References

Baker, H. N., Gotto, A. M., and Jackson, R. L. (1975), *J. Biol. Chem. 250,* 2725.
Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466.
Brewer, H. B., Lux, S. E., Ronan, R., and John, K. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1304.
Jackson, R. L., and Gotto, A. M. (1972), *Biochim. Biophys. Acta 285,* 36.
Jackson, R. L., Gotto, A. M., Lux, S. E., John, K. M., and Fleischer, S. (1973a), *J. Biol. Chem. 248,* 8449.
Jackson, R. L., Mao, S. J. T., and Gotto, A. M. (1974a), *Biochem. Biophys. Res. Commun. 61,* 1317.
Jackson, R. L., Morrisett, J. D., and Gotto, A. M. (1976), *Physiol. Rev. 56,* 529.
Jackson, R. L., Morrisett, J. D., Gotto, A. M., and Segrest, J. P. (1975), *Cell. Mol. Biochem. 6,* 43.
Jackson, R. L., Morrisett, J. D., Pownall, H. J., and Gotto, A. M. (1973b), *J. Biol. Chem. 248,* 5218.
Jackson, R. L., Morrisett, J. D., Sparrow, J. T., Segrest, J. P., Pownall, H. J., Smith, L. C., Hoff, H. F., and Gotto, A. M. (1974b), *J. Biol. Chem. 249,* 5314.
Lux, S. E., John, K. M., Fleischer, S., Jackson, R. L., and Gotto, A. M. (1972a), *Biochem. Biophys. Res. Commun. 49,* 23.
Lux, S. E., John, K. M., Ronan, R., and Brewer, H. B. (1972b), *J. Biol. Chem. 247,* 7519.
Mao, S. J. T., Gotto, A. M., and Jackson, R. L. (1975), *Biochemistry 14,* 4127.
Morrisett, J. D., David, J. S. K., Pownall, H. J., and Gotto, A. M. (1973), *Biochemistry 12,* 1290.
Morrisett, J. D., Jackson, R. L., and Gotto, A. M. (1977), *Biochim. Biophys. Acta* (in press).
Segrest, J. P., Jackson, R. L., Morrisett, J. D., and Gotto, A. M. (1974), *FEBS Lett. 38,* 247.
Sparrow, J. T. (1976), *J. Org. Chem. 41,* 1350.

Proton Nuclear Magnetic Resonance Study of the Decay of Transbilayer Compositional Asymmetry Generated by a Phosphatidylcholine Exchange Protein†

J. Michael Shaw, William C. Hutton,‡ B. R. Lentz,§ and T. E. Thompson*

ABSTRACT: Transbilayer compositional asymmetry was generated in single-lamellar vesicles formed from $-N(CD_3)_3$ egg phosphatidylcholine by incubation with erythrocyte ghost membranes in the presence of a purified phosphatidylcholine exchange protein prepared from beef liver. In a series of experiments, between 50 and 85% of the $-N(CD_3)_3$ phosphatidylcholine in the external face of the bilayer vesicles was replaced by $-N(CH_3)_3$ phosphatidylcholine from ghost membranes after a 24-h incubation at 37 °C. Proton NMR studies utilizing Pr^{+3} as a shift reagent showed that 82 to 89% of the exchanged $-N(CH_3)_3$ phosphatidylcholine was found on the external face of the vesicle wall. The decay of the transbilayer compositional asymmetry by exchange migration of $-N(CH_3)_3$ and $-N(CD_3)_3$ phosphatidylcholines, as followed by NMR spectroscopy over a 5-day period at 23 °C, was shown to be a slow process with a half-time of 26 days. The half-times consistent with ±1 standard deviation were 16 and 69 days. Appropriate controls established the integrity of the vesicles throughout the 5-day period. The cholesterol content of the erythrocyte ghost membranes used to prepare the asymmetric vesicles was reduced 40 to 60% by prior incubation with phosphatidylcholine vesicles. During preparation of the asymmetric vesicles, spontaneous cholesterol movement from the cholesterol-depleted ghosts resulted in final cholesterol concentrations in the asymmetric vesicles of between 5 and 16 mol %.

Three approaches have been utilized for measuring transbilayer migration of phosphatidylcholine in small, single-lamellar vesicles. Spin-labeled phosphatidylcholine has been incorporated into phosphatidylcholine vesicles and the outer bilayer spin-label paramagnetism abolished with ascorbate (Kornberg and McConnell, 1971). The rate of accessibility to external ascorbate of the remaining spin label initially on the inner bilayer surface suggested a half-time for transbilayer equilibration of spin label as short as 6.5 h at 30 °C. Second, a chemical-labeling technique has been utilized in which the outer monolayer phosphatidylethanolamine present in phosphatidylcholine vesicles is converted to its amidine derivative by reaction with isethionyl acetimidate. Transbilayer movement of the unreacted inner monolayer phosphatidylethanolamine, measured with 2,4,6-trinitrobenzenesulfonic acid, showed the half-time to be greater than 80 days at 22 °C (Roseman et al., 1975). A third approach introduced by Johnson and co-workers (1975) and by Rothman and Dawidowicz (1975) employed a phosphatidylcholine exchange protein to generate a compositional asymmetry of isotopically labeled phospholipid in a vesicle bilayer. The subsequent "exchange-out" kinetics of the labeled phospholipid was used to establish the half-time for the transbilayer equilibration of this lipid. Johnson and co-workers (1975) reported a half-time

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received March 25, 1977.* This investigation was supported by a grant from the National Institutes of Health, United States Public Health Service (GM-14628) and a Postdoctoral Fellowship (GM 05190) awarded to J.M.S.

‡ Department of Chemistry, University of Virginia, Charlottesville, Va. 22901.

§ Current address: Department of Biochemistry and Nutrition, University of North Carolina School of Medicine, Chapel Hill, N.C., 27514.

of 4 to 15 days at 37 °C, while Rothman and Dawidowicz (1975) showed that the half-time must be greater than 11 days at this temperature.

In this paper, we report the determination of the kinetics of transbilayer lipid migration in vesicles by a new procedure utilizing NMR spectroscopy and vesicle compositional asymmetry generated by a phosphatidylcholine exchange protein. The basic design of the experiment is as follows. Using the beef liver exchange protein, phosphatidylcholine from normal erythrocyte ghost membranes is incorporated into the outer bilayer surface of vesicles formed from egg phosphatidylcholine which has the *N*-methyl protons completely replaced by deuterium. Since the *N*-methyl deuterons do not give rise to a proton NMR signal in the spectrometer operating at 100 MHz, only the resonance line of the *N*-methyl protons exchanged into the surface of the vesicle is seen. The association of these protons with externally or internally located phosphatidylcholines can be established by the introduction into the vesicle dispersion of the impermeant paramagnetic ion Pr^{+3}. When this is done, most of the *N*-methyl proton signal is shifted downfield. If aliquots of this asymmetric vesicle system are then examined in the NMR spectrometer as a function of time, both in the absence and presence of Pr^{+3}, the appearance of internalized phosphatidylcholine with protonated *N*-methyl groups can be readily detected. Preliminary accounts of this study have been presented elsewhere (Shaw et al., 1977; Thompson, 1977).

Materials and Methods

Preparation of Phosphatidylcholine and Phosphatidylethanolamine. Both phosphatidylcholine and phosphatidylethanolamine were isolated and purified from hen egg yolks using two-step silicic acid (Silicar CC-4, Mallinckrodt) column chromatography (Litman, 1973). An alumina column was employed to remove any lysophosphatidylethanolamine from the silicic acid purified phosphatidylcholine (Singleton et al., 1965). The purified phospholipids were washed with an ethylenediaminetetraacetic acid–ethylene glycol–bis(β-amino ethyl ether)-*N,N'*-tetraacetic acid mixture (pH 7.2) as described by Suurkuusk et al. (1976). Both phospholipids were passed through Teflon filters (0.2 μm fluoropore, Millipore) for removal of any residual silicic acid and then precipitated two times from spectral grade chloroform with spectral grade acetone. The purified phospholipids 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 two methods. (1) Phosphatidic acid was prepared by phospholipase D (Cabbage, Sigma) treatment of egg phosphatidylcholine (Dawson and Hemington, 1967). Choline deuterated in the *N*-methyl group was synthesized from 0.45 mL of redistilled ethanolamine and 4 g of CD_3I (Merck and Co.) dissolved in 15 mL of methanol and maintained at pH 11 for 2 days at room temperature. After removal of solvent under reduced pressure, the residue was extracted with ethanol/ether (1:1) and the crystals of choline iodide were collected by filtration. Conversion of choline iodide to the acetate form has been described by Sears et al. (1976). The [^{2}H]choline acetate and phosphatidic acid were condensed in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (Aneja and Chadha, 1971) and purified by silicic acid column and applied to an alumina column followed by subsequent elution with increasing concentrations of methanol in chloroform. The resulting phosphatidylcholine was shown to be free from phosphatidyl-*N,N*-dimethylethanolamine by using the thin-layer chromatography system chloroform/methanol/acetic acid/water (25:14:4:2). Phosphatidyl[*methyl*-^{2}H]-choline prepared in the above manner was a gift of Dr. Charles F. Schmidt of this laboratory. Proton NMR spectra of vesicles chromatography. (2) —$N(CD_3)_3$ phosphatidylcholine was also prepared by the methylation of phosphatidylethanolamine with CD_3I in the dark as described by Stockton et al. (1974). The chloroform/methanol phase was removed under reduced pressure and the reactant lipids were dissolved in chloroform of —$N(CD_3)_3$ phosphatidylcholine prepared by both procedures were identical. No resonance arising from —$N(CH_3)_3$ protons was detectable.

Preparation of [^{3}H]Phosphatidylcholine. Phosphatidyl-[*methyl*-^{3}H]choline was prepared from phosphatidic acid derived from egg phosphatidylcholine and [*methyl*-^{3}H]choline acetate (Aneja and Chadha, 1971) and purified as described above. [*palmitoyl*-^{3}H]Phosphatidylcholine derived from rat liver was a gift of Dr. Y. Barenholz. [*oleoyl*-^{3}H]Phosphatidylcholine was synthesized as described by Cubero Robles and Van den Berg (1969) from L-α-glycerophosphorylcholine prepared from egg phosphatidylcholine (Chadha, 1970), oleic acid (K salt), and [^{3}H]oleoyl anhydride (Selinger and Lapidot, 1966). Dipalmitoylphosphatidyl[*methyl*-^{14}C]choline was purchased from New England Nuclear.

Isolation and Purification of Phosphatidylcholine Exchange Protein. A phosphatidylcholine exchange protein was isolated from 6 lb of beef liver as described by Kamp et al. (1973). Several modifications in the work-up included the utilization of 0.1 mM dithiothreitol in place of 0.01 M mercaptoethanol in the various buffers and a partial concentration step after the CM-52 column using a Bio-fiber 50 beaker (Bio-Rad) and further concentration in a dialysis bag against 70% sucrose. The initial volumes from separate exchange protein preparations were 240 or 295 mL following the CM-52 column. The final concentrated volumes were 67 or 53 mL, respectively. The concentrating steps did not result in aggregation of the protein as described by Kamp et al. (1973). The column dimensions when starting with 6 lb of beef liver were 4 × 45 cm (diethylaminoethylcellulose-52, Whatman), 2.8 × 44 cm (carboxymethylcellulose-52, Whatman), and 3 × 95 cm (Sephadex G-50). The volume applied to the Sephadex G-50 column represented no more than 2 to 3.5% of the column volume. The exchange protein was stored at −20 °C as the appropriate fractions from the Sephadex G-50 column diluted 1/1 with cold glycerol. Exchange protein was assayed after exhaustive dialysis against 10 mM Tris–0.1 M KCl, pH 7.4, and in the presence of 0.1–0.2 mg/mL bovine serum albumin (Rothman and Dawidowicz, 1975). For routine assay of the exchange protein, a slight modification of the procedure described by Rothman and Dawidowicz (1975) was utilized. Erythrocyte ghosts (150 μL) (350 μg of protein and 60 nmol of phosphatidylcholine) were placed in a centrifuge tube. To this tube was added 250 to 650 μL of 10 mM Tris–0.1 M KCl, pH 7.4, 50 μL of 100 μg of bovine serum albumin (Sigma), 100 μL of egg phosphatidylcholine vesicles (40 nmol of phosphorus) containing either phosphatidylcholine (600 000 dpm/μmol) with the nonexchangeable marker [^{14}C]cholesterol oleate (50–60 μCi/μmol) (New England Nuclear) or trioleoyl[^{14}C]glycerol (30–60 μCi/μmol) (Amersham/Searle) representing less than 0.5 mol % of the vesicle composition, and 50 to 400 μL of exchange protein or column fraction. Column fractions were also assayed after dialysis against 10 mM

[1] Abbreviations used are: PC, phosphatidylcholine; —$N(CH_3)_3$ phosphatidylcholine, phosphatidyl[*methyl*-H]choline; —$N(CD_3)_3$ phosphatidylcholine, phosphatidyl[*methyl*-^{2}H]choline; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Tris–0.1 M KCl, pH 7.4. The assay suspensions (1 mL total volume) were vortexed and capped under an argon atmosphere and assayed at 37 °C. The reaction was stopped by centrifugation at 15 000*g* for 15 min in a Type 50 Beckman rotor. The ghost pellet was washed one or two times with buffer and finally measured for radioactivity in a Beckman LS-230 scintillation counter. The transfer of radioactivity to ghost was calculated by subtracting the "dpm of phosphatidylcholine transferred" in the absence of exchange protein from the "dpm of phosphatidylcholine transferred" in the presence of exchange protein using the following equation: dpm of phosphatidylcholine transferred = dpm of phosphatidylcholine in ghosts − (dpm of nonexchangeable marker in ghosts × (dpm of phosphatidylcholine/dpm of nonexchangeable marker) in original vesicles). One unit of exchange protein is defined as the initial rate of exchange of 1 nmol of phosphatidylcholine/min at 37 °C.

The purified exchange protein was labeled with ^{125}I using chloramine-T (Moore et al., 1974). It was shown to be approximately 90% homogeneous and to have an apparent molecular weight between 20 000 and 25 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Moore et al., 1976). We are grateful to Dr. Norman Moore, Unit of Invertebrate Virology, Oxford University, Oxford, England, for performing the ^{125}I labeling and electrophoresis. It is also significant that the dialyzed exchange protein did not measurably perturb the bilayer of phosphatidylcholine vesicles containing carboxyfluorescein (Weinstein et al., 1977). No dye was released over a 6-h incubation at 37 °C (Shaw, unpublished results).

Preparation of Erythrocyte Ghosts. Erythrocyte ghosts were prepared from outdated human blood by the method of Hanahan and Ekholm (1974). The ghosts were depleted of approximately 40 to 60% of their cholesterol by incubation with an excess of egg phosphatidylcholine vesicles using 25 μmol of vesicle phosphorus/μmol of ghost cholesterol (Shaw and Thompson, manuscript in preparation). The resulting cholesterol-depleted ghosts were washed with a large excess of 10 mM Tris–0.1 M KCl–1 mM EDTA–0.02% NaN_3, pH 7.5, and for most experiments pelleted through a 20% buffered sucrose solution and again washed with buffer.

Preparation of Vesicles. Single-lamellar vesicles were prepared by the procedure of Huang and Thompson (1974) in 10 mM Tris–0.1 M KCl, pH 7.4–7.6, at 4 °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. Cholesterol-depleted ghost suspensions containing 100–300 mg of protein and 45 to 100 μmol of total phosphatidylcholine in 35 to 41 mL of 10 mM Tris–0.1 M KCl–1 mM EDTA–0.02% NaN_3, pH 7.5, were incubated with 0.8 to 3.4 mL of 10 to 20 μmol of $—N(CD_3)_3$ phosphatidylcholine vesicles (in 10 mM Tris–0.1 M KCl, pH 7.5) in the presence of 85 to 115 units of dialyzed exchange protein (21 to 29 mL). The depleted ghosts used in these experiments contained high levels of phosphatidylcholine, as a result of "fusion" of egg phosphatidylcholine vesicles to the ghosts during the cholesterol depletion (Shaw and Thompson, manuscript in preparation). All buffers were saturated with argon and the 10 mM Tris–0.1 M KCl–1 mM EDTA–0.02% NaN_3, pH 7.5, accounted for 65 to 75% of the total buffer volume. Incubations were carried out under argon in sealed containers with stirring at 37 °C for 12 h. At this point an additional 60 to 90 units of dialyzed exchange protein (17 to 28 mL) was added and the reaction continued for another 12 h. An identical assay was carried out in the absence of exchange protein. Total volumes in three separate experiments varied from 81 to 97 mL. After 24 h the assay suspensionswere *either* centrifuged twice for 20 min at 26 000*g* for removal of all erythrocyte ghosts (preparation I) *or* overlayed on top of 20% sucrose–10 mM Tris–0.1 M KCl–1 mM EDTA–0.02% NaN_3 solutions and centrifuged in a SW-27 rotor at 25 000 rpm for 90 min (preparation II). The supernatant from preparation I and the layer above the sucrose pad in preparation II were concentrated using an Amicon diaflo apparatus with XM-50 filter. At this point some of the samples were either (1) further centrifuged for 30 min at 126 000*g* for removal of any vesicle aggregates, (2) passed through a Sepharose 6B column and again concentrated by Amicon filtration, or (3) maintained without centrifugation or column treatment. Next the vesicle solutions were exhaustively dialyzed against 50 mM KCl in 99.8% D_2O (Bio-Rad). After dialysis, vesicle samples were placed in sealed containers fitted with a Teflon slide valve through which dry argon was flushed for about 5 min each day. Samples were incubated in the dark at 23 °C in a desiccator containing D_2O and examined over a 9-day period by NMR spectroscopy.

NMR Spectroscopy. Measurements were performed at 23 °C using a JEOL PS-100P/EC-100 Fourier transform spectrometer at 100 MHz for proton and 40.5 MHz for ^{31}P. For proton experiments the dynamic range problem caused by the residual HDO solvent was eliminated using a $(180°-\tau-90°-T)$ pulse sequence (Pat and Sykes, 1972). Typical values for τ and T were 0.95 and 1.05 s, respectively. These values were chosen such that the *N*-methyl proton intensities were not significantly weakened. Typically, 600 to 2000 transients were required for adequate signal to noise ratios. The 90° pulse width was 30 μs. Peak areas representing the inner and outer monolayer signals were determined by planimetry or by weighing cutout tracings. Phosphorus NMR spectra were recorded as previously described (Yeagle et al., 1976).

Vesicle samples containing 1 to 7 mM phosphorus were examined before and after the addition of $PrCl_3$ (Alfa Ventron). The Pr^{+3}/phosphatidylcholine mole ratio varied from 0.05 to 0.35. High Pr^{+3}/phosphatidylcholine mole ratios (0.5–1.0) were necessary when certain samples contained approximately 0.5 mg of protein, identified mainly as albumin (incubation component, see Materials and Methods). In this case the Pr^{+3} was scavanged by the protein so that free Pr^{+3}/phosphatidylcholine mole ratios were probably much lower. Some turbidity was observed at the higher ratios. However, the ratio of outer to inner bilayer surface phosphatidylcholine did not vary as a function of Pr^{+3} concentration. The majority of the results for outside/inside ratios reported here were obtained at a Pr^{+3}/phosphatidylcholine mol ratio of between 0.15 and 0.25. For ^{31}P NMR, the Pr^{+3}/phosphatidylcholine mole ratio varied from 0.01 to 0.03.

Miscellaneous Methods. Phosphorus was determined by the method of Bartlett (1959) on aqueous lipid dispersions or after Bligh and Dyer extraction (Bligh and Dyer, 1959). Cholesterol was measured using cholesterol oxidase by a modification of the procedure of Allain et al. (1974). Protein was determined by the method of Lowry et al. (1951). Sepharose 6B chromatography was performed using 2 × 23 cm columns after preequilibration of the column with $—N(CD_3)_3$ phosphatidylcholine vesicles.

Results

Proton NMR of Asymmetric Vesicles. The transfer of phosphatidylcholine between erythrocyte ghosts and single-lamellar vesicles by the exchange protein used in these studies has been examined by Rothman and Dawidowicz (1975) and

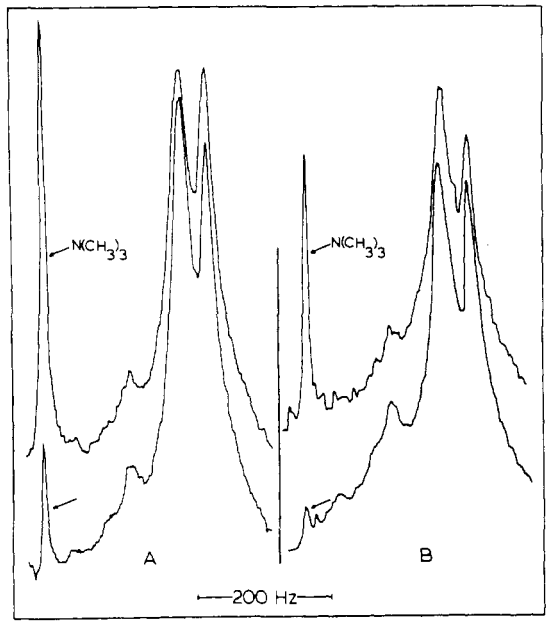

FIGURE 1: Proton NMR spectra of $-N(CD_3)_3$ phosphatidylcholine vesicles in 50 mM KCl in D_2O after incubation at 37 °C with partially cholesterol-depleted erythrocyte ghosts in the presence (upper spectra) or absence (lower spectra) of a phosphatidylcholine exchange protein. The vesicles of spectra A were 5–7 mM in phospholipid and contained 14–16 mol % cholesterol and 0.3 to 0.5 mg of protein/μmol of total lipid. Vesicles given in spectra B contained 2 mM phospholipid, 5–7 mol % cholesterol, and 0.2 mg of protein/μmol of total lipid.

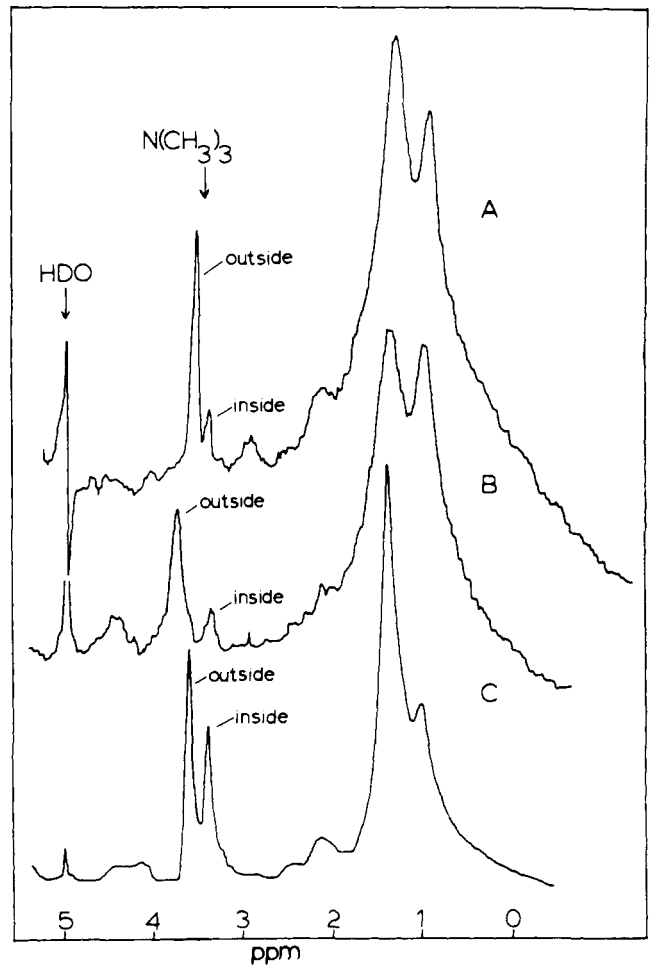

FIGURE 2: The effect of Pr^{+3} on the $-N(CH_3)_3$ phosphatidylcholine transferred to $-N(CD_3)_3$ phosphatidylcholine vesicles after incubation with a phosphatidycholine exchange protein and partially, cholesterol-depleted erythrocyte ghosts A and B. The outside monolayer *N*-methyl protons (shifted by Pr^{+3}) and inside monolayer *N*-methyl protons signals are labeled. The sample shown in spectrum A contains 2.3 mM phospholipid, 7 mol % cholesterol, 0.2 mg of protein/μmol of total lipid and has a Pr^{+3}/PC mol ratio of 0.19. The vesicles in spectrum B contain 4.6 mM phospholipid, 16 mol % cholesterol, 0.5 mg of protein/μmol of total lipid, and have a Pr^{+3}/PC mol ratio of 0.80. Spectrum C is obtained from egg $-N(CH_3)_3$ phosphatidylcholine vesicles examined at a Pr^{+3}/PC mol ratio of 0.12.

by Bloj and Zilversmit (1976) under several sets of conditions. However, in order to obtain maximum precision in the determination of the kinetics of transbilayer lipid migration by the present technique, it was necessary to establish an initial transmembrane lipid asymmetry which was as large as possible. The conditions utilized to accomplish this, which are outlined under Materials and Methods, were established in pilot experiments using [^{3}H]phosphatidylcholine. Based on these experiments, it can be estimated that between 50 and 85% of the $-N(CD_3)_3$ phosphatidylcholine in the outer surface of the vesicle was replaced by $-N(CH_3)_3$ phosphatidylcholine at the end of the incubation with the exchange protein. A similar degree of replacement (56–82%) was calculated by comparing the intensity of the $N(CH_3)_3$ proton NMR signal relative to the combined intensities of the methyl–methylene proton signals in the asymmetric vesicles to the same ratio obtained for vesicles comprised only of $-N(CH_3)_3$ phosphatidylcholine.

Figure 1 shows the proton NMR spectra from two experiments in which $-N(CD_3)_3$ phosphatidylcholine vesicles were incubated with cholesterol-depleted ghosts in the presence and absence of exchange protein. These spectra are high-resolution signals typical of small vesicles. The slightly broadened methyl–methylene signals and the somewhat reduced intensity of the methylene signal are due to the cholesterol in the vesicles (Darke et al., 1972). The data in Figure 1A were obtained in an initial series of experiments with vesicles containing 14–16 mol % cholesterol derived by spontaneous transfer from cholesterol-depleted ghosts during incubation with the phospholipid exchange protein. In subsequent experiments (Figure 1B) the cholesterol content in the isolated vesicles reached a level of only 6 mol %. This was accomplished by using depleted ghosts containing less than 50% of their normal cholesterol content.

The *N*-methyl proton signal in the presence of exchange protein amounts to about 84% (Figure 1A) and 91% (Figure 1B) of the signal found in the absence of exchange protein as estimated on the basis of signal intensity normalized to the combined methyl and methylene intensities. The $N(CH_3)_3$ signal (lower spectra, Figure 1) observed after incubation without exchange protein may be due to spontaneous movement of $-N(CH_3)_3$ phosphatidylcholine from ghost to vesicles. Spontaneous migration of phospholipids has in fact been observed between vesicles and retinal rod outer segment disk and vesicular stomatitis virus preparations (Shaw, Smith, and Litman, unpublished results; Shaw, Moore, and Patzer, unpublished results). It seems unlikely, however, that spontaneous phosphatidylcholine exchange is the explanation, since the $N(CH_3)_3$ signal observed in the absence of exchange protein is eliminated by broadening, but not shifted by Pr^{+3}. If the signal were due to $N(CH_3)_3$ protons on the external bilayer surface, the interaction with Pr^{+3} would be expected to shift the signal downfield without broadening. In addition, control experiments have been performed by incubating $-N(CH_3)_3$ phosphatidylcholine vesicles *separately* with 10 mM Tris–0.1 M KCl–0.02% sodium azide–1 mM EDTA, pH 7.5, buffer, purified exchange protein or bovine serum albumin. After

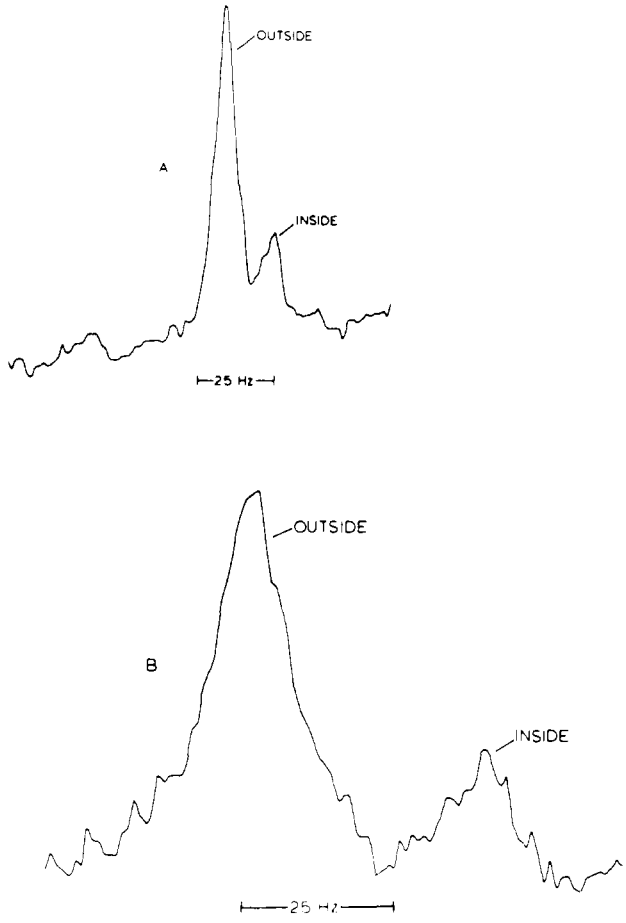

FIGURE 3: The *N*-methyl region of $-N(CH_3)_3$ phosphatidylcholine in $-N(CD_3)_3$ phosphatidylcholine vesicles from Figure 2. The spectra shown in A and B are the outside-inside $N(CH_3)_3$ regions from Figure 2A,B, respectively.

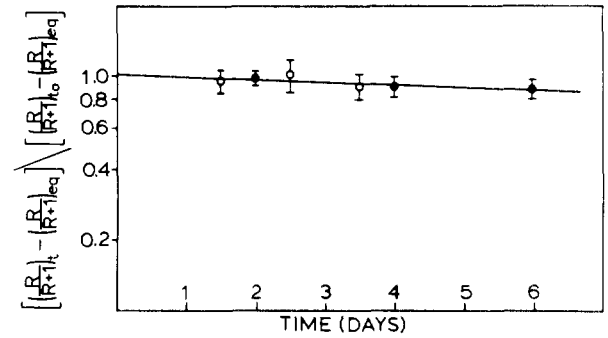

FIGURE 4: Decay of transbilayer asymmetry of $-N(CH_3)_3$ phosphatidylcholine in $-N(CD_3)_3$ phosphatidylcholine vesicles generated by a phosphatidylcholine exchange protein. Cholesterol-depleted erythrocyte ghosts, $-N(CD_3)_3$ phosphatidylcholine vesicles, and a phosphatidylcholine exchange protein were incubated at 37 °C for 24 h. The vesicles were isolated from the supernatant after *low-speed centrifugation* (26 000*g*) of the reaction mixture or after being overlayed on top of a 20% sucrose solution followed by centrifugation at 25 000 rpm (SW-27 rotor). Vesicles were examined after Amicon concentration, or further processed by *high-speed centrifugation* (126 000*g*) or *Sepharose column chromatography* (see Methods). The various vesicle samples were dialyzed against seven changes of 50 mM KCl in D_2O then incubated at 23 °C under argon. Aliquots were taken over a 5-day period and examined by NMR in the presence or absence of Pr^{+3}. The percentage of inner and outer monolayer $-N(CH_3)_3$ phosphatidylcholine after Pr^{+3} addition was measured and expressed as the outside/inside ratio. The expression on the ordinate of the figure can be found in the text. The slope was fitted using linear regression by the method of least squares. The open (○) and filled (●) circles represent data from separate experiments.

isolation of vesicles as described under Materials and Methods, no abnormal broadening effect or change in the outside/inside ratio of the $N(CH_3)_3$ proton signal after Pr^{+3} was observed. It seems most likely that the signal observed in the absence of exchange protein is due to the presence of material originating from the ghost membrane.

Determination of Compositional Asymmetry. When Pr^{+3}, a lanthanide to which the vesicle bilayer is impermeable, is added to the vesicle dispersion, $N(CH_3)_3$ protons on the outer surface of the bilayer are shifted downfield. The $N(CH_3)_3$ protons on the inner surface, which cannot interact with the Pr^{+3}, are unshifted. This is illustrated by the spectra of vesicles shown in Figure 2A,B which were obtained following incubation with exchange protein. The ratio of outer to inner monolayer $-N(CH_3)_3$ phosphatidylcholine is 4.6 in experiment 1, shown in Figure 2B, and 8.1 in experiment 2, shown in Figure 2A. It is clear that highly asymmetric bilayers can be generated with the exchange protein under the conditions employed in this study, since a ratio of 2.1 would be expected if the labeled phosphatidylcholine were equally distributed between both bilayer surfaces (Hutton et al., 1977; Israelachvili et al., 1976).

It is interesting to note, however, that the asymmetry is apparently not complete. In both experiments a small but finite $N(CH_3)_3$ signal remains unshifted in the presence of Pr^{+3}. This signal was present at the first time point for NMR examination and most likely occurred during the 24 h incubation period. We do not consider the unshifted signal to be the result of spontaneous transbilayer movement. This unshifted signal may have one of several origins. (1) It could in principle be the signal observed in the absence of exchange protein as seen in the lower spectra of Figure 1A,B. This seems highly unlikely, since this signal is completely broadened by low concentrations of Pr^{+3} as pointed out above. (2) A more likely possibility is that, during incubation of the vesicles with the exchange protein, some internalization of $-N(CH_3)_3$ phosphatidylcholine actually occurred. A number of obvious mechanisms for internalization suggest themselves, including perturbation of the bilayer by the exchange protein and limited vesicle fusion. (3) A possible but less likely explanation is that the unshiftable signal arises from the outer vesicle surface from $N(CH_3)_3$ protons which are for some reason masked from interactions with Pr^{+3}. Experiments are currently in progress to establish the origin of this small unshiftable signal.

Kinetics of Transbilayer Phospholipid Migration. Enlargements of the spectra shown in Figure 2 A,B are presented in Figure 3. Using enlarged spectra of this type, changes in the inner and outer surface $N(CH_3)_3$ proton spectra resulting from transbilayer migration have been determined by peak area measurements over a 5-day period at 23 °C. If it is assumed that the kinetics of transbilayer movement of phosphatidylcholine is reversible first order (Kornberg and McConnell, 1971), then the following equation obtains:

$$\frac{\left(\frac{R}{R+1}\right)_t - \left(\frac{R}{R+1}\right)_{eq}}{\left(\frac{R}{R+1}\right)_{t_0} - \left(\frac{R}{R+1}\right)_{eq}} = e^{-(k_0+k_i)t} \qquad (1)$$

Here R denotes the ratio of the number of molecules of $-N(CH_3)_3$ phosphatidylcholine in the outer surface to that in the inner surface of the vesicle bilayer. The subscripts t and eq denote, respectively, time and equilibrium. The t_0 value represents a time point immediately after the 24-h incubation. The outside/inside ratio at t_0 was estimated from a log plot of

TABLE I: Vesicle Suspensions Examined during the NMR Experiment.[a]

	Vesicles after low-speed centrifugation (15 000g)	Vesicles after high-speed centrifugation (127 000g)	Vesicles from Sepharose 6B
Phosphatidylcholine	2.32 μmol/mL, 90%	1.83 μmol/mL, 92%	1.2 μmol/mL, 92%
Cholesterol	0.19 μmol/mL, 7.3%	0.10 μmol/mL, 5.1%	0.07 μmol/mL, 5.1%
Sphingomyelin plus phosphatidylethanolamine	0.07 μmol/mL, 2.7%	0.05 μmol/mL, 2.7%	0.04 μmol/mL, 2.8%
Protein	0.5 mg/mL	0.4 mg/mL	0.2 mg/mL

[a] See Figure 4 for incubation conditions and isolation of vesicles.

the outside/inside ratios of the vesicles vs. time. The equilibrium value of R was taken to be 2.0. k_0 and k_i are the rate constants for transbilayer equilibration from inner to outer surface and the converse. Thus

$$PC_0 \underset{k_0}{\overset{k_i}{\rightleftharpoons}} PC_i$$

where PC_0 and PC_i are the number of molecules of $-N(CH_3)_3$ phosphatidylcholine in the outer and inner bilayer surfaces.

The half-time for transbilayer migration given by $t_{1/2} = 0.693/(k_0 + k_i)$ can be obtained from the slope of a plot of:

$$\log\left[\frac{\left(\frac{R}{R+1}\right)_t - \left(\frac{R}{R+1}\right)_{eq}}{\left(\frac{R}{R+1}\right)_{t_0} - \left(\frac{R}{R+1}\right)_{eq}}\right]$$

vs. time. The derivation of this equation is analogous to the treatment presented by Kornberg and McConnell (1971). The data plotted in this manner in Figure 4 give a half-time of 26 days. The possible half-times consistent with ±1 standard deviation in the slope of this line are 16 and 69 days. The primary sources of error involved in calculating the half-time are the signal to noise ratio and the inconsistency in establishing baselines. These difficulties are apparent in Figure 3.

Vesicle Composition and Integrity. Although before incubation with exchange protein and erythrocyte ghosts the vesicles were composed of pure $-N(CD_3)_3$ phosphatidylcholine, after incubation the presence of other lipids was detected. The compositions of several vesicle suspensions after incubation are given in Table I. It is apparent that the principal contaminant is cholesterol contributed by the cholesterol-depleted ghosts by spontaneous exchange. Very small amounts of sphingomyelin (< 2%) plus phosphatidylethanolamine (< 2%) were also detectable. This level of sphingomyelin was not sufficient to cause a measurable error in the estimation of the amount of $-N(CH_3)_3$ phosphatidylcholine in the system. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the low levels of protein present in the vesicle suspensions were due almost entirely to albumin added during the incubation with exchange protein. It is also important to note that extraction of the vesicle suspension followed by thin-layer chromatography on silicic acid on the 8th day of the experiments revealed no trace of lysophosphatidylcholine. This material was, however, detectable in the vesicles after 8 days.

Since it is obvious that the decrease with time of the ratio of PC_0/PC_i could be an artifact due to loss of vesicle integrity, it was important to assess this parameter throughout the time course of NMR examination. This was done in several ways. The impermeability of the vesicle population to Pr^{+3} was assessed after a 5-day period by determining the ratio of intensities of ^{31}P resonances in the outer and inner bilayer faces in

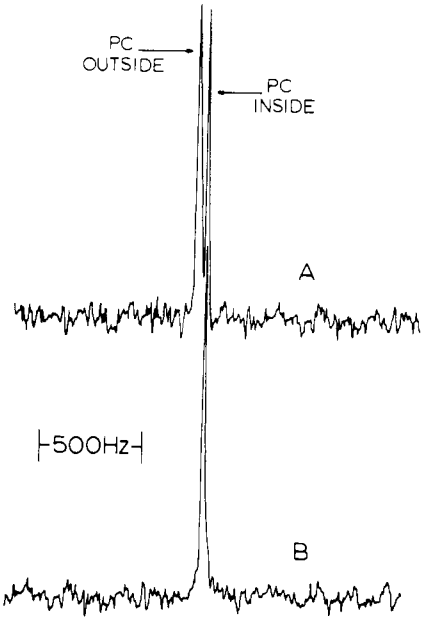

FIGURE 5: ^{31}P NMR of $-N(CD_3)_3$ phosphatidylcholine vesicles in the presence (A) or absence (B) of Pr^{+3}. Single-lamellar vesicles, representing 24 μmol of $-N(CD_3)_3$ phosphatidylcholine (1.5 mL), were incubated with 1 mL of 3.5 mg of protein of erythrocyte ghosts (0.6 μmol of PC and not depleted of cholesterol), 3 mL of 10 mM Tris–0.1 M KCl–1 mM EDTA–0.02% NaN_3, pH 7.5, 100 μL of 200 μg of bovine serum albumin, and 1 mL of dialyzed phosphatidylcholine exchange protein, representing 12 units. The total volume was 7.2 mL. After 21 h at 37 °C, the incubation was centrifuged at 17 000g and the supernatant recentrifuged. After Amicon concentration, the vesicles were dialyzed seven times against 50 mM KCl in D_2O and then incubated at 23 °C under argon and examined by ^{31}P NMR after a 5-day period. The *outside* monolayer phosphatidylcholine (shifted by Pr^{+3}) and *inside* monolayer phosphatidylcholine signals are labeled. The final percentage of cholesterol in the phosphatidylcholine vesicle suspension was 1.6 mol %.

control vesicle dispersions. Figure 5 shows the ^{31}P NMR spectra of control vesicles with and without added Pr^{+3}. The ratio of the signals arising from ^{31}P in the outer to inner bilayer surface calculated from this data is 1.9. In addition, this ratio remained unchanged over a period of 6 h in the presence of Pr^{+3}. Thus, not only did the vesicles in the absence of Pr^{+3} remain impermeable to this ion over a 5-day period, but they remained impermeable in the presence of the ion over a period of 1 or 2 h which is the duration of most proton NMR measurements. Furthermore, the ^{31}P NMR spectrum after 5 days was consistent with a small vesicle population with 65% of their phosphate groups exposed to Pr^{+3} on the outer surface. The vesicle suspensions upon assay revealed a cholesterol level of 1.6 mol %. Similar experiments on control vesicles comprised of $-N(CH_3)_3$ phosphatidylcholine completely substantiated these results. In addition, no significant changes in absorbance ratios measured in the region 300 to 400 nm were detected over

TABLE II: Half-Times for Transbilayer Migration of Phospholipids and Phospholipid Analogues in Single-Bilayer Vesicles.

	System	Method	$t_{1/2}$	Molecule studied[f]	Temp (°C)	Duration of the expt
(1)	Egg phosphatidylcholine vesicles	Exchange protein plus shift reagent	26 days[a]	PC	23	5 days
(2)	Dioleoylphosphatidylcholine vesicles	Exchange protein	>11 days[b]	PC	37	5 days
	Rat liver phosphatidylcholine vesicles		4–15 days[c]	PC	37	2 days
(3)	Egg phosphatidylcholine vesicles	Spin-label reduction	6.5 h[d]	S-PC	30	5 h
(4)	Egg phosphatidylcholine/phosphatidylethanolamine/amidinoylphosphatidylethanolamine	Chemical titration	>80 days[e]	PE	22	12 days

[a] This report. [b] Rothman and Dawidowicz (1975). [c] Johnson et al. (1975). [d] Kornberg and McConnell (1971). [e] Roseman et al. (1975). [f] Abbreviations: PC, phosphatidylcholine; S-PC, spin-labeled phosphatidylcholine; PE, phosphatidylethanolamine.

the 5-day period. Furthermore, phosphatidylcholine vesicles which were utilized to deplete cholesterol from erythrocyte ghosts were also tested for sidedness with Pr^{+3}, and routinely revealed outside to inside ratios of 1.8 to 2.0. The cholesterol concentration in these vesicles varied from 5 to 10 mol %. Therefore, several experiments which vary in the quantity of ghost, vesicle, or exchange protein utilized support the contention that the vesicles are not leaky to Pr^{+3}.

It should be noted that under the conditions of the experiment some evidence for a loss of vesicle integrity was detectable. This became quite apparent after NMR examination on day 8. For this reason, the time course of the transbilayer migration experiments was limited to 5 days.

Discussion

Table II compares the results obtained in this study (1) with the available literature values for the half-time of transbilayer lipid migration in single-bilayer vesicles. The results obtained by Johnson and co-workers (1975) and by Rothman and Dawidowicz (1975), listed as entry 2, were obtained with compositionally asymmetric vesicles formed by action of the exchange protein. In this respect, these experiments and ours are similar. However, although Johnson and co-workers were able to obtain evidence for transbilayer migration, the half-times reported by Rothman and Dawidowicz are lower limit values set by the precision of the data. The duration of the experiments given in column 6 shows that, although the Johnson experiments were of relatively short duration (2 days), the Rothman–Dawidowicz experiment is of the same duration as ours. It should be noted here that the limitation of the Johnson and co-workers and Rothman–Dawidowicz type of experiment is the assumption that the two kinetically defined pools of [^{14}C]phosphatidylcholine are in fact located on opposite faces of the vesicle bilayer. This limitation is overcome in our experimental design. With this design, sidedness of the vesicle system is established by Pr^{+3} shiftability, an operational definition which is independent of kinetic parameters.

Examination of all of the results in Table II suggests that the value of $t_{1/2} \simeq 6.5$ h determined by ascorbate reduction of spin-labeled phospholipid is probably too small. It seems clear that transbilayer lipid migration in simple bilayer vesicles is a slow process. The half-time for the exchange of chemically distinct species of phospholipids (Table II, entry 4), as determined by the study of Roseman and co-workers (1975), is in excess of 80 days. The self-exchange of identical lipid molecules across the bilayer may be a somewhat faster process, as suggested by the results of the study reported herein. It will be interesting to examine the dependence of these two processes on parameters such as temperature and bilayer composition.

It would also be of interest to examine how the net movement of cholesterol affects the asymmetry of phosphatidylcholine transferred into vesicles by the exchange protein. In our studies, for example, the unshifted signal of Figure 2A represents 11% of the $-N(CH_3)_3$ signal and the vesicles contain 5 to 7 mol % cholesterol. In contrast, the vesicles of Figure 2B show an unshifted signal representing 18% of the $-N(CH_3)_3$ signal and contain 14 to 16 mol % cholesterol. Unfortunately, the origin of the unshifted inner monolayer $-N(CH_3)_3$ signal after incubation for 24 h with exchange protein is not immediately clear and no definitive conclusions can be drawn. Presumably, the cholesterol exchanged into these vesicles is located in the outer monolayer and no significant increase in the size of the vesicles has occurred (see Results). Cholesterol reportedly does not undergo rapid transbilayer movement in vesicles (Poznansky and Lange, 1976), although recent work indicates that cholesterol transbilayer movement in erythrocyte membranes occurs on the order of minutes to hours (Lange et al., 1977).

Measurement of transbilayer movement of phosphatidylcholine or its analogues in biological membranes has revealed half-times which vary from minutes to days depending upon the technique utilized and/or the membrane examined. For example, the spin-label technique originally introduced by Kornberg and McConnell has recently been examined by Rousselet et al. (1976a,b). These workers found the values for transbilayer movement when using ascorbate reduction to be on the order of 4 and 7 h in mitochondrial inner membrane and human erythrocyte membrane, respectively, when the spin label was located on the acyl chain. When an endogenous component[2] served to reduce the polar head-group spin-labeled phosphatidylcholine with ensuing measurement of transbilayer movement, the half-time became undetectable for the erythrocyte membrane. In addition, introduction of acyl-chain spin-labeled phosphatidylcholine into inner mitochondrial membrane particles via the exchange protein revealed after ascorbate reduction half-times greater than 25 days. It would appear from these studies that the placement of the nitroxide radical and/or the method for reduction should be carefully considered, especially in view of the rather rapid half-times (minutes) suggested for transbilayer movement of spin-labeled phospholipids in certain biological membranes (Grant and McConnell, 1973; McNamee and McConnell, 1973). Nev-

[2] Glutathione, an endogenous component of mitochondria and erythrocytes, has been suggested as the reductant (Rousselet et al., 1976a).

ertheless, exchange experiments with the phosphatidylcholine exchange protein and erythrocyte ghosts (Bloj and Zilversmit, 1976) and phospholipase digestion of ^{32}P-labeled external phospholipid in rat erythrocytes (Renooij et al., 1976) each have revealed half-times for transbilayer movement between 2 and 6 h. The transbilayer movement of phosphatidylcholine in biological membranes may in fact be more rapid than in single-bilayer vesicles.

Acknowledgments

We thank Ms. Maria Freire for helpful assistance and Drs. Y. Barenholz and M. Roseman for several valuable discussions.

References

Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W., and Fu, P. C. (1974), *Clin. Chem. (Winston-Salem, N.C.) 20,* 470.

Aneja, R., and Chadha, J. S. (1971), *Biochim. Biophys. Acta 248,* 455.

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

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466.

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37,* 911.

Bloj, B., and Zilversmit, D. B. (1976), *Biochemistry 15,* 1277.

Chadha, J. S. (1970), *Chem. Phys. Lipids 4,* 104.

Cubero Robles, E., and Van den Berg, D. (1969), *Biochim. Biophys. Acta 187,* 520.

Darke, A., Finer, E. G., Glook, A/ G., and Phillips, M. C. (1972), *J. Mol. Biol. 63,* 265.

Dawson, R. M. C., and Hemington, N. (1967), *Biochem. J. 102,* 76.

Grant, C. W. M., and McConnell, H. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1238.

Hanahan, D. J., and Ekholm, J. E. (1974), *Methods Enzymol. 31A,* 168.

Huang, C., and Thompson, T. E. (1974), *Methods Enzymol. 32,* 485.

Hutton, W. C., Yeagle, P., and Martin, R. B. (1977), *Chem. Phys. Lipids* (in press).

Israelachvili, J. N., Mitchell, D. J., and Ninham, B. W. (1976), *J. Chem. Soc., Faraday Trans. 1 72,* 1525.

Johnson, L. W., Hughes, M. E., and Zilversmit, D. B. (1975), *Biochim. Biophys. Acta 375,* 176.

Kamp, H. H., Wirtz, K. W. A., and Van Deenen, L. L. M. (1973), *Biochim. Biophys. Acta 318,* 313.

Kornberg, R. D., and McConnell, H. M. (1971), *Biochemistry 10,* 111.

Lange, Y., Cohen, C. M., and Poznansky, M. J. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 1538.

Litman, B. J. (1973), *Biochemistry 12,* 2545.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

McNamee, M., and McConnell, H. M. (1973), *Biochemistry 12,* 295.

Moore, N. F., Barenholz, Y., and Wagner, R. (1976), *J. Virol. 19,* 126.

Moore, N. F., Delley, J. M., and Wagner, R. R. (1974), *Virology 61,* 292.

Pat, S. L., and Sykes, B. D. (1972), *J. Chem. Phys. 56,* 3182.

Poznansky, M., and Lange, Y. (1976), *Nature (London) 259,* 420.

Renooij, W., Van Golde, L. M. G., Zwaal, R. F. A., and Van Deenen, L. L. M. (1976), *Eur. J. Biochem. 61,* 53.

Roseman, M., Litman, B. J., and Thompson, T. E. (1975), *Biochemistry 14,* 4826.

Rothman, J. E., and Dawidowicz, E. A. (1975), *Biochemistry 14,* 2809.

Rousselet, A., Colbeau, A., Vignais, P. M., and Devaux, P. F. (1976a), *Biochim. Biophys. Acta 426,* 372.

Rousselet, A., Guthmann, C., Matricon, J., Bienvenue, A., and Devaux, P. F. (1976b), *Biochim. Biophys. Acta 426,* 357.

Sears, B., Hutton, W. C., and Thompson, T. E. (1976), *Biochemistry 15,* 1635.

Selinger, Z., and Lapidot, Y. (1966), *J. Lipid Res. 7,* 174.

Shaw, J. M., Hutton, W. C., Lentz, B. R., and Thompson, T. E. (1977), *Biophys. J. 17,* 83a.

Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), *J. Am. Oil Chem. Soc. 42,* 53.

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

Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., and Thompson, T. E. (1976), *Biochemistry 15,* 1393.

Thompson, T. E. (1977), in Molecular Specialization and Symmetry in Membrane Function, Solomon, A. K., Giebisch, G., and Hoffman, J., Ed., Cambridge, Mass., Harvard University Press (in press).

Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., and Hagins, W. A. (1977), *Science 195,* 489.

Yeagle, P. L., Hutton, W. C., Martin, R. B., Sears, B., and Huang, C. (1976), *J. Biol. Chem. 251,* 2110.