reduced the increase in evolution of methanol and duration of smooth swimming caused by addition of aspartate.

The methylation profiles resulting from the addition of different amino acid attractants to the bacteria may also shed light on the "jamming capillary assays" performed on *B. subtilis* (Ordal et al., 1977). In those experiments it was found that aspartate and glutamate were able to compete with all the amino acids tested as "victims" in a capillary assay. It is interesting that these two amino acids had the most dramatic effects, compared to the other amino acids, on the methylation of *B. subtilis* MCPs.

Time course experiments in which the rate of methylation of the MCPs was measured indicate that there is no significant change upon addition of attractant (unpublished data). There may be changes in the rate of methylation of individual proteins, but inadequate resolution makes quantitation difficult.

If, as it appears, the attractants mediate their effects via demethylation of MCPs, then one might expect that there exists chemicals that would result in inhibition of methanol production and thus be repellents. We are currently examining repellents for *B. subtilis* to determine their effect on methanol production.

Acknowledgments

We thank Cedric Pearce for help with gas chromatography of the volatile radioactive product and Jeffrey A. Ahlgren and Anthony Burgess-Cassler for the preparation of antiflagellar antibody. We are also grateful to the members of Dr. Ordal's laboratory for their critical reading of the manuscript.

References

Berg, H. C., & Tedesco, P. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3235-3239.

Goldman, D. J., & Ordal, G. W. (1981) *J. Bacteriol. 147*, 267-270.

Goy, M. F., Springer, M. S., & Adler, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4964-4968.

Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335-341.

Macnab, R. M. (1978) *CRC Crit. Rev. Biochem. 5*, 291-341.

Mesibov, R., Ordal, G. W., & Adler, J. (1973) *J. Gen. Physiol. 62*, 203-223.

Ordal, G. W., Villani, D. P., & Gibson, K. J. (1977) *J. Bacteriol. 129*, 156-165.

Paoni, N. F., & Koshland, D. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3693-3697.

Silverman, M., & Simon, M. (1974) *Nature (London) 249*, 73-74.

Springer, M. S., Goy, M. F., & Adler, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3312-3316.

Spudich, J. L., & Koshland, D. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 710-713.

Toews, M. L., & Adler, J. (1979) *J. Biol. Chem. 254*, 1761-1764.

Ullah, A. H. F., & Ordal, G. W. (1981) *J. Bacteriol. 145*, 958-965.

Zukin, R. S., Strange, P. G., Heavey, L. R., & Koshland, D. E. (1977) *Biochemistry 16*, 381-386.

Effect of Phospholipid Oxidation Products on Transbilayer Movement of Phospholipids in Single Lamellar Vesicles†

J. Michael Shaw‡ and T. E. Thompson*

ABSTRACT: Single lamellar phosphatidyl[*methyl*-^{2}H]choline vesicles were incubated with an excess of unlabeled phosphatidylcholine vesicles or phosphatidylcholine–cholesterol vesicles containing 8 mol % glucuronosyldiglyceride. Incubation of the two vesicle populations was performed in the presence or absence of a purified phosphatidylcholine exchange protein. The negatively charged glycolipid donor vesicles could be completely removed by column chromatography on DEAE-Sephacel. Following incubation with exchange protein and subsequent fractionation, the $-N(CD_3)_3$ phosphatidylcholine acceptor vesicles exhibited a 61–73% enrichment of the unlabeled phosphatidylcholine in the outer monolayer. Upon incubation in an air atmosphere, no appreciable transbilayer movement of the outer monolayer $-N(CH_3)_3$ phosphatidylcholine was observed for at least 5 days. Between days 5 and 7, however, extensive transbilayer movement occurred, leading to an outer monolayer/inner monolayer phosphatidylcholine ratio of 2.1 on day 7. In phosphatidylcholine–6 mol % cholesterol vesicles treated similarly, the outside/inside ratio of the unlabeled phospholipid was 6.7, suggesting a much smaller percentage of transbilayer movement. The loss of transbilayer asymmetry which occurred during a 36-h period after day 5 could be estimated at the upper limit, $t_{1/2} \sim 7.3$ h for phosphatidylcholine vesicles and $t_{1/2} \sim 53$ h for phosphatidylcholine–cholesterol vesicles. The actual rates for transbilayer movement, however, were likely more rapid. Transbilayer movement occurred at a time period when oxidized phospholipid breakdown products had reached critical levels.

Transbilayer and transmembrane movement of phospholipid has been examined in a variety of synthetic single lamellar vesicles and specific biological membranes. In single lamellar vesicles, transbilayer movement of phosphatidylcholine (Johnson et al., 1975; Rothman & Dawidowicz, 1975; Shaw et al., 1977), lysophosphatidylcholine (Van Den Besselaar et al., 1977; DeKruijff et al., 1977), and phosphatidylinositol (Low & Zilversmit, 1980) is generally slow, with half-times on the order of many days. An increase in the transbilayer rate of movement of phospholipid can occur if the acyl chain composition of the two monolayers comprising the bilayer is

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very different. For example, the half-time for dioleoylphosphatidyl[*N*-($^{13}CH_3$)$_3$]choline transbilayer movement from outer monolayer to an inner monolayer occupied by dimyristoylphosphatidylcholine was found to be <12 h (DeKruijff & Wirtz, 1977). A maximal rate for transbilayer movement has also been shown to occur at the phase transition of the phospholipid. The half-time for dimyristoylphosphatidylcholine at ~19 °C was about 4 h (DeKruijff & Van Zoelen, 1978). Finally, preparation of a bilayer vesicle containing a transmembrane protein such as glycophorin enhanced the transbilayer movement of dioleoylphosphatidylcholine or palmitoyllysophosphatidylcholine by about 2 orders of magnitude with a half-time about 1.5 h or less (DeKruijff et al., 1978; Van Zoelen et al., 1978). Furthermore, Gerritsen et al. (1980) have observed enhanced transbilayer movement of phosphatidylcholine when both glycophorin and partially purified band 3 were reconstituted into phospholipid vesicles. In contrast, Dicorleto & Zilversmit (1979) could not show enhancement of transbilayer movement of phosphatidylcholine in cytochrome oxidase containing vesicles which consisted of a mixture of phospholipids.

In biological membranes the majority of studies concerned with transmembrane movement have yielded rapid half-times on the order of minutes to hours [see Table II or Shaw et al. (1979)]. Initially, the presence of protein transmembrane channels was suggested as a possible means for rapid transmembrane movement. More recently, the finding that phospholipid can occur in different states of organization other than "bilayer" is a popular alternative (Van Den Besselaar et al., 1978; Cullis & McLaughlin, 1977; DeKruijff et al., 1980a,b). Work by P.R. Cullis, B. DeKruijff, A.J. VerKleij, and co-workers has led to studies of nonbilayer lipid structures in synthetic membranes. Such structures, if present in biological membranes, may account for rapid transmembrane movement (DeKruijff et al., 1980a,b).

In the present paper, we wish to describe the preparation of phospholipid and phospholipid–cholesterol single lamellar vesicles in which complete transbilayer compositional asymmetry was generated and maintained for a finite period. Upon exposure to air, the accumulation of acyl chain oxidized phospholipid, breakdown products, and slowing increasing levels of lysophosphatidylcholine led to a critical point at which transbilayer movement of phospholipid rapidly occurred. The presence of cholesterol at low levels in the vesicles significantly reduced the transbilayer movement of phospholipid. Preliminary accounts of this study have been presented elsewhere (Shaw & Thompson, 1978).

Materials and Methods

Preparation of Unlabeled Lipids. Egg yolk phosphatidylcholine was isolated and purified from hen egg yolks as previously described (Shaw et al., 1977). Cholesterol was purchased from J. T. Baker Chemical Co. and further purified according to Estep et al. (1978). Glucuronsyldiglyceride was extracted with total lipids from late log growth *Pseudomonas diminuton* by the procedure of Bligh & Dyer (1959). The glycolipid could be isolated and purified by one-step chromatography on silica gel thin layers. If thin layers were not overloaded, the band corresponding to glucuronosyldiglyceride represented a purity of about 95%, with the major contaminant being phosphatidylglycerol. Glucuronosyldiglyceride was passed through a Teflon filter (0.2 μm fluoropore, Millipore) for removal of any residual silicic acid. Greater detail on the growth of *P. diminuta* and isolation of glycolipids is available (Shaw & Pieringer, 1977a,b). All lipids were stored at −20 °C under argon in spectral grade chloroform.

Preparation of $-N(CD_3)_3$ Phosphatidylcholine.[1] Phosphatidylcholine enriched in the *N*-methyl group with deuterium (greater than 99%) was prepared by the following procedure. Phosphatidylethanolamine purified from egg yolk was methylated with CD_3I to its choline analogue in the dark as described by Stockton et al. (1974). The chloroform/methanol phase was removed under reduced pressure, dissolved in chloroform, and applied to an alumina column followed by subsequent elution with increasing concentrations of methanol. Further purification was accomplished on a silicic acid column. The resulting phosphatidylcholine was shown to be free from phosphatidyl-*N*,*N*-dimethylethanolamine (Sigma) by using the thin-layer chromatography (TLC) system chloroform/methanol/acetic acid/water (25:14:4:2). Upon examination by proton NMR, no resonance arising from *N*-methyl protons was detectable.

Preparation of Isotopically Labeled Lipids. Phosphatidyl[*methyl*-^{14}C]choline was prepared as described above for $-N(CD_3)_3$ phosphatidylcholine with the exception that $^{14}CH_3I$ was utilized. Trioleoyl[2-^{3}H]glycerol (30–60 μCi/μmol, Amersham/Searle) was repurified by thin-layer chromatography before use with the solvent system 80:20:1 petroleum ether/diethyl ether/acetic acid. Phosphatidyl[*methyl*-^{14}C]choline was also prepared by exchange of [*methyl*-^{14}C]choline for the unlabeled choline in phosphatidylcholine by a reaction using cabbage phospholipase D (Sigma) using a modified procedure of Yang et al. (1967). Glucuronosyl[*glycerol*]-2-^{3}H]diglyceride was prepared from *P. diminuta* grown in the presence of [2-^{3}H]glycerol.

Isolation and Purification of Phosphatidylcholine Exchange Protein. The isolation and purification of a phosphatidylcholine exchange protein from beef liver has been previously described (Shaw et al., 1977). The only devivation from the protocol was exhaustive dialysis of the exchange protein against 20 mM Tris–acetate, 0.25 M sucrose, and 0.5 mM EDTA, a procedure used by Zilversmit and coworkers (Zilversmit & Hughes, 1977). Consequently, albumin was not utilized during the exchange protein dialysis. One unit of exchange protein is defined as the initial rate of exchange of 1 nmol of PC per min at 37 °C when assayed as previously described (Shaw et al., 1977).

Preparation of Single Lamellar Vesicles. All lipids to be included in the preparation of vesicles were added to a tube in chloroform. After evaporation of chloroform, benzene was added and the sample frozen in an alcohol dry ice bath followed by lyophilization under vacuum. The colyophilized lipids were suspended by vortexing in 20 mM Tris-HCl–25 mM NaCl, pH 7.4. Single lamellar vesicles were prepared by the procedure of Huang & Thompson (1974) at ~12 °C using a Heat Systems W-350 sonifier. Small vesicles were separated from large vesicles by high-speed centrifugation (Barenholz et al., 1977).

Preparation of Compositionally Asymmetric Vesicles. Donor single lamellar vesicles composed of 108 μmol of unlabeled phosphatidylcholine, 2.5 μmol of phosphatidyl[*methyl*-^{14}C]choline (~580 000 dpm/μmol), 9.5 μmol of glucuronosyldiglyceride, and <0.05 mol % trioleoyl[2-^{3}H]glycerol in 3.7 mL of 20 mM Tris-HCl–25 mM NaCl, pH 7.4, were incubated with 2.4 mL of acceptor $-N(CD_3)_3$ phosphatidylcholine vesicles (42 μmol) in the presence of ~48 units of dialyzed exchange protein (5.5 mL). An additional 5 mL of

[1] Abbreviations: PC, phosphatidylcholine; $-N(CD_3)_3$ phosphatidylcholine, phosphatidyl[*methyl*-^{2}H]choline; Pr^{3+}, praseodymium chloride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

20 mM Tris-HCl–25 mM NaCl–0.02% sodium azide was included. Control tubes contained 5.5 mL of the buffer utilized for dialysis of the exchange protein. The incubation mixture was sealed under argon and shaken at 37 °C for 8 h. Incubations were also performed with donor vesicles as described above which contained in addition 19.5 μmol of cholesterol.

Following incubation of donor and acceptor vesicles with or without exchange protein, the entire reaction mixture was loaded at room temperature on a DEAE-Sephacel (Pharmacia) column containing a small amount of Sephadex G-50 overlaying the DEAE-Sephacel. The 2-cm diameter column contained 50 mL of DEAE-Sephacel and 2.5 cm in depth of Sephadex G-50. Prior to the fractionation of the reaction mixture, each column was carefully equilibrated against 20 mM Tris-HCl–25 mM NaCl, pH 7.5, and prewashed with 20 μmol of $-N(CD_3)_3$ phosphatidylcholine vesicles.

Five-milliliter fractions were collected. Acceptor vesicles were normally found between fractions 5 and 9. The donor vesicles containing the glucuronosyldiglyceride adsorbed to the column. The sucrose and exchange proteins present in the reaction mixture were also retarded on the Sephadex–DEAE-Sephacel relative to acceptor vesicles.

Pooled fractions were concentrated by using an Amicon diaflo apparatus with an XM-50 filter to a volume of 1.5–2 mL. The concentrated vesicle solutions were placed in $^1/_4$-in. dialysis tubing and attached to a Teflon-coated magnetic bar. Each acceptor vesicle sample was dialyzed with rapid rotation of the tubing in six separate vials of 12 mL of 25 mM NaCl in 99.8% D_2O (Bio-Rad). After dialysis, vesicle samples were placed in sealed containers with a Teflon slide valve through which dry argon or air could be passed. Samples were incubated in the dark room temperature in a desiccator containing D_2O and examined over an 8-day period by proton NMR spectroscopy.

NMR Spectroscopy. Proton NMR was performed at 34 °C by using a continuous wave Varian EM-390, 90-MHz NMR. The maximum sweep time for any one sample was 10 min, and care was taken to avoid saturation of the signal at the R_f power and sweep time employed. The phosphorous concentration of all vesicle samples was ~16 mM. The percentage of unlabeled phosphatidylcholine in each vesicle preparation amounted to 42% when exchange protein was utilized. Consequently, the phosphorous concentration of phosphatidylcholine in these proton experiments was 6.7 mM.

^{31}P NMR spectra were obtained by using a JEOL PS 100P/EC100 Fourier transform spectrometer at 23 °C and 40.48 MHz. Spectra were obtained with successive 90° pulses (19 μs) with a delay of 4–5 T_1 between pulses. The spectral width was 5 kHz, and between 400 and 1000 transients were performed for each spectra. All spectra were obtained with the proton decoupler gated to remove the nuclear Overhauser effect.

The acceptor vesicle samples were examined by proton NMR before and after the addition of $PrCl_3$ (Alfa Ventron). The Pr^{+3}/phosphatidylcholine mole ratio varied from 0.15 to 0.30. For ^{31}P NMR, the Pr^{+3}/phosphatidylcholine mole ratio was 0.01.

Fatty Acid Analysis. Fatty acid methyl esters were prepared by the procedure of Kates (1972) using methanolic HCl. Methyl esters were analyzed by using a Shimadzu GC-4B gas chromatograph with a FID-4C flame ionization detector. Fatty acid methyl esters were fractionated on a glass column packed with 10% silar, 10 C 100–200 mesh on Gas Chrom Q (Applied Science). Methyl ester peaks were quantitated relative to the internal standard, pentadecanoic acid, with a Shimadzu chromatopac EIA integrator.

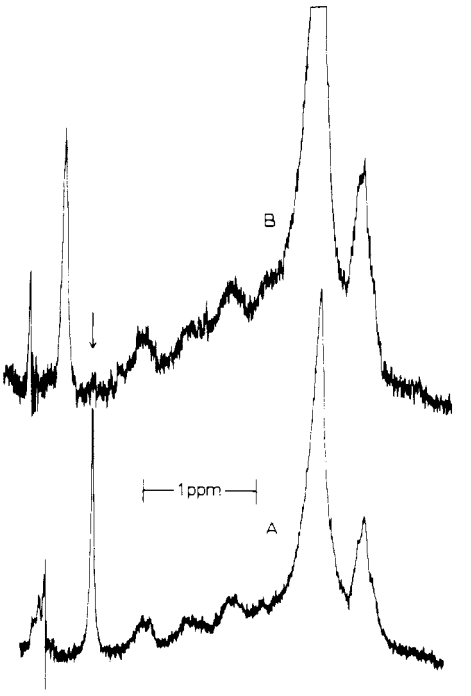

FIGURE 1: Effect of Pr^{3+} on the unlabeled phosphatidylcholine transferred to $-N(CD_3)_3$ phosphatidylcholine acceptor vesicles after incubation with a phosphatidylcholine exchange protein and phosphatidylcholine donor vesicles containing 8 mol % glucuronosyldiglyceride. Spectrum A was performed in the absence of Pr^{3+} whereas spectrum B in the presence of Pr^{3+}. The arrow indicates the region in which the $-N(CH_3)_3$ proton resonance occurs in the absence of Pr^{3+}. The peak to the left of the arrow represents the downfield shifted $-N(CH_3)_3$ protons in the presence of Pr^{3+}. All spectra were similar between days 1 and 5. The total lipid phosphorus concentration was 16 mM whereas the phosphorus concentration of phosphatidylcholine was 6.7 mM. The Pr^{3+}/phosphatidylcholine mole ratio was 0.30.

Miscellaneous Methods. Phosphorus was determined by the method of Bartlett (1959) on aqueous lipid dispersions or after Bligh and Dyer extraction (Bligh & Dyer, 1959). Glucuronosyldiglyceride was quantitated by determination of glucuronic acid as described by Blumenkrantz & Hansen (1973). Protein was determined by the method of Lowry et al. (1951). Cholesterol was determined by the cholesterol oxidase method as described by Moore et al. (1977).

Results

Preparation of Asymmetric Vesicles. Compositionally asymmetric vesicles containing unlabeled phosphatidylcholine in the outer monolayer of $-N(CD_3)_3$ phosphatidylcholine single lamellar vesicles were prepared from negatively charged glycolipid–phosphatidylcholine vesicles in the presence and absence of a purified phosphatidylcholine exchange protein. This system has proven to be a considerable improvement over our earlier system in which erythrocyte ghosts were utilized as the donor for phosphatidylcholine (Shaw et al., 1977). In the presence of exchange protein, a 61–73% enrichment of phosphatidylcholine occurred in the outer monolayer of $-N(CD_3)_3$ phosphatidylcholine acceptor vesicles (Figure 1A). This was determined by comparing both isotopically labeled phospholipid transfer and intensities of the $-N(CH_3)_3$ proton NMR signal relative to the combined intensities of the methyl–methylene proton signals as previously described (Shaw et al., 1977). In the absence of exchange protein, a 15–22% enrichment of unlabeled phosphatidylcholine occurred in the outer monolayer of the acceptor vesicles after the 8-h incubation (Figure 2A). Cholesterol, a 15 mol % component of one of the donor vesicle bilayers, gave at equilibrium a 6–7%

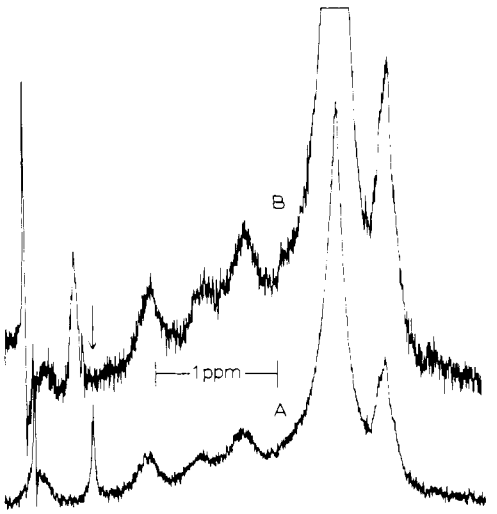

FIGURE 2: Effect of Pr^{3+} on the unlabeled phosphatidylcholine spontaneously transferred to $-N(CD_3)_3$ phosphatidylcholine acceptor vesicles after incubation with phosphatidylcholine donor vesicles containing 8 mol % glucuronosyldiglyceride. Spectrum A was performed in the absence of Pr^{3+} whereas spectrum B in the presence of Pr^{3+}. Refer to Figure 1 for additional details. The Pr^{3+}/PC mole ratio was 0.15.

cholesterol level in acceptor vesicles as a result of spontaneous exchange. In overall spectral appearance, acceptor vesicles containing 6–7 mol % cholesterol were indistinguishable from vesicles containing no cholesterol. Acceptor vesicles incubated with donor vesicles containing cholesterol exhibited a 10% higher percentage transfer of unlabeled phosphatidylcholine by exchange protein than from donor vesicles containing no cholesterol.

Both protein-catalyzed exchange and spontaneous exchange of phosphatidylcholine resulted in an introduction unlabeled phosphatidylcholine into acceptor vesicles. The resulting signal was of narrow line width and completely characteristic for single lamellar vesicles (Figures 1 and 2). It is significant that upon addition of the lanthanide shift reagent, Pr^{3+}, the $-N(CH_3)_3$ signal was completely shifted in both protein-catalyzed and spontaneous exchange experiments. As a result both exchange processes led to phospholipid insertion into the outer monolayer of $-N(CD_3)_3$ phosphatidylcholine vesicles (Figures 1B and 2B).

Glucuronosyldiglyceride initially present in donor vesicles did not undergo exchange to acceptor vesicles which eluted from the DEAE-Sephacel column. This was assessed by observing the absence of [^{14}C]glycerol-labeled glucuronosyldiglyceride in the recovered acceptor vesicles. Consequently, glucuronosyldiglyceride does not participate in spontaneous exchange unless acceptor vesicles adsorbed to the DEAE-Sephacel column contained the glycolipid. Recovery of acceptor vesicles from the column was about 75%.

Compositional Asymmetry and Transbilayer Movement. The asymmetric phosphatidylcholine and phosphatidylcholine–cholesterol acceptor vesicles were incubated at room temperature, and on day 2 air was introduced into the samples, with additional aeration on all following days. No transbilayer movement of the unlabeled choline was detected over 5 days. On day 7, examination in the presence and absence of Pr^{+3} revealed the spectra shown in Figure 3. A typical spectrum for phosphatidylcholine vesicles in the absence of shift reagent is shown in Figure 3A. Upon addition of Pr^{+3}, the vesicles containing only phosphatidylcholine revealed an outside/inside ratio of 2.1 (Figure 3B). Since these vesicles have an external diameter of about 210 Å (Huang, 1969), this ratio indicates a complete equilibration of the unlabeled phosphatidylcholine

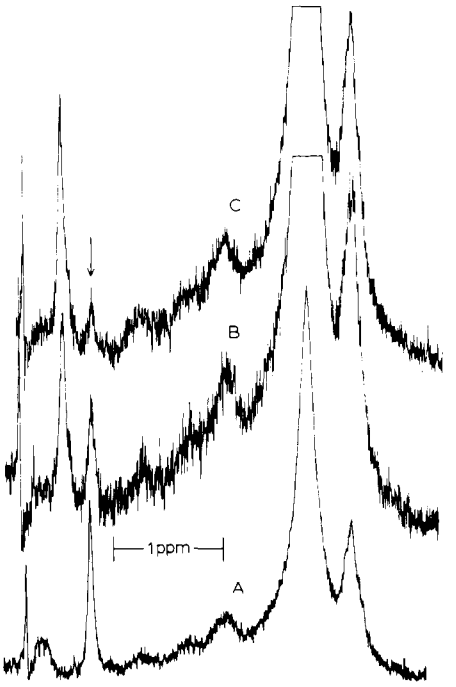

FIGURE 3: Decay of transbilayer asymmetry on day 7 after accumulation of critical levels of phospholipid peroxidation products. Spectrum A represents unlabeled phosphatidylcholine in $-N(CD_3)_3$ phosphatidylcholine vesicles in the absence of Pr^{3+}. Spectrum B represents unlabeled phosphatidylcholine in $-N(CD_3)_3$ phosphatidylcholine vesicles in the presence of Pr^{3+}. Spectrum C represents unlabeled phosphatidylcholine in phosphatidylcholine–6 mol % cholesterol. The phosphorous concentration of phosphatidylcholine in the $-N(CD_3)_3$ phosphatidylcholine–6 mol % cholesterol vesicles was 7.4 mM. Additional concentrations are described in Figure 1.

between inner and outer monolayers. The acceptor vesicles which contained phosphatidylcholine and 6 mol % cholesterol had an outside/inside ratio of 6.7 (Figure 3C). Both spectra in the presence of Pr^{+3} gave sharp and clearly resolved outside/inside monolayer signals. In addition, the spectra show a higher noise level suggestive of a loss in the percentage of phospholipid participating in the signal response.

Upper limit half-times for the transbilayer movement which occurred between days 5 and 7, a time period of 36 h, were estimated by using the previously described equation for reversible first-order kinetics (Shaw et al., 1977):

$$\frac{[R/(R+1)]_{t_7} - [R/(R+1)]_{eq}}{[R/(R+1)]_{t_0} - [R/(R+1)]_{eq}} = e^{-(k_0 + k_i)t}$$

R represents the number of unlabeled phosphatidylcholine molecules in the outer monolayer to the number in the inner monolayer of the vesicle. R at t_0 was given a value of 100/1 since the $-N(CH_3)_3$ signal was virtually completely asymmetric between days 1 and 5. The R at t_7 was either 2.1 or 6.7. The equilibrium value of R was set at 2.0. The half-time for transbilayer movement of unlabeled phosphatidylcholine was found to be 7.3 h in phosphatidylcholine vesicles and 53 h in phosphatidylcholine–6 mol % cholesterol vesicles. This rate represents an upper limit only since time points during the period of transbilayer movement were not taken.

Aging Effects on Vesicle Integrity. During the experiments with the asymmetric phosphatidylcholine and phosphatidylcholine–6 mol % cholesterol vesicles, air was introduced during sample manipulation beginning on day 2 and each day thereafter. Autooxidation intermediates and products should begin to accumulate in the system at this time. The fatty acid composition of the egg phosphatidylcholine used in these experiments contained 16.5% 18:2 and 2.6% 20:4 fatty acids.

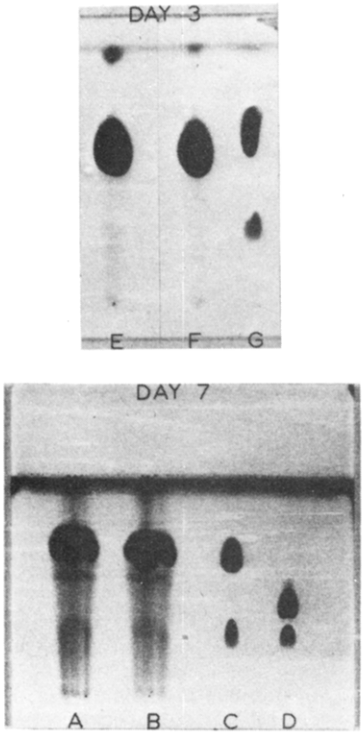

FIGURE 4: Silicic acid thin-layer chromatograms of extracted lipids from vesicle preparations at day 3 (E and F) and day (A and B). (A) and (F) represent lipids extracted from unlabeled phosphatidylcholine in $-N(CD_3)_3$ phosphatidylcholine vesicles. (B) and (E) represents lipids extracted from phosphatidylcholine in $-N(CD_3)_3$ phosphatidylcholine–6 mol % cholesterol. The thin layers were developed by using chloroform/methanol/ammonia/water (65:25:3.5:1.5) and stained by using iodine vapors. Between 1 and 2 μmol of lipid phosphorus was applied to the thin layers. (C) and (G) represent the standards, phosphatidylcholine (upper spot) and lysophosphatidylcholine (lower spot). (D) represents the standards, sphingomyelin (upper spot) and lysophosphatidylcholine (lower spot).

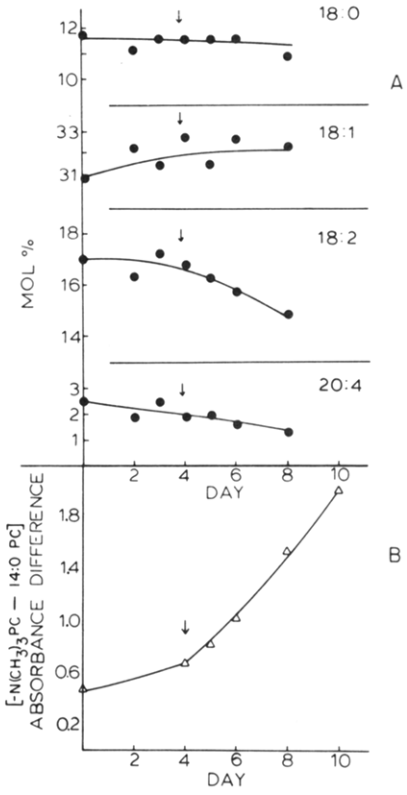

FIGURE 5: (A) Gas–liquid chromatography of fatty acid methyl esters prepared from egg phosphatidylcholine vesicles and aged for up to 8 days under similar conditions as vesicles utilized in the NMR experiments. The standard deviation upon injecting the sample 3 times was ±1%, and separate transesterification followed by GLC analysis varied up to ±10%. (B) Absorbance difference spectra at 230 nm between unlabeled phosphatidylcholine and dimyristylphosphatidylcholine dissolved in ethanol. The phospholipids were prepared from vesicles aged for up to 10 days under similar conditions as the phosphatidylcholine/$-N(CD_3)_3$ phosphatidylcholine vesicles utilized in the NMR experiments. At the time of analysis, an aqueous aliquot of the vesicle preparation was dissolved in ethanol and scanned in an Aminco DWII scanning spectrometer between 210 and 340 nm. The absorbance difference between the peak at 230 nm for dimyristylphosphatidylcholine (a phospholipid not subject to peroxidation) was subtracted from the peak at 230 nm for unlabeled phosphatidylcholine (a phospholipid sensitive to peroxidation). The arrows in (A) and (B) represent time points when air was bubbled through the samples. Duplicate samples were also analyzed as described above but contained small amounts of sodium azide. The results were analogous to samples not containing sodium azide.

The egg $-N(CD_3)_3$ phosphatidylcholine used in the experiments was prepared from egg phosphatidylethanolamine (see Materials and Methods) and contained 13.5% 18:2, 13.9% 20:4, and 6.4% higher saturated or polyunsaturated fatty acids.

The presence of autooxidation products in the system was determined by thin-layer chromatography at time periods during the experiment. The results for days 3 and 7 are shown in Figure 4. On day 3 a phosphorus determination of areas below the major phosphatidylcholine band from the two experimental samples revealed 1% phosphorus in the region where lysophosphatidylcholine normally migrates. Other areas below the major phosphatidylcholine band were unidentified autooxidation products and accounted for 3% of the phosphorus in phosphatidylcholine vesicles and 4% in phosphatidylcholine–cholesterol vesicles. On day 7 lysophosphatidylcholine had increased to 6% in the phosphatidylcholine vesicles and 4% in phosphatidylcholine–cholesterol vesicles. Phosphorus-containing autooxidation products increased to 11% in phosphatidylcholine vesicles and 7% in phosphatidylcholine–cholesterol vesicles. In separate studies with unlabeled phosphatidylcholine vesicles incubated under identical conditions, losses in the content of 18:2 and 20:4 fatty acids present in phosphatidylcholine correlated with an increased absorbance at 230 nm of ethanol solubilized phospholipid vesicles (Figure 5). The absorbance ratio (230/215 nm) or Klein index for the dimyristylphosphatidylcholine and egg phosphatidyl, respectively, of Figure 5B was 0.39 and 0.49 for day 1, 0.46 and 0.78 for day 5, 0.47 and 1.01 for day 8, and 0.36 and 1.24 for day 10. The ratio for egg phosphatidylcholine increased steadily and was comparable at day 1 with dimyristylphosphatidylcholine. The Klein index lost its usefulness at about day 6 since the apparent variety of carbonyls and peroxides produced a rather broad peak at 230 nm which distorted the ester-linked carbonyls at 215 nm. It should be realized that we have examined an aqueous aliquot containing small amounts of sodium azide dissolved in ethanol and not a chloroform-soluble lipid as are most reports for the Klein index in the literature.

The vesicle bilayer was found to be impermeable to Pr^{+3}, the lanthanide shift reagent. For example, on day 4 of the experiments, aliquots from all four NMR samples incubated in the presence and absence of exchange protein were pooled for a total volume of 0.8 mL at ~16 mM phospholipid concentration. Phosphorus NMR spectra were recorded in the presence and absence of Pr^{+3} and supported the contention of an impermeable bilayer (Figure 6). The outside/inside ratio varied from 2.0 at initial time periods to 1.8 after being maintained in the presence of Pr^{+3} for 22 h. In fact, to abolish the differential interaction of Pr^{+3} with phosphate moieties present on the outer monolayer relative to inner monolayer,

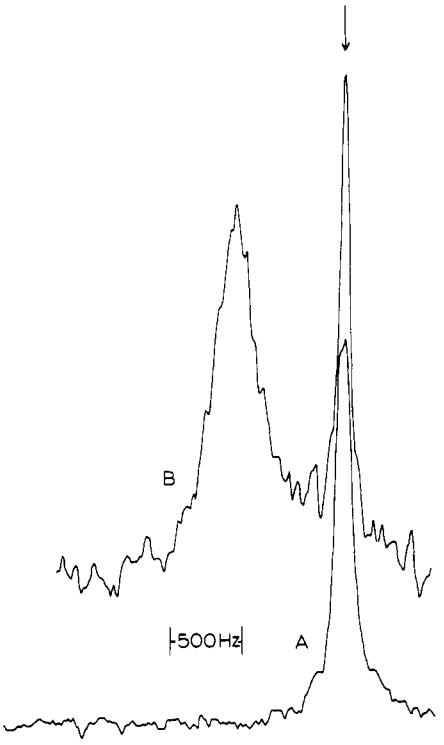

FIGURE 6: ^{31}P NMR of a mixture of the acceptor phospholipid and phospholipid–cholesterol vesicles utilized in the proton NMR experiments. The samples were pooled on day 4 for a total quantity of 12 μmol in 0.8 mL (15 mM). (A) represents the spectrum in the absence of Pr^{3+}. (B) represents the spectrum taken in the presence of a 0.01 mole ratio of Pr^{3+}/phosphatidylcholine. The arrow represents the outer monolayer phosphorus, and the peak to the left of the arrow is the inner monolayer phosphorus. The total time period required for accumulating transients varied from 1 to 2 h.

it was necessary to add 9% ethanol and place the sample in a boiling water bath for 1 min.

The proton NMR spectra obtained on day 7 (Figure 3) showed the decrease of asymmetry of unlabeled phosphatidylcholine in the two vesicle populations. These observations are not consistent with leakiness to Pr^{+3} since both outside and inside signals were maintained during the NMR measurements of about 1 h on day 7. Consequently, the outside/inside ratio of 6.7 for phosphatidylcholine–6 mol % cholesterol vesicles may have reached a semistable asymmetric arrangement in contrast to complete equilibration in phosphatidylcholine vesicles (i.e. outside/inside = 2.1). The $-N(CH_3)_3$ choline signals after Pr^{+3} addition were sharp and not those of fused or leaky vesicles. In separate studies with unlabeled phosphatidylcholine vesicles or unlabeled phosphatidylcholine–6 mol % cholesterol vesicles, fusion aggregation and leakiness to Pr^{+3} correlated with poorly resolved outside/inside $-N(CH_3)_3$ signals (Figure 7B) and/or Pr^{+3} shiftability of inner monolayer unlabeled phosphatidylcholine (Figure 7A). The examples illustrated in Figure 7 represent phosphatidylcholine vesicles (Figure 7A) or phosphatidylcholine–6 mol % cholesterol vesicles (Figure 7B) aged for 21 days in the presence of aerated azide-containing buffers.

Discussion

Asymmetric single lamellar vesicles with unlabeled phosphatidylcholine in the outer monolayer and $-N(CD_3)_3$ phosphatidylcholine in the inner monolayer have been prepared from negatively charged donor vesicles and acceptor vesicles composed of $-N(CD_3)_3$ phosphatidylcholine. The experiments are simple in design and avoid the use of a biological membrane donor such as erythrocyte ghosts which contain cholesterol, numerous proteins, and possibly small lipid, lipid–protein

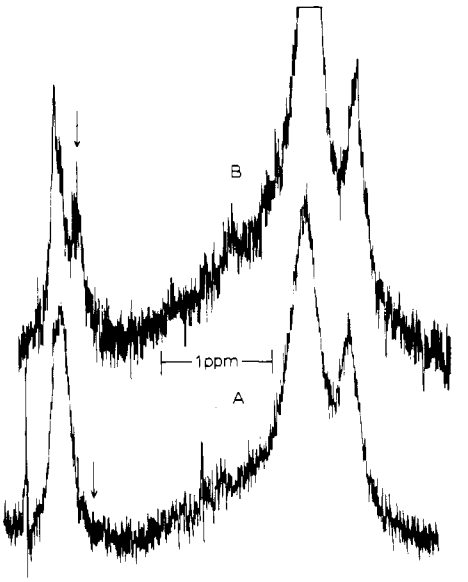

FIGURE 7: Effect of Pr^{3+} on the outer and inner monolayer $-N(CH_3)_3$ choline signals in phospholipid vesicles aged for 21 days. (A) represents unlabeled phosphatidylcholine vesicles aged for 21 days and examined in the presence of Pr^{3+}. The vesices are completely leaky to Pr^{3+}; therefore no inner monolayer signal is observed. In (B), phosphatidylcholine–6 mol % cholesterol vesicles were aged for 21 days and examined in the presence of Pr^{3+}. The Pr^{3+}/PC ratio in (A) was 0.25 and in (B) 0.30. Higher ratios of Pr^{3+}/PC revealed no discernible differences. Both samples contained 0.2% sodium azide.

vesicles after incubation. Furthermore, Cook et al. (1980) have recently demonstrated transfer of erythrocyte membrane proteins to phospholipid vesicles. The transfer process was rapid and extensive when recipient phospholipid vesicles were above their phase transition. The exchange catalyzed by a phosphatidylcholine exchange protein is a reasonably well-studied phenomenon, and it was not surprising in light of our earlier work (Shaw et al., 1977) to find unlabeled phosphatidylcholine asymmetrically located in the outer monolayer. We consistently found a higher percentage transfer of phosphatidylcholine to acceptor vesicles (~10%) with donor vesicles which contained 14 mol % cholesterol. The donor vesicles in question, phosphatidylcholine–6 mol % cholesterol, contained a slightly higher percentage of the acidic glycolipid than phosphatidylcholine vesicles, namely, 7.9% relative to 6.8%. Such subtle variations in the percentages of glucuronosyldiglyceride may be sufficient to alter exchange protein activity if the increased percentage is confined only to the outer monolayer of donor vesicles or if cholesterol influences glycolipid distribution in the bilayer. The nature of the role of bilayer surface charge in exchange protein activity was first studied by Van Den Besselaar et al. (1975). These workers found a decreased apparent dissociation for the exchange protein–liposome complex when increasing quantities of acidic phospholipids were present in the bilayer. In contrast, Dicorleto et al. (1977) has found stimulation of phosphatidylcholine transfer by acidic phospholipids in two out of three assay systems studied.

Several groups have been concerned with the mechanism by which phospholipids exchange between lipid bilayer vesicles (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Thilo, 1977; Roseman & Thompson, 1980). Phospholipid exchange can in principle occur through the aqueous phase as a monomer, exchange via micelles, collision of vesicles, or fusion. A substantial occurrence of spontaneous exchange resulted during the 8-h incubation of acceptor and donor vesicles (see Figure 2). Since the present work shows the absence of transbilayer movement until oxidation products

accumulate (i.e., after 5 days), the spontaneous exchange between vesicles must occur between the outer monolayers of donor and acceptor vesicles. The spontaneous exchange likely occurs between donor and acceptor vesicles in both directions. Otherwise, a one way directional transfer would have led to lateral expansion of the outer monolayer of the acceptor vesicles with ensuing transbilayer movement. Under the assumption that reversible first-order kinetics applies during the intervesicular exchange between outer monolayers for the 8-h incubation (see Figure 2), the $t_{1/2}$ for spontaneous exchange is equal to 11.6 h whereas the $t_{1/2}$ for exchange protein stimulated intervesicular exchange equals 1.8 h.[2]

The presence of a stable asymmetric bilayer with unlabeled phosphatidylcholine in only the outer monolayer for 5 days suggests that for transbilayer asymmetry to decay rapidly, a perturbation must occur. We chose to test the effect of exposure to air on the bilayer system since significant unsaturation was present. Lipid peroxidation is a most complex phenomena. When hydrogen abstraction occurs on an unsaturated acyl chain, a series of events leads to a variety of free radical intermediates, acyl chain peroxides, and small chain scission products, such as malondialdehyde (Quinn & Williams, 1978; Vladimirov et al., 1980). Many of the acyl chain oxidation products will remain covalently bonded to the glycerophosphorylcholine backbone; hence, a series of streaked phosphorus-containing products will be seen on thin-layer chromatography as observed in Figure 4. We have observed streaked areas on silica gel thin layers when unsaturated phospholipids which are subject to oxidation are used. Dimyristylphosphatidylcholine (14:0) vesicles upon aging in an air atmosphere revealed only a distinct band of lysomyristylphosphatidylcholine but no streaked areas. The increase in the streaked thin-layer products also correlated with a marked elevation in the absorbance at 230 nm, and in separate studies elevated malondialdehyde levels were present in phospholipid vesicle samples which showed greater streaking upon thin-layer chromatography on chloroform-soluble products. The streaked areas are hydrocarbon in nature since they stain with iodine and the areas possess phosphorus. Crosslinked compounds induced by products of oxidation may also be present in the streaked areas. We would expect phospholipids which contain intact acyl chains with hydroperoxide, aldehyde, alkene aldehyde, or conjugated diene acyl chains to fractionate more closely to the unmodified phosphatidylcholine area. Some of these products will also migrate with an R_f very similar to that of the intact unoxidized phospholipid as shown by Chakrabarti & Khorana (1975) and Poghossian & Nalbandyan (1980).

In phospholipid bilayer vesicles and certain biological membranes, lipid peroxidation in the presence of Fe^{2+}–ascorbic acid–air can lead to a more ordered microenvironment as detected by physical techniques using fluorescent probes (Dobretsov et al., 1977; Shaw et al., 1980). Furthermore, Barsukov et al. (1980) have recently found that lipid peroxidation can promote transbilayer movement in phospholipid vesicles. These authors provide ^{31}P NMR data from oxidized phospholipid vesicles that suggest nonbilayer structures, particularly highly isotropic inverted micellar structures, are produced upon oxidation of vesicles. It has previously been suggested by DeKruijff et al. (1980a,b) that transbilayer movement might occur via nonbilayer, micellar structures. An interesting aspect of the present unlabeled phosphatidylcholine/$-N(CD_3)_3$ phosphatidylcholine vesicle system is that there is an asymmetry between the outer and inner monolayers with respect to unsaturation. The $-N(CD_3)_3$ phosphatidylcholine is more subject to peroxidation since it is more highly unsaturated. Furthermore, the $-N(CD_3)_3$ phosphatidylcholine is found on the inner monolayer (100%) and the outer monolayer at a level of only about 32%. Consequently, peroxidation products may accumulate at higher percentages on the inner monolayer. This unequal accumulation of oxidation products might be an important perturbation for transbilayer movement of the more saturated phosphatidylcholine probe which is located initially only in the outer monolayer (68%).

In other experiments on $-N(CD_3)_3$ phosphatidylcholine vesicles containing unlabeled phosphatidylcholine in the outer monolayer (J.M. Shaw and T.E. Thompson, unpublished results), we have abused the bilayer with Fe^{2+}–ascorbic acid–air, a powerful oxidizing agent (Hamilton, 1964; Vladimirov et al., 1980). Levels of malondialdehyde between 12 and 30 nmol/μmol of phospholipid were produced over short time periods (<1 h); however, no transbilayer movement of the outer monolayer phosphatidylcholine occurred. Only 3 nmol of malondialdehyde per μmol of phospholipid was produced during similar time periods (<1 h) with air alone. We conclude that either oxidation products were not at critical levels or the specific oxidized compounds produced by Fe^{2+}–ascorbic acid–air treatment were insufficient to stimulate transbilayer movement (J. M. Shaw and T. E. Thompson, unpublished results). The time periods the bilayer is exposed to intermediates during the short time periods with Fe^{2+}–ascorbic acid air vs. the longer time periods in the present study with air oxidation alone may also be critical. The use of Fe^{2+}–ascorbic acid–air is known to enhance chain propagation and chain branching of the radical process (Vladimirov et al., 1980); therefore, it follows that a larger percentage of water-soluble breakdown products will be present relative to air oxidation alone. It is clear from our present work that critical levels of peroxidation products must be reached before rapid transbilayer movement occurs. In this regard, cholesterol in the present system prevented complete equilibration of the unlabeled phosphatidylcholine (i.e., O/I = 6.7) (Figure 3C). Consequently, cholesterol may provide a stabilizing effect on bilayer structure and thus reduce transbilayer movement.

The upper limit half-times reported here for unlabeled phosphatidylcholine vesicles ($t_{1/2} \sim 7.3$ h) and phosphatidylcholine–6 mol % cholesterol vesicles ($t_{1/2} \sim 53$ h) are similar to half-times found in biological membranes [see Table II of Shaw et al. (1979)]. Of special interest is the rapid transmembrane migration found in microsomal membranes (Zilversmit & Hughes, 1977; Van Den Besselaar et al., 1978). Microsomal membranes are highly subject to lipid peroxidation (Wills, 1969; Hogberg et al., 1973; Svingen et al., 1979) and contain low percentages of cholesterol. Consequently, the possible occurrence of lipid peroxidation in biological membranes and the effect of those products on the transbilayer movement of phospholipid should be considered when membrane systems are examined.

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References

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. (1977) *Biochemistry 16*, 2806.

[2] The reversible first-order equation described under Results was utilized except R equals the percentage of unlabeled phosphatidylcholine enriched in the outer monolayer of the acceptor vesicles (R = 0.67 with exchange protein, 0.185 minus exchange protein, 0.0 at time zero, and 0.72 at equilibrium).

Barsukov, L. I., Victorov, A. V., Vasilenko, I. A., Evstigneeva, R. P., & Bergelson, L. D (1980) *Biochim. Biophys. Acta 598*, 153.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.
Blumenkrantz, N., & Hansen, G. A. (1973) *Anal. Biochem. 54*, 484.
Chakrabarti, P., & Khorana, H. G. (1975) *Biochemistry 14*, 5021.
Cook, S. L., Bouma, S. R., & Huestis, W. H. (1980) *Biochemistry 19*, 4601.
Cullis, P. R., & McLaughlin, A. C. (1977) *Trends Biochem. Sci. (Pers. Ed.) 2*, 196.
DeKruijff, B., & Wirtz, K. W. (1977) *Biochim. Biophys. Acta 468*, 318.
DeKruijff, B., & Van Zoelen, E. J. (1978) *Biochim. Biophys. Acta 514*, 105.
DeKruijff, B., & Van Den Besselaar, A. M., & Van Deenen, L. L. (1977) *Biochim. Biophys. Acta 465*, 443.
DeKruijff, B., Van Zoelen, E. J., & Van Deenen, L. L. (1978) *Biochim. Biophys. Acta 509*, 337.
DeKruijff, B., Cullis, P. R., & Verkleij, A. J. (1980a) *Trends Biochem. Sci. (Pers. Ed.) 5*, 79.
DeKruijff, B., Rietveld, A., & Van Echteld, C. J. A. (1980b) *Biochim. Biophys. Acta 600*, 597.
Dicorleto, P. E., & Zilversmit, D. B. (1979) *Biochim. Biophys. Acta 552*, 114.
Dicorleto, P. E., Fakharzadeh, F. F., Searles, L. L., & Zilversmit, D. B. (1977) *Biochim. Biophys. Acta 468*, 296.
Dobretsov, G. E., Borschevskaya, T. A., Petrov, V. A., & Vladimirov, Y. A. (1977) *FEBS Lett. 84*, 125.
Duckwitz-Peterlein, G., Eilenberger, G., & Overath, P. (1977) *Biochim. Biophys. Acta 469*, 311.
Estep, T. N., Mountcastle, D. B., Biltonen, R. L., & Thompson, T. E. (1978) *Biochemistry 17*, 1984.
Gerritsen, W. J., Henricks, P. A., DeKruijff, B., & Van Deenen, L. L. M. (1980) *Biochim. Biophys. Acta 600*, 607.
Hamilton, G. A. (1964) *J. Am. Chem. Soc. 86*, 3391.
Hogberg, J., Bergstrand, A., & Jakobsson, S. V. (1973) *Eur. J. Biochem. 37*, 51.
Huang, C. (1969) *Biochemistry 8*, 344.
Huang, C., & Thompson, T. E. (1974) *Methods Enzymol. 32*, 485.
Johnson, L. W., Hughes, M. E., & Zilversmit, D. B. (1975) *Biochim. Biophys. Acta 375*, 176.
Kates, M. (1972) *Techniques of Lipidology* (Work, T. S., & Work, E., Eds.) pp 362–367, North-Holland Publishing Co., Amsterdam.
Low, M., & Zilversmit, D. B. (1980) *Biochim. Biophys. Acta 596*, 223.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Martin, F. J., & MacDonald, R. C. (1976) *Biochemistry 15*, 321.
Moore, N. F., Patzer, E. J., Barenholz, Y., & Wagner, R. R. (1977) *Biochemistry 16*, 4708.
Poghossian, G. G., & Nalbandyan, R. M. (1980) *Lipids 15*, 591.
Quinn, P. J., & Williams, W. P. (1978) *Prog. Biophys. Mol. Biol. 34*, 156.
Roseman, M. A., & Thompson, T. E. (1980) *Biochemistry 19*, 439.
Rothman, J. E., & Dawidowicz, E. A. (1975) *Biochemistry 14*, 2809.
Shaw, J. M., & Pieringer, R. A. (1977a) *J. Biol. Chem. 252*, 4391.
Shaw, J. M., & Pieringer, R. A. (1977b) *J. Biol. Chem. 252*, 4395.
Shaw, J. M., & Thompson, T. E. (1978) *Biophys. J. 21*, 122a.
Shaw, J. M., Hutton, W. C., Lentz, B. R., & Thompson, T. E. (1977) *Biochemistry 16*, 4156.
Shaw, J. M., Moore, N. F., Patzer, E. J., Correa-Freire, M. C., Wagner, R. R., & Thompson, T. E. (1979) *Biochemistry 18*, 538.
Shaw, J. M., Shaw, K. V., Bembeneck, M. E., Litman, B. J., & Konigsberg, I. R. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2098.
Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., & Smith, I. C. (1974) *Biochem. Biophys. Res. Commun. 60*, 844.
Svingen, B. A., Buege, J. A., O'Neal, F. O., & Aust, S. D. (1979) *J. Biol. Chem. 254*, 5892.
Thilo, L. (1977) *Biochim. Biophys. Acta 469*, 326.
Van Den Besselaar, A. M., Helmkamp, G. M., & Wirtz, K. N. (1975) *Biochemistry 14*, 1852.
Van Den Besselaar, A. M., Van Den Bosch, H., & Van Deenen, L. L. (1977) *Biochim. Biophys. Acta 465*, 454.
Van Den Besselaar, A. M., DeKruijff, B., Van Den Bosch, H., & Van Deenen, L. L. (1978) *Biochim. Biophys. Acta 510*, 242.
Van Zoelen, E. J., DeKruijff, B., & Van Deenen, L. L. (1978) *Biochim. Biophys. Acta 508*, 97.
Vladimirov, Y. A., Olenev, V. I., Suslova, J. B., & Cheremisina, Z. P. (1980) *Adv. Lipid Res. 17*, 174.
Wills, E. D. (1969) *Biochem. J. 113*, 315.
Yang, S. F., Freer, S., & Benson, A. A. (1967) *J. Biol. Chem. 242*, 477.
Zilversmit, D. B., & Hughes, M. E. (1977) *Biochim. Biophys. Acta 469*, 99.