaluated directly from the powder-type spectra. No computer simulations of the spectra were performed.

Results

Figure 1 shows deuterium NMR spectra (at 13.8 MHz) of unsonicated multibilayers of $^{+}NCH_2CD_2$-DPPC containing various amounts of cholesterol. Although the quadrupole splitting remains approximately constant up to 50 mol % cholesterol, the spectra are unusual in that the signal splits into two doublets at higher cholesterol concentrations. This cholesterol effect is unique to $^{+}NCH_2CD_2$-DPPC; a single spectral component is observed for all other segments of the phosphocholine group and all segments of the phosphoethanolamine group, including the phosphate moieties (spectra not shown). Figure 2 illustrates the variation of the phosphorus-31 chemical shielding anisotropy, $\Delta\sigma$, and the residual deuterium quadrupole splittings, $\Delta\nu$, of bilayers of DPPC with cholesterol

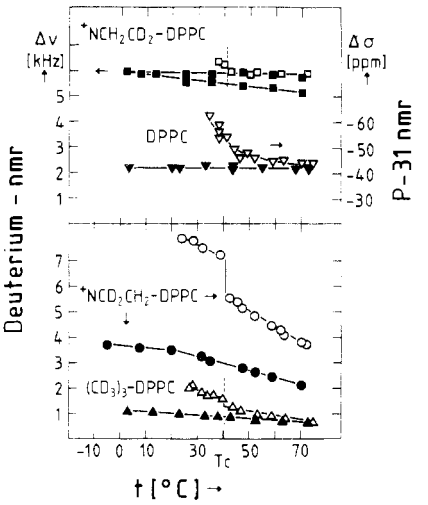

FIGURE 3: Variation of the deuterium quadrupole splittings and phosphorus-31 chemical shielding anisotropy of bilayers composed of DPPC and cholesterol with temperature. Open symbols: pure DPPC. Filled symbols: DPPC–cholesterol mixture (1:1 molar ratio).

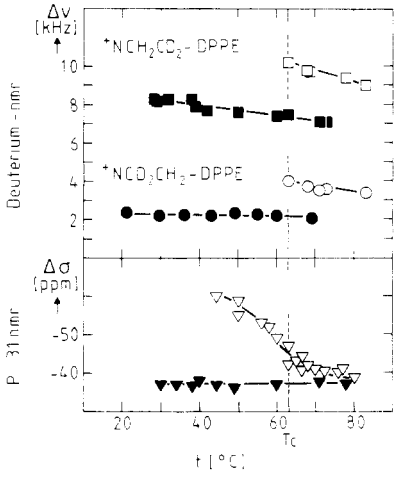

FIGURE 4: Dependence of the spectral parameters of bilayers composed of DPPE and cholesterol on temperature. Open symbols: pure DPPE. Filled symbols: DPPE–cholesterol mixture (1:1 molar ratio).

concentration. Approximately straight lines are obtained as a function of cholesterol concentration; however, since the concentration intervals are rather large, *small* discontinuities are not ruled out by the data presented. A concentration profile was not obtained for DPPE. Figures 3 and 4 summarize the temperature dependence of the chemical shielding anisotropies and residual quadrupole splittings of bilayers of DPPC and DPPE containing 50 mol % cholesterol.

Discussion

The position of cholesterol in membranes composed of natural egg-yolk lecithin has been determined to a high degree of accuracy using x-ray and neutron diffraction techniques (Franks, 1976; Worcester and Franks, 1976). The cholesterol molecules are found to be immersed in the hydrocarbon region of the bilayer with their OH groups in the vicinity of the ester carbonyl groups of the phospholipids. It is likely that a similar situation exists in bilayers of DPPC and DPPE. The well-defined location of cholesterol in the hydrocarbon region of bilayer membranes leads to disruption of cooperative interactions between the fatty acyl side chains. Pure DPPC and DPPE in excess water exhibit sharp gel-to-liquid crystal phase

transitions at 41 and 63 °C, respectively, which are accompanied by calorimetric transition enthalpies of about 8 kcal mol^{-1} (van Dijck et al., 1976). Incorporation of increasing amounts of cholesterol gradually abolishes the transition enthalpy in both systems (Ladbrook et al., 1968; Hinz and Sturtevant, 1972; van Dijck et al., 1976; Mabrey et al., 1977). At 50 mol % cholesterol the phase transition, as detected by differential scanning calorimetry, is completely eliminated. The presence of the rigid sterol frame further restricts the flexing motions of the neighboring fatty acyl chain segments, leading to an increase in the population of trans conformations and hence a decrease in the average molecular area per phospholipid (Oldfield et al., 1971; Lippert and Peticolas, 1971; Mendelsohn, 1972; Schreier-Muccillo et al., 1973; Stockton et al., 1976; Gally et al., 1976). This ordering effect is, however, limited to the rigid steroid backbone, since the hydrocarbon tail of cholesterol and the corresponding fatty acyl chain segments undergo considerable flexing motions (Stoffel et al., 1974; Kroon et al., 1975; Opella et al., 1976; Stockton and Smith, 1976). The effects of cholesterol on the structure of the polar head group region of membranes have been much less extensively investigated. Little or no effect of cholesterol is observed on the motion of the choline methyl groups, as indicated by carbon-13 NMR (Keough et al., 1973) and deuterium NMR studies (Stockton et al., 1974; Gally et al., 1976). Likewise, cholesterol does not appear to affect the motion of the phosphate segment in mixed phosphatidylcholine-cholesterol bilayers, as judged from measurements of the ^{31}P [^{1}H] nuclear Overhauser effect (Yeagle et al., 1975) and the phosphorus-31 chemical shielding anisotropy (Cullis et al., 1976). A somewhat different conclusion, however, has been reached from studies of the infrared dichroism of oriented lecithin multibilayers, which have been interpreted in terms of a configurational rearrangement of the phosphocholine group induced by cholesterol (Verma and Wallach, 1973). No spectroscopic data seems to be available regarding the interaction of cholesterol with bilayers of DPPE or other phosphatidylethanolamines.

The precise phase diagram of DPPC-cholesterol-water mixtures appears to be a point of some contention in the literature and a phosphatidylethanolamine-cholesterol phase diagram has not yet been determined. The earlier literature regarding the presence of phase boundaries in DPPC-cholesterol mixtures is reviewed and interpreted in the recent paper by Haberkorn et al. (1977). The following discussion refers to cholesterol concentrations between 33 and 50 mol %. In this region of the DPPC-cholesterol phase diagram all of the phospholipids are thought to be in direct steric contact with cholesterol (Engelman and Rothman, 1972) and there does not appear to be evidence of significant phase separation of pure cholesterol regions (Shimshick and McConnell, 1973). Our results for cholesterol-free bilayers of DPPC and DPPE show that the phosphorus chemical shielding anisotropies, $\Delta\sigma$, and the residual deuterium quadrupole couplings, $\Delta\nu$, exhibit distinct changes at the gel-to-liquid crystal phase transition temperatures (Figures 3 and 4). Addition of 50 mol % cholesterol completely abolishes these discontinuities and leads to an almost linear temperature dependence of the spectroscopic parameters, consistent with the previously mentioned differential scanning calorimetric studies. However, while differential scanning calorimetry measures the bulk properties of the lipid-water mixtures, the NMR results reflect the behavior of individual molecular segments and clearly demonstrate that the phase transition is eliminated in the polar head group region.

The two quadrupole doublets present in the deuterium NMR spectra of bilayers of $^{+}NCH_2CD_2$-DPPC containing 50 mol % cholesterol (Figure 1) may originate from (a) two separate phases or environments with differing cholesterol:DPPC ratios, (b) magnetic inequivalence of the two deuterons, or (c) the presence of two relatively long-lived glycerol backbone conformations (Seelig and Seelig, 1975). We consider the first possibility unlikely, since the relative proportions of the two components do not vary with cholesterol concentration or temperature in a manner consistent with the presence of separate phases. A more likely explanation is that the two components have the same origin as the two quadrupole splittings observed for DPPC deuterated at the C-3 segment of the glycerol moiety (Gally et al., 1975) or at the 2′ position of the fatty acid esterified to the C-2 carbon of the glycerol backbone (Seelig and Seelig, 1975). This is supported by the observation that, if cholesterol-free bilayers of $^{+}NCH_2CD_2$-DPPC are measured at a deuterium resonance frequency of 41.4 MHz, the improved resolution compared with 13.8 MHz shows that the spectrum actually consists of two doublets with slightly different quadrupole splittings (difference ~0.2 kHz). Haberkorn et al. (1977) have recently confirmed our observation that the methylene segment immediately following the ester linkage of the 2 chain gives rise to two distinct quadrupole splittings and have further shown that these splittings persist in DPPC bilayers containing up to 50 mol % cholesterol. These observations suggest that cholesterol enhances the difference between the two quadrupole splittings of $^{+}NCH_2CD_2$-DPPC; however, a distinction between explanations b and c is not possible at the present time, so that this effect will not be further discussed here.

Regarding the phosphocholine and phosphoethanolamine head group conformations in the presence of cholesterol, we note that some parameters, e.g., the phosphorus-31 chemical shielding anisotropy, show little or no change compared to cholesterol-free bilayers, while other parameters, such as the residual quadrupole splittings of $^{+}NCD_2CH_2$-DPPC and $^{+}NCD_2CH_2$-DPPE, are reduced by almost a factor of two. If only a single parameter were selected to characterize the effect of cholesterol on the structure of the polar head group region, the data could be used to support either of two conflicting points of view, viz., that there is (Verma and Wallach, 1973) or is not (Stockton et al., 1974; Cullis et al., 1976) a change in the head group structure upon addition of cholesterol. However, the phosphorus-31 and deuterium NMR data taken together provide a set of parameters which can be used to rather precisely define the average orientation and mobility of the polar head groups. In order to obtain a quantitative estimate of the changes induced by addition of cholesterol, we have applied the same model as described for the interpretation of the phosphorus-31 and deuterium NMR spectra of DPPC (Seelig et al., 1977) and DPPE (Seelig and Gally, 1976). Considering first the data obtained for DPPC, it can be concluded from the rather small changes in the phosphorus-31 chemical shielding anisotropy and the quadrupole splittings of $^{+}NCH_2CD_2$-DPPC that the average orientation and motion of these head groups segments are not significantly affected by cholesterol. The changes in the residual quadrupole splittings of the remaining segments further removed from the glycerol backbone, i.e., the β and γ positions, must be due to a change in the torsion angle (α_5) of the O-C-C-N bond system, since the $(CD_3)_3N^{+}$ group has a threefold rotational symmetry axis. Addition of 50 mol % cholesterol reduces $|\Delta\nu_\beta|$ from 5.1 to 2.7 kHz and $|\Delta\nu_\gamma|$ from 1.15 to 0.8 kHz (at 49 °C). The quite large fractional changes in $|\Delta\nu_\beta|$ and $|\Delta\nu_\gamma|$, however, do not necessarily imply an extensive reorganization of the phosphocholine head group, since the residual quadrupole

splittings are rather sensitive to small variations in torsion angle (Seelig and Gally, 1976). For the cholesterol-free bilayer the torsion angle α_5 was determined to be $\pm 81°$, yielding calculated quadrupole coupling constants of $|\Delta\nu_\beta| = 4.9$ kHz and $|\Delta\nu_\gamma| = 1.25$ kHz. Employing the same model but increasing the torsion angle to $\alpha_5 = \pm 83.5°$ leads to quadrupole couplings of $|\Delta\nu_\beta| = 2.8$ kHz and $|\Delta\nu_\gamma| = 0.87$ kHz, in good agreement with the experimental observations. A similar calculation can be made for DPPE, except that changes in the quadrupole splittings of both $^+NCH_2CD_2$-DPPE and $^+NCD_2CH_2$-DPPE have to be taken into account. Only small changes in α_4 and α_5 account for the experimentally observed changes in the residual quadrupole splittings.

The important result of these calculations is not the absolute accuracy of the torsion angles but the fact that both head group structures are only slightly modified by the presence of cholesterol. Addition of cholesterol therefore leads to opposite effects in the hydrocarbon and polar head group regions of membranes; the well-known condensing effect of cholesterol due to an increased number of trans conformations in the hydrocarbon region is not accompanied by corresponding conformational changes in the polar head groups. These considerations lead to a picture of the polar region of biological membranes in which the zwitterionic phosphocholine and phosphoethanolamine dipoles are aligned parallel to the surface of the bilayer, regardless of whether they are in contact with cholesterol or not. In cholesterol-free bilayers the packing density of the liquid crystalline state probably allows weak electrostatic or hydrogen-bonding interactions between adjacent head groups. Direct experimental evidence for such intermolecular interactions comes from nuclear Overhauser effect studies (Yeagle et al., 1975, 1976, 1977) and also from the crystal structure of 1,2-dilauroyl-DL-phosphoethanolamine (Hitchcock et al., 1974). These interactions could lead to a slight twisting or distortion of the head group torsion angles from their lowest internal energy configurations. It is unlikely that incorporation of cholesterol entails any new head group interactions, since the cholesterol OH group is anchored at the level of the fatty acid carbonyl groups (Franks, 1976; Worcester and Franks, 1976). On the contrary, with respect to the polar head group region, cholesterol probably acts as a spacer molecule which increases the separation between head groups and eliminates intermolecular interactions. The latter effect is again evidenced by nuclear Overhauser effect studies (Yeagle et al., 1977). Due to the removal of small constraints on the torsion angles provided by intermolecular interactions, the head groups could relax to their energetically most favorable intrinsic conformations, thereby explaining the changes observed in the quadrupole splittings.

References

Brown, M. F., and Seelig, J. (1977), *Nature (London) 269,* 721.

Büldt, G., Gally, H. U., Seelig, A., Seelig, J., and Zaccai, G. (1978), *Nature (London)* (in press).

Chapman, D. (1975), *Q. Rev. Biophys. 8,* 185.

Cullis, P. R., de Kruyff, B., and Richards, R. E. (1976), *Biochim. Biophys. Acta 426,* 433.

Demel, R. A., and de Kruyff, B. (1976), *Biochim. Biophys. Acta 457,* 109.

Engelman, D. M., and Rothman, J. E. (1972), *J. Biol. Chem. 247,* 3694.

Franks, N. P. (1976), *J. Mol. Biol. 100,* 345.

Gally, H. U., Niederberger, W., and Seelig, J. (1975), *Biochemistry 14,* 3647.

Gally, H. U., Seelig, A., and Seelig, J. (1976), *Hoppe Seyler's Z. Physiol. Chem. 357,* 1447.

Haberkorn, R. A., Griffin, R. G., Meadows, M. D., and Oldfield, E. (1977), *J. Am. Chem. Soc. 99,* 7353.

Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 3697.

Hitchcock, P. B., Mason, R., Thomas, K. M., and Shipley, G. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3036.

Keough, K. M., Oldfield, E., Chapman, D., and Beyman, P. (1973), *Chem. Phys. Lipids 10,* 37.

Kroon, P. A., Kainosho, M., and Chan, S. I. (1975), *Nature (London) 256,* 582.

Ladbrooke, B. D., Williams, R. M., and Chapman, D. (1968), *Biochim. Biophys. Acta 150,* 333.

Lippert, J. L., and Peticolas, W. L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1572.

Mabrey, S., Mateo, P. L., and Sturtevant, J. M. (1977), *Biophys. J. 17,* 82a.

Mendelsohn, R. (1972), *Biochim. Biophys. Acta 290,* 15.

Oldfield, E., Chapman, D., and Derbyshire, W. (1971), *FEBS Lett. 16,* 102.

Opella, S. J., Yesinowski, J. P., and Waugh, J. S. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3812.

Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H., and Smith, I. C. P. (1973), *Chem. Phys. Lipids 10,* 11.

Seelig, A., and Seelig, J. (1975), *Biochim. Biophys. Acta 406,* 1.

Seelig, A., and Seelig, J. (1977), *Biochemistry 16,* 45.

Seelig, J., and Gally, H. U. (1976), *Biochemistry 15,* 5199.

Seelig, J., Gally, H. U., and Wohlgemuth, R. (1977), *Biochim. Biophys. Acta 467,* 109.

Shimshick, E. J., and McConnell, H. M. (1973), *Biochem. Biophys. Res. Commun. 53,* 446.

Stockton, G. W., and Smith, I. C. P. (1976), *Chem. Phys. Lipids 17,* 251.

Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., and Smith, I. C. P. (1974), *Biochem. Biophys. Res. Commun. 60,* 844.

Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., and Smith, I. C. P. (1976), *Biochemistry 15,* 954.

Stoffel, W., Tunggal, B. D., Zierenberg, O., Schreiber, E., and Binczek, E. (1974), *Hoppe Seyler's Z. Physiol. Chem. 355,* 1367.

Sundaralingam, M. (1972), *Ann. N.Y. Acad. Sci. 195,* 324.

van Dijck, P. W. M., de Kruijff, B., van Deenen, L. L. M., de Gier, J., and Demel, R. A. (1976), *Biochim. Biophys. Acta 455,* 576.

Verma, S. P., and Wallach, D. F. H. (1973), *Biochim. Biophys. Acta 330,* 122.

Worcester, D. L., and Franks, N. P. (1976), *J. Mol. Biol. 100,* 359.

Yeagle, P. L., Hutton, W. C., Huang, C., and Martin R. B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3477.

Yeagle, P. L., Hutton, W. C., Huang, C., and Martin, R. B. (1976), *Biochemistry 15,* 2121.

Yeagle, P. L., Hutton, W. C., Huang, C., and Martin, R. B. (1977) *Biochemistry 16,* 4344.