Inside–Outside Transitions of Phospholipids in Vesicle Membranes*

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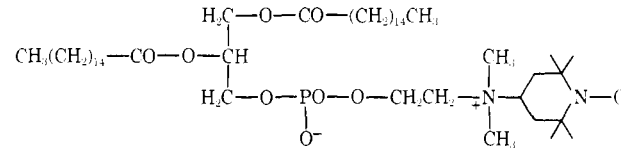
ABSTRACT: Spin-labeled phosphatidylcholine is a paramagnetic analog of phosphatidylcholine. The vesicle which results from prolonged sonication of egg phosphatidylcholine and spin-labeled phosphatidylcholine in salt solution has an aqueous compartment and a bilayer membrane. Sodium ascorbate at 0° abolishes the paramagnetism of spin-labeled molecules in the external monolayer of the vesicle membrane (65% of the total paramagnetism of a vesicle) without affecting the paramagnetism of internal molecules. The consequent asymmetry in the distribution of paramagnetic molecules between the two monolayers of the vesicle membrane decays with a half-time of 6.5 hr at 30°. It follows that the probability of a spin-labeled phosphatidylcholine molecule passing from the internal monolayer of the vesicle membrane to the external monolayer is 0.07/hr at 30°, and the probability of a spin-labeled phosphatidylcholine molecule passing from the external monolayer of the vesicle membrane to the internal monolayer is 0.04/hr at 30°.

Transport of ions and molecules through a membrane must require motions of the membrane constituents. The motions may be parallel to the membrane surface (longitudinal) and perpendicular to it (transverse).

Langmuir (1938) investigated transverse motions of lipids. He inferred from the properties of barium stearate multilayers that "thermal agitation causes individual molecules to overturn from time to time." A closely related thermal process in stearate bilayers was studied by Deamer and Branton (1967). They demonstrated by a freeze-fracture technique that ^{14}C-labeled stearate exchanges between the two stearate monolayers with a half-time of 25 min at 25°, and suggested than an exchange process involving the lipids in fluid regions of biological membranes should occur more rapidly. Pagano and Thompson (1968) cited the exchange process in stearate bilayers in support of their view that a phospholipid–chloride ion complex mediates the permeation of chloride ion through phospholipid bilayers. They proposed a carrier-mediated diffusion mechanism, adapted from Hanai *et al.* (1965), to account for the saturation kinetics of the chloride ion flux and the excess of isotopic over electrical chloride ion flux. But an exchange of phospholipids between the two sides of a membrane as a consequence of carrier-mediated transport of ions or molecules has not been demonstrated.

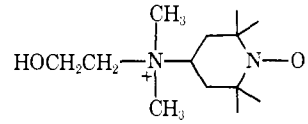
Langmuir anticipated from "an almost instantaneous reversal of orientation" of barium stearate monolayers under some conditions, the possibility of overturning of whole lipid layers in a biological membrane. This concerted motion of lipid molecules would result from a "change of potential or of chemical composition of the liquid on one side" of the membrane. Papahadjopoulos and Ohki (1969) have speculated that the "inversion" of phosphatidylserine molecules or clusters of phosphatidylserine molecules from one side of a bilayer to the other is responsible for the rupture of phosphatidylserine bilayers in gradients of calcium ion concentration. The transverse motion of phospholipids which Papahadjopoulos and Ohki called inversion we call phospholipid flip-flop.

We report here a direct method of measuring the flip-flop rate of any phospholipid whose polar part includes the paramagnetic nitroxide group. We report, in particular, the rate of exchange of spin-labeled phosphatidylcholine (molecule I) between two monolayers which constitute the bilayer membrane of a phosphatidylcholine vesicle.



Materials and Methods

Tempocholine (Molecule II) Chloride. *N,N*-Dimethyl-*N*-



(2′,2′,6′,6′-tetramethyl-4′-piperidyl)-2-acetoxyethylammonium bromide was prepared by the reaction of 4-(*N,N*-dimethylamino)-2,2,6,6-tetramethylpiperidine with 2-bromoethyl acetate and then hydrolyzed, oxidized, and ion exchanged to tempocholine bicarbonate. An aqueous solution of tempocholine bicarbonate was acidified with HCl to yield tempocholine chloride. This procedure was partly due to Dr. Wayne L. Hubbell.

(a) 4-(*N,N*-Dimethylamino)-2,2,6,6-tetramethylpiperidine was prepared by the method of Icke and Wisegarver (1955). 4-Amino-2,2,6,6-tetramethylpiperidine (Aldrich Chemical Co.) (25 g) was added with magnetic stirring to 38 g of 98% formic acid in an ice bath. The mixture was removed from the ice bath, combined with 28.5 g of 37% formaldehyde, refluxed for about 10 hr, cooled in an ice bath, and combined with 18.2 g of concentrated HCl. Formic acid, formal-

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dehyde, and water were removed under reduced pressure. The residue was combined with about 100 ml of water and about 50 g of solid NaOH. The lower phase was extracted twice with diethyl ether. The upper phase and ether extracts were dried over barium oxide. The ether was removed by distillation at atmospheric pressure. Vacuum distillation (at 29–36° and less than or about 1 mm) yielded 18 g (67%) of clear, colorless liquid. *Anal.* Calcd for $C_{11}H_{24}N_2$: C, 71.67; H, 13.13; N, 15.20. Found: C, 71.55; H, 12.99; N, 14.93.

(b) 2-Bromoethyl acetate was prepared by the reaction of 2-bromoethanol with acetic anhydride. Excess acetic anhydride (about 100 g) was added with magnetic stirring to 100 g of 2-bromoethanol (Aldrich Chemical Co.) in an ice bath. The mixture was heated on a steam bath for 1 hr, cooled in an ice bath, and repeatedly extracted with cold 1% $NaHCO_3$ until the pH of the upper phase was about 7. The lower phase was then dried over anhydrous sodium sulfate. Vacuum distillation yielded a clear, colorless liquid: infrared (film), band at 1740 cm^{-1} (carboxylic ester).

(c) A mixture of 8.52 g of 4-(*N*,*N*-dimethylamino)-2,2,6,6-tetramethylpiperidine and 15.5 g of 2-bromoethyl acetate was kept in the dark at room temperature for 3 days. The white solid product was dispersed in anhydrous diethyl ether, filtered and washed with anhydrous diethyl ether by suction, and dried to 11.6 g (71%) under vaccuum over P_2O_5. A mixture of 6.15 g of the dry solid, 1.70 g of EDTA, 0.93 g of NaOH, 2 g of $Na_2WO_4 \cdot 2H_2O$, 25 ml of 30% hydrogen peroxide, and 100 ml of water was kept at room temperature. After 1 day, solid NaOH was added to bring the pH to about 10 and then another 25 ml of 30% hydrogen peroxide was added. After 2 days the remaining hydrogen peroxide was destroyed by vigorous magnetic stirring. The water was removed under reduced pressure. Benzene was added and then removed under reduced pressure. The residual solids were dispersed in about 500 ml of absolute ethanol and filtered. The filtrate was evaporated under reduced pressure and redissolved in 20 ml of water Part of this solution (15 ml) was applied to a column containing 60 ml of AG 50W-X8 (Bio-Rad Laboratories, NH_4^+ form), and eluted with 250 ml of 0.25 M NH_4HCO_3 and 400 ml of 1 M NH_4HCO_3. The first 300 ml of 1 M NH_4HCO_3 eluate was evaporated under reduced pressure and the residual solids were dispersed in 100 ml of absolute ethanol and filtered. The filtrate was evaporated under reduced pressure and the residual solids were twice dispersed in 100 ml of absolute ethanol and evaporated under reduced pressure. The pH of a solution of the residual solids in 50 ml of water was adjusted to 2.9 by the addition of 6 ml of 1.2 M HCl. The water was removed under reduced pressure. The orange solid was dried to 2.1 g under vacuum over P_2O_5. It appeared as a single spot on a cellulose thin-layer chromatogram which was developed in propanol–NH_4OH (14.8 M)–water (6:3:1, v/v) and stained with iodine vapor. *Anal.* Calcd for $C_{13}H_{28}ClN_2O_2$: C, 55.80; H, 10.09; N, 10.01; Cl, 12.67. Found: C, 54.76; H, 9.97; N, 9.95; Cl, 12.48.

Spin-Labeled Phosphatidylcholine (*Molecule I*). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoric acid was prepared by the action of phospholipase D on dipalmitoylphosphatidylcholine and condensed with tempocholine. The condensation was mediated by 2,4,6-triisopropylbenzenesulfonyl chloride (Lohrmann and Khorana, 1966) in a mixture of pyridine and chloroform which contained tempocholine as solid tempocholine chloride. Aneja and Davies (1970) have also employed 2,4,6-triisopropylbenzenesulfonyl chloride in the synthesis of a spin-labeled phospholipid.

(a) Dipalmitoylphosphatidylcholine (2 g), a gift of Drs. H. M. Verheij (derived from egg phosphatidylcholine by the methods of Brockerhoff and Yurkowski (1965) and Baer and Buchnea (1959)), was dispersed in 50 ml of water with a Bronwill 20-kHz sonicator and intermittent cooling in an ice bath. The sonication was continued until further sonication did not produce further clearing of the homogeneous opalescent dispersion. A mixture of 3.3 ml of 1 M pH 5.6 acetic acid–calcium acetate buffer, 100 ml of Savoy cabbage supernatant (freshly prepared by the method of Davidson and Long (1958)), 100 ml of alcohol-free diethyl ether (freshly distilled from P_2O_5), and 20 ml of water was shaken with the dipalmitoylphosphatidylcholine dispersion for 13 hr at room temperature, then shaken with 35 g of citric acid (monohydrate) and 170 ml of water, and centrifuged. The aqueous phase was extracted three times with 500-ml portions of chloroform. The combined ethereal phase and chloroform extracts were evaporated under reduced pressure. The residue was dissolved in absolute ethanol, and then the ethanol and last traces of water were removed under reduced pressure. The residue was dissolved in 15 ml of chloroform and added dropwise to a magnetically stirred solution of 20 g of barium acetate (monohydrate) and 400 ml of methanol–water (1:1, v/v). The precipitate was collected by centrifugation, shaken for about 10 min at room temperature with 250 ml of 0.5 N H_2SO_4 and 250 ml of chloroform, and centrifuged. The lower phase was extracted once more with 250 ml of 0.5 N H_2SO_4, once with 500 ml of methanol–water (1:1, v/v), combined with an extract of the upper phase from the first 0.5 N H_2SO_4 extraction (the upper phase from the first 0.5 N H_2SO_4 extraction was extracted with 500 ml of chloroform–methanol (1:1, v/v), and the resulting lower phase was extracted once with 250 ml of 0.5 N H_2SO_4 and once with 250 ml of water), and evaporated under reduced pressure. Traces of water were removed from the residue by repeated evaporation of absolute ethanol, and traces of ethanol were removed by repeated evaporation of toluene, yielding 1.83 g (103%) of white solid which was dissolved in 10 ml of anhydrous chloroform. An oxalic acid impregnated silica thin-layer chromatogram of this solution was developed in chloroform–methanol–HCl (12 M) 87:13:0.5, v/v). It showed one spot with the same R_F value as phosphatidic acid derived from egg phosphatidylcholine by the method of Davidson and Long (1958).

(b) One-half of the anhydrous chloroform solution which resulted from the procedure in part a was transferred to a two-necked flask, equipped for mechanical overhead stirring, which contained about 45 g of glass beads (roughly 2 mm in diameter), 2.5 ml of anhydrous pyridine, 0.55 g of tempocholine chloride, and 1.21 g of 2,4,6-triisopropylbenzenesulfonyl chloride. The mixture was vigorously stirred for 5 hr at room temperature. Water (10 ml) was added with cooling and then the mixture was stirred for 3 hr at room temperature, shaken with 250 ml of chloroform and 150 ml of 0.5 N H_2SO_4, and centrifuged. The lower phase was washed successively with 150 ml of 0.5 N H_2SO_4, 150 ml of water, 225 ml of methanol–$NaHCO_3$ (2%) (1:2, v/v), and 225 ml of methanol–water (1:2, v/v), evaporated under reduced pressure, dissolved in 50 ml of chloroform–methanol (1:1, v/v), applied to a column containing 600 ml of alumina (E. Merck, activity I, washed extensively with chloroform–methanol (1:1, v/v) for the removal of fines, activated for 3 hr at 110°, and packed in the column as a slurry in chloroform), and eluted with chloroform–methanol (1:1, v/v). The first 25 ml of orange eluate was discarded. The next 250 ml of orange

eluate was concentrated to a small volume under reduced pressure, diluted with absolute ethanol, and centrifuged for removal of alumina particles. The ethanol was removed under reduced pressure. The orange solids were dissolved in 20 ml of chloroform. This spin-labeled phosphatidylcholine solution contained 914 μmoles of phosphorus (78% of the total phosphorus in the solution which was applied to the alumina column). Silica thin-layer chromatograms of the solution were developed in chloroform–methanol–NH_4OH (14.8 M)–water (70:30:4:1, v/v), chloroform–methanol–water (65:25:4, v/v), and chloroform–methanol–acetic acid–water (50:25:7:3, v/v). In each case there was only one spot. There were, however, two spots (R_F values 0.42 and 0.47) on a silica-impregnated paper chromatogram developed in diisobutyl ketone–acetic acid–water (8:5:1, v/v) and sprayed with the phosphate reagent of Dittmer and Lester (1964). The spot corresponding to an R_F value of 0.42 was about 10% as intense as the other spot. A 12% contamination of the spin-labeled phosphatidylcholine solution by some nonparamagnetic phospholipid would explain why the ratio of nitroxide/phosphorus was lower than expected. *Anal.* Nitroxide/phosphorus, 0.88; palmitic acid/phosphorus, 2.00. The nonparamagnetic impurity was removed by silica column chromatography. Part of the $HCCl_3$ solution (4 ml) of spin-labeled phosphatidylcholine was applied to a bed of silica (Mallinckrodt 100 mesh silicic acid, sieved to 60–140 mesh, activated for 2 days at 110°) in chloroform, roughly 50 cm in height, and eluted successively with 300 ml of chloroform, 800 ml of 5% methanol in chloroform, 400 ml of 10%, 800 ml of 12.5%, 800 ml of 15%, and 2.5 l. of 17.5%. The second liter of 17.5% methanol in chloroform contained about 125 μmoles of phosphorus. The spot on the silica-impregnated paper chromatogram corresponding to an R_F value of 0.42 was about 1% as intense as the other spot. *Anal.* Nitroxide/phosphorus, 1.04; palmitic acid/phosphorus, 2.00. The quantitative hydrolysis of spin-labeled phosphatidylcholine by phospholipase D (2 μmoles of spin-labeled phosphatidylcholine, 0.5 ml of alcohol-free diethyl ether, 0.02 ml of 1 M pH 5.6 acetic acid–calcium acetate buffer, and 0.5 ml of cabbage supernatant were shaken for 15 hr at room temperature) yielded only phosphatidic acid (identified on an oxalic acid impregnated silica thin-layer chromatogram developed in chloroform–methanol–HCl (12 M) (87:13:0.5, v/v) by comparison to phosphatidic acid derived from egg phosphatidylcholine by the method of Davidson and Long (1958)) and tempocholine (identified on a cellulose thin-layer chromatogram developed in propanol–NH_4OH (14.8 M)–water (6:3:1, v/v) by comparison with tempocholine chloride). The R_F values of spin-labeled phosphatidylcholine on silica chromatograms in basic and acidic solvents were greater than the corresponding R_F values of phosphatidylcholine (R_F values of 0.67 and 0.40 for spin-labeled phosphatidylcholine and phosphatidylcholine on a silica thin-layer chromatogram developed in chloroform–methanol–NH_4OH (14.8 M)–water (70:30:4:1, v/v), and R_F values of 0.47 and 0.42 for spin-labeled phosphatidylcholine and phosphatidylcholine on a silica-impregnated paper chromatogram developed in diisobutyl ketone–acetic acid–water (8:5:1, v/v)); that is to say, spin-labeled phosphatidylcholine was less polar than phosphatidylcholine by the criterion of silica chromatography.

Phospholipid Dispersions. Egg phosphatidylcholine and mixtures of egg phosphatidylcholine and spin-labeled phosphatidylcholine were sonicated in buffered salt solutions with titanium probes and then centrifuged. This procedure was adapted from the methods of Attwood and Saunders (1965) and Huang (1969). Egg phosphatidylcholine for preparation A was isolated by the method of Singleton *et al.* (1965). It had the following fatty acid composition: palmitic (16:0), 28.5 mole %; palmitoleic (16:1), 0.5%; stearic (18:0), 11.5%; oleic (18:1), 25.2%; linoleic (18:2), 12.5%. Egg phosphatidylcholine for preparations B–G was a gift of Mr. N. Miller and Dr. A. D. Bangham. It had, according to Mr. Miller, the following fatty acid composition: palmitic (16:0), 26.5 mole %; palmitoleic (16:1), 1.77%; stearic (18:0), 13.3%; oleic (18:1), 32.3%; linoleic (18:2), 12.9%; arachidonic (20:4), 4.25%; (22:4), 3.03%; (22:6), 4.24%. In every preparation, the sum of egg phosphatidylcholine and spin-labeled phosphatidylcholine concentrations was 40 μmoles/ml. 5% of this sum was spin-labeled phosphatidylcholine in preparation A, and 3.3% was spin-labeled phosphatidylcholine in preparations C–G; preparation B did not contain spin-labeled phosphatidylcholine. The buffer was 0.01 M pH 8.0 Tris-Cl in preparation A, 0.05 M pH 8.0 Tris-Cl in preparations B–F, and 0.05 M pH 7.1 potassium phosphate in preparation G. Every preparation contained 0.1 M NaCl. The concentrations ($10^3 \times$ M) and radioactivity ($10^{-6} \times$ cpm/ml) of ^{14}C-labeled disaccharides (Calatomic) in the various preparations were as follows: A (lactose) 0.3 and 1.1; B (sucrose), 5.0 and 3.4; C (sucrose), 5.0 and 4.5; D-F, 0.0; G (sucrose), 5.0 and 0.6. Preparation B contained 10^{-3} M tempocholine chloride; none of the other preparations contained tempocholine chloride. In every case the buffered salt solution was flushed with argon through a gas dispersion tube before the phospholipids were introduced. Preparation A (10 ml) was sonicated for 120 min with the 0.25-in. tip of an MSE 100W ultrasonic disintegrator in a 10-ml Rosett cell cooled in an ice bath. Preparations B–G (5 ml) were sonicated for 90 min with the 0.5-in. tip of a Branson Model W185D sonifier cell disrupter at output control setting 6 under argon in a $^5/_8$-in. i.d. glass tube cooled in an ice bath. The sonicated preparations were centrifuged for 60 min at 80,000*g* in the cold, and stored under argon on ice.

Paramagnetic Resonance Spectra. Samples in 50-μl glass capillaries were mounted in the variable-temperature accessory of a Varian E-4 spectrometer. The spectra were integrated and normalized by means of a Fabri-Tek 1070 Series signal-averaging system which was donated for this work by Fabri-Tek Instruments, Inc.

The analysis of paramagnetic resonance line shapes (see text relating to Figures 2 and 3a,b) is incidental to the main purpose of this work, so the analysis has not been justified in detail. The reader is referred to more complete discussions of paramagnetic resonance line shapes in the reviews by Hamilton and McConnell (1968), Griffith and Waggoner (1969), and McConnell and McFarland (1970).

Analytical Methods. Total phosphate was determined by the method of Ames and Dubin (1960) unless otherwise indicated. Fatty acids were determined by the method of Scandella and Kornberg (1969). Nitroxide in spin-labeled phosphatidylcholine samples was determined by comparing the amplitudes of the low-field lines of the paramagnetic resonance spectra of absolute ethanol solutions of tempocholine chloride (at known concentration) and spin-labeled phosphatidylcholine. The widths of these lines were not perceptibly different. To the extent that an imperceptible difference in widths had a significant effect on the amplitudes, the results of these analyses for nitroxide were lower limits on the actual values. pH's of solutions containing Tris-Cl were measured at room temperature with a Beckman 39030

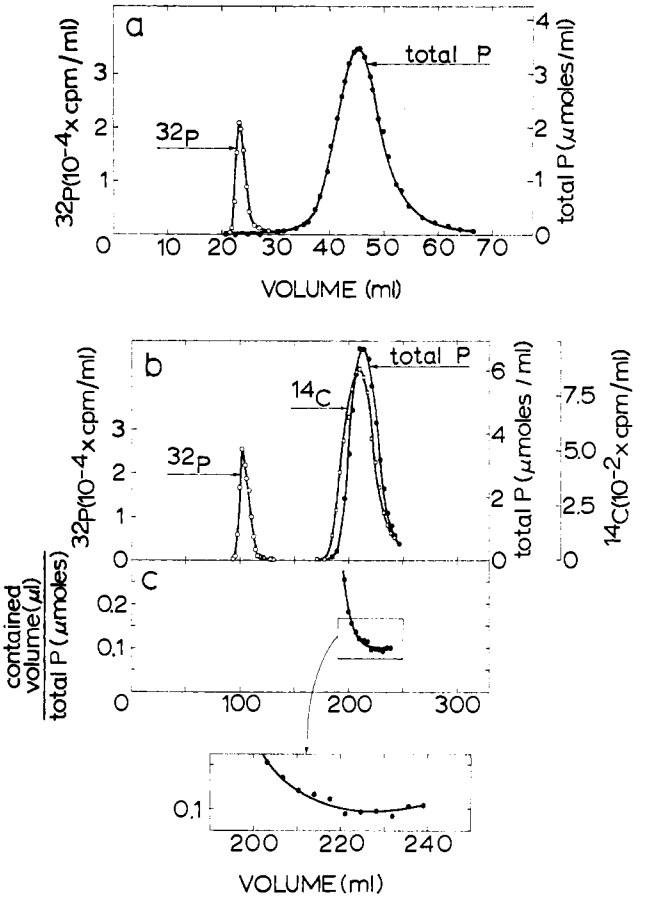

FIGURE 1: Elution profiles of spin-labeled phosphatidylcholine vesicles on Sepharose 4B at 3°. The procedure was adapted from the method of Huang (1969). (a) Preparation D (1 ml) was applied under hydrostatic pressure to the bottom of a column of Sepharose 4B (Pharmacia), 65 × 1.2 cm, in 0.05 M pH 8.0 Tris-Cl–0.1 M NaCl, and eluted through the top of the column with the same buffered salt solution (the column was previously treated by identical procedures with 1 ml each of preparations B and C). Total phosphate in the eluate was determined by a minor modification of the method of Gerlach and Deuticke (1963). The void volume of the column was determined in a separate experiment with ^{32}P-labeled bacteriophage λDNA (molecular weight 3×10^7), a gift of Dr. D. A. Jackson. (b and c) Preparation A (8 ml) was applied to the bottom of a Sepharose 4B column, 70 × 2.5 cm, in 0.01 M pH 8.0 Tris-Cl–0.1 M NaCl, and eluted through the top of the column with the same buffered salt solution. The void volume of the column was determined as described in part a.

Combination Electrode. Silica thin-layer chromatograms were sprayed with the phosphate reagent of Dittmer and Lester (1964) and charred.

Results

Spin-Labeled Phosphatidylcholine Particles Are Vesicles: Their Sizes and Shapes. We analyzed the particle size homogeneity of our phospholipid dispersions by Sepharose 4B chromatography. Every dispersion appeared as a symmetrical peak at 2 void volumes (Figure 1a). Our dispersions were identical in this respect with a fraction of phosphatidylcholine particles which Huang (1969) collected as the 2 void volume eluate of a Sepharose 4B column. Huang also found a fraction of larger phosphatidylcholine particles which emerged at the void volume of the Sepharose 4B column. Our dispersions contained none of these larger

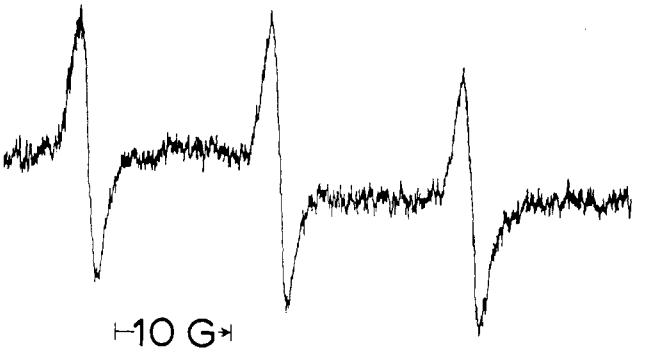

FIGURE 2: Paramagnetic resonance spectrum of tempocholine inside phosphatidylcholine vesicles at 0°. Preparation B (0.35 ml) was passed twice through a column of Sephadex, G-25 coarse (Pharmacia), in 0.05 M pH 8.0 Tris-Cl–0.1 M NaCl at about 2°. The diameter and void volume of the column were 8 mm and 2.5 ml. The base-line shift in the paramagnetic resonance spectrum is due to some paramagnetic impurity in the microwave cavity.

particles. In this respect, our observations more closely resemble the finding of Attwood and Saunders (1965) by light-scattering methods that prolonged sonication results in a limiting fragmentation of phosphatidylcholine particles.

A phospholipid particle is a vesicle if it isolates an aqueous volume. We demonstrated that the particles in our dispersions were vesicles by including a radioactive disaccharide in the sonication mixture. The aqueous volume contained in phospholipid vesicles is given then by the ratio of radioactivity associated with phospholipids after gel filtration to radioactivity in the sonication mixture. The results of one such experiment are shown in Figure 1b,c. It is clear from the pattern of contained volume per micromole of phospholipid that the unfractionated dispersion was partially heterogeneous. But the same data indicate a homogeneity with respect to size and shape in the preponderance of the vesicle population.

Many structural features of the vesicles in the homogeneous fractions of Sepharose 4B eluate are uncertain. The measurements of contained volumes of vesicles which we report here are pertinent to the question of vesicle shape. Attwood and Saunders interpreted the results of their light-scattering and viscosity measurements in terms of "elongated spheroids" of axial ratios between 2 and 3 and weight-average particle weight 2.0×10^6 daltons. Huang, on the other hand, concluded from analytical gel filtration experiments and electron microscopic observations that the vesicles are spherical lipid bilayer structures of outside diameter 250 Å. We find a contained volume for disaccharides of 0.10 μl/μmole of phospholipid (Figure 1c) which is incompatible with a spherical shape for vesicles of the dimensions reported by Huang: a spherical vesicle of outside diameter 250 Å and bilayer thickness 50 Å has a contained volume for disaccharides of at least 0.25 μl/μmole of phospholipid (the calculation assumes a vesicle weight of 2.0×10^6 daltons, an egg phosphatidylcholine molecular weight of 770 daltons, and an upper limit of 20 Å on the diameter of a disaccharide molecule). Our measurement seems then to require an outside diameter of less than 250 Å or a deformation of the spherical structure (an elongated spheroid, perhaps, or a biconcave disk (Bangham, 1968)).

Sodium Ascorbate at 0° Creates an Asymmetry in the Distribution of Paramagnetic Molecules between the Two Monolayers of a Vesicle Membrane. Ascorbic acid reduces

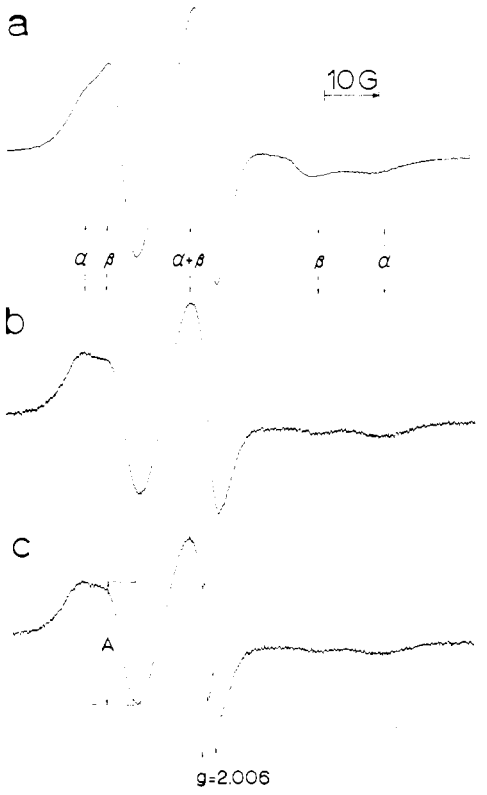

FIGURE 3: Paramagnetic resonance spectra of spin-labeled phosphatidylcholine vesicles at 0°. (a) Before treatment with sodium ascorbate (preparation F). Relative gain, 1. The designations α and β are explained in the text. (b) After treatment with sodium ascorbate at 0°. Relative gain, 6. A mixture of 0.45 ml of preparation C and 0.1 ml of 0.35 M pH 6.9 sodium ascorbate was kept for 10 min on ice and then passed through a column of Sephadex G-25 coarse (Pharmacia), in 0.05 M pH 8.0 Tris-Cl–0.1 M NaCl (previously flushed with argon through a gas dispersion tube) at about 2°. The diameter and void volume of the column were 8 mm and 2.3 ml. (c) After further addition of sodium ascorbate at 0° to the sample described in part b. Relative gain, 6. Cold 0.35 M pH 6.9 sodium ascorbate (5 μl) was added to 50 μl of the sample described in part b. The resulting paramagnetic resonance spectrum did not change during 30 min. The designation $g = 2.006$ corresponds to the center point of a spectrum of 10^{-3} M tempocholine chloride in water (the isotropic g factor of di-*t*-alkyl nitroxides in water is 2.006 (Briere *et al.*, 1965).

the nitroxide spin label 1-oxyl-2,2,6,6-tetramethyl-4-piperidinol to the nonparamagnetic hydroxylamine 1-hydroxyl-2,2,6,6-tetramethyl-4-piperidinol (W. G. Struve, unpublished results). Even at 0° a tenfold excess of sodium ascorbate diminishes the amplitude of the paramagnetic resonance spectrum of 3.3×10^{-3} M tempocholine by 99.9% in 2 min (5 μl of 0.35 M pH 7.5 sodium ascorbate and 50 μl of 3.3×10^{-3} M tempocholine (molecule II) chloride). However, the paramagnetic resonance of tempocholine inside phosphatidylcholine vesicles at 0° (Figure 2) is not affected by the addition of 1000-fold excess of sodium ascorbate (adding 5 μl of cold 0.035 M pH 6.8 sodium ascorbate to 50 μl of the sample whose paramagnetic resonance spectrum is recorded in Figure 2 diminished the amplitude of the paramagnetic resonance spectrum by 8% in 2 min and produced no further change during 20 min). We conclude that the vesicle membrane is impermeable to sodium ascorbate at 0°. The line shape of the paramagnetic resonance of tempocholine inside phosphatidylcholine vesicles indicates that the tempocholine is not bound to the vesicle membrane (the paramagnetic resonance spectrum of 10^{-4} M tempocholine chloride in water at 0° is indistinguishable from the spectrum in Figure 2).

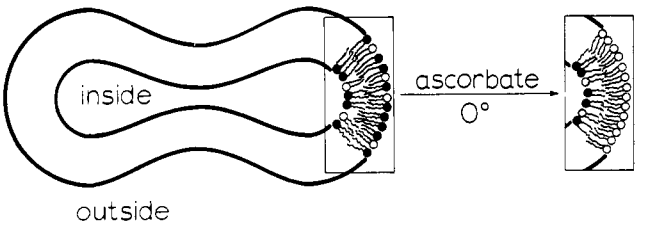

FIGURE 4: Schematic representation of the effect of sodium ascorbate at 0° on the distribution of paramagnetic molecules in a spin-labeled phosphatidylcholine vesicle. A biconcave vesicle is shown in cross section (the shapes of vesicles in our preparations are not known: they may have been spherical). The filled and open circles represent the polar parts of paramagnetic and nonparamagnetic molecules (the proportion of filled circles is much greater than the actual proportion of paramagnetic molecules in our vesicle preparations). The wiggley lines represent the hydrocarbon chains.

Paramagnetic resonance spectra of spin-labeled phosphatidylcholine vesicles at 0°, before and after treatment with sodium ascorbate at 0°, are shown in Figure 3. Because the vesicle is impermeable to sodium ascorbate at 0°, all of the spin-labeled phosphatidylcholine molecules in the internal monolayer of the vesicle membrane contribute to the paramagnetic resonance spectrum in Figure 3b; because a further addition of sodium ascorbate at 0° has no effect on the paramagnetic resonance spectrum in Figure 3b (the amplitude designated A in Figure 3c is 97% of the corresponding amplitude in Figure 3b) we know that the reaction with sodium ascorbate is complete, so none of the paramagnetic resonance spectrum in Figure 3b is due to spin-labeled phosphatidylcholine molecules in the external monolayer of the vesicle membrane. We conclude that the total spin-label paramagnetism of a sodium ascorbate treated vesicle is equivalent to the internal spin-label paramagnetism of an untreated vesicle (Figure 4).

We define a quantity γ by

$$\gamma \equiv \frac{N_i}{N_i + N_o} \tag{1}$$

where N_i and N_o are the numbers of paramagnetic molecules in the internal and external monolayers of a vesicle membrane. γ specifies the distribution of paramagnetic molecules between the two monolayers of a vesicle membrane. γ can be measured for any spin-labeled vesicle preparation[1] because

$$\gamma = \frac{I_{\text{internal}}}{I_{\text{total}}} D \tag{2}$$

where I_{total} is the integrated paramagnetic resonance absorption of the vesicle preparation (the integrated paramagnetic resonance absorption of a sample is proportional to the number of paramagnetic centers in the sample), I_{internal}

[1] The γ of eq 1 is a property of a single vesicle (N_i is the number of internal paramagnetic molecules in a single vesicle). The γ of eq 2 is a property of a large vesicle population (I_{internal} is proportional to the total number of internal paramagnetic molecules in a vesicle population whose paramagnetic resonance is detectable). If the vesicle population is homogeneous then these γ's are equivalent.

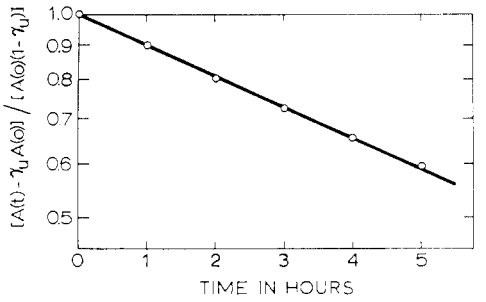

FIGURE 5: Time dependence of the internal spin-label paramagnetism of sodium ascorbate treated vesicles at 30°. A mixture of 1.0 ml of preparation D and 0.2 ml (2.2 × 10^5 cpm) of 0.35 M pH 7.1 ^{14}C-labeled sodium ascorbate (Calatomic) was kept for 10 min on ice and then passed through a column of Sephadex G-25 fine (Pharmacia), in 0.05 M pH 8.0 Tris-Cl–0.1 M NaCl (previously flushed with argon through a gas dispersion tube), at about 2°. The diameter and void volume of the column were 11 mm and 6.6 ml; 50 μl of the eluate contained less than 5 cpm so the removal of ascorbate was greater than 99.5% complete. Part of the eluate was kept at 30° under argon. After an interval of time, t, at 30°, 50 μl of the eluate was chilled and mixed with 5 μl of cold 0.35 M pH 7.1 sodium ascorbate. The paramagnetic resonance spectrum of this mixture was recorded at 0°. The amplitude corresponding to A in Figure 3c was designated $A(t)$. The expression on the ordinate in this figure is the left side of eq 12.

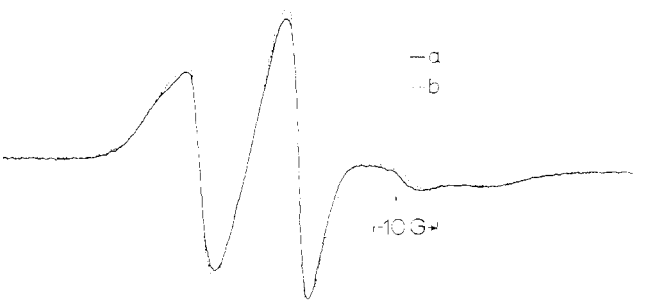

FIGURE 6: Paramagnetic resonance spectra of spin-labeled phosphatidylcholine vesicles at 0° (preparation G). (a) Before treatment with sodium ascorbate. (b) After treatment with sodium ascorbate at 0° and then 4 hr at 40°. The procedure was as described in Figure 5 except that the Sephadex G-25 eluate was kept for 4 hr at 40° under argon with no further manipulation. The paramagnetic resonance spectra have been normalized to facilitate the comparison of line shapes (the amplitude of spectrum b would other wise have been about 0.4 times the amplitude of spectrum a).

is the integrated paramagnetic resonance absorption of the vesicle preparation after addition of sodium ascorbate at 0° and Sephadex G-25 chromatography (the chromatography eliminates a contribution to the paramagnetic resonance from an ascorbate free radical[2]), and D is a correction factor for the effect on I_{internal} of dilution during Sephadex G-25 chromatography ($D = A_{\text{before}}/A_{\text{after}}$ where A_{after} is the same as A in Figure 3c, and A_{before} is the corresponding amplitude in the paramagnetic resonance spectrum of a mixture of 5 μl of cold 0.35 M sodium ascorbate and 50 μl of the vesicle preparation). We denote by γ_u the value of γ for an untreated vesicle preparation. γ_u would be 0.26 for a homogeneous preparation of spherical vesicles of outside diameter 250 Å and bilayer thickness 50 Å under the assumption of equal surface concentrations of spin-labeled phospholipid molecules in both monolayers of the vesicle membrane;[3] γ_u for vesicle preparation G was 0.35. The experimental value of γ_u was larger than the calculated value in part at least because preparation G was heterogeneous (the contained volume of the vesicles in preparation G was 0.28 μl/μmole of phospholipid (based on radioactivity in the eluate from Sephadex G-25) which indicates a contamination by larger vesicles of the otherwise homogeneous population of vesicles of contained volume 0.10 μl/μmole of phospholipid; if γ_u for the homogeneous part of the vesicle population were 0.26 then γ_u for the whole vesicle population would be larger than 0.26).

The line shapes of the paramagnetic resonance spectra in Figure 3 contain information about local structure in phosphatidylcholine vesicles. In each case there are two components. α designates a contribution to the paramagnetic resonance from strongly immobilized spin labels, and β designates a contribution to the paramagnetic resonance from weakly immobilized spin labels. Figure 3b indicates a relative preponderance of strongly immobilized spin-labeled phosphatidylcholine molecules in the internal monolayer of the vesicle membrane, which could be due to closer packing of phospholipids in the internal monolayer of the vesicle membrane (there might be a conformational equilibrium between strongly and weakly immobilized states for every spin label with an equilibrium constant that depends on the local density of phospholipid molecules), or the close approach of internal surfaces in a biconcave disk (the two classes of spin labels might be spatially distinct, the strongly immobilized spin labels located in near contact regions of the internal monolayer and the weakly immobilized spin labels located in the other regions of the internal monolayer).

An Exchange Process Randomizes the Paramagnetic Molecule Distribution in a Sodium Ascorbate Treated Vesicle. The asymmetry in the spin-label paramagnetism of a sodium ascorbate treated vesicle (all of the paramagnetic resonance arises from spin-labeled molecules in the internal monolayer of the vesicle membrane) does not persist indefinitely: the internal spin-label paramagnetism decays exponentially with a half-time of 6.5 hr at 30° (Figure 5). It may be that the internal population of spin-labeled molecules is depleted by exchange with the external population of nonparamagnetic molecules, or that the spin-label paramagnetism is abolished by the combination of spin labels with transient free radicals in the preparation (for example, free-radical intermediates in the peroxidation of unsaturated fatty acids). If the exchange process occurs, then the line shape of the paramagnetic resonance spectrum of sodium ascorbate treated vesicles (Figure 3b) should revert to the line shape of the paramagnetic resonance spectrum of untreated vesicles (Figure 3a). In fact the paramagnetic resonance spectrum of sodium ascorbate treated vesicles whose internal spin-label paramagnetism has 90% decayed to equilibrium is nearly identical with the paramagnetic resonance spectrum of untreated vesicles (Figure 6).

The time rate of change of the internal spin-label paramagnetism of a spin-labeled vesicle depends on the exchange

[2] Piette *et al.* (1961) observed, during the oxidation of ascorbic acid by turnip peroxidase and hydrogen peroxide, a transient ascorbate free radical whose paramagnetic resonance spectrum was a doublet. The splitting and center point g factor of the doublet were about 3 gauss and about 2.003, respectively. This doublet was always evident in the spectrum (for example, Figure 3c) of a mixture of sodium ascorbate and spin-labeled vesicles.

[3] We have no means of evaluating this assumption. The main conclusions of the paper do not depend on it.

of molecules between monolayers through the kinetic properties of both inward and outward translocation processes:

$$\text{paramagnetic molecule in internal monolayer} \underset{k_i}{\overset{k_o}{\rightleftharpoons}} \text{paramagnetic molecule in external monolayer}$$

If every inward or outward translocation of a paramagnetic molecule is an independent event, then the time rate of change of the internal spin-label paramagnetism is given by

$$\frac{dN_i}{dt} = k_i N_o - k_o N_i \tag{3}$$

If the gross structural features (such as size and shape) of a vesicle are constant, then k_i and k_o will be constant and eq 3 implies

$$k_i N_o^{eq} = k_o N_i^{eq} \tag{4}$$

where the superscript "eq" designates an equilibrium value. Equations 3 and 4, together with the fact that $N_i + N_o = N_i^{eq} + N_o^{eq}$, lead to

$$\frac{d(N_i - N_i^{eq})}{dt} = -k(N_i - N_i^{eq}) \tag{5}$$

where

$$k \equiv k_i + k_o \tag{6}$$

If the probability per unit time of a translocation event is uniform within each population of paramagnetic molecules, then eq 5 can be integrated[4] to

$$\frac{N_i - N_i^{eq}}{N_i^0 - N_i^{eq}} = e^{-kt} \tag{7}$$

where the superscript "0" designates an initial value.

Our supposition of no change in the gross structural features of a vesicle during the exchange experiment implies that the equilibrium distribution of paramagnetic molecules in a sodium ascorbate treated vesicle is identical with the distribution in an untreated vesicle. This means that the equilibrium value of the distribution parameter, γ, for a sodium ascorbate treated vesicle is equal to γ_u, the value of the distribution parameter for an untreated vesicle. That is to say, for a sodium ascorbate treated vesicle

$$\frac{N_i^{eq}}{N_i^{eq} + N_o^{eq}} = \gamma_u \tag{8}$$

Equation 8 together with the fact that $N_i^0 = N_i^{eq} + N_o^{eq}$ for a sodium ascorbate treated vesicle leads to

$$N_i^{eq} = \gamma_u N_i^0 \tag{9}$$

[4] If the probability per unit time of a translocation event were not uniform within, for example, the internal population of paramagnetic molecules, then the first-order kinetics would be multiphasic and the integration of eq 5 to eq 7 would not be valid.

TABLE I: Rate Constants and Activation Energies for the Exchange of Spin-Labeled Phosphatidylcholine between Two Monolayers Which Constitute the Bilayer Membrane of a Phosphatidylcholine Vesicle.[a]

Prepn	Age (Days)	k (hr^{-1}) 20°	30°	35°	40°	E_a (kcal/Mole)
C	0		0.319			
	1		0.272			
D	2		0.106		0.316	19.7
E	0				0.427	
	1		0.185	0.326		22.7
F	0		0.098		0.234	15.6
	1		0.189	0.379		27.6
	2		0.266		0.900	22.0
G	0	0.196	0.379			12.0
	1	0.138	0.403		0.987	19.3, 16.2

[a] The procedures were as described in Figure 5 except that preparations C–G were used and the Sephadex G-25 eluates were kept at 20, 30, 35, and 40°.

Substituting from eq 9 into eq 7 yields

$$\frac{N_i - \gamma_u N_i^0}{N_i^0(1 - \gamma_u)} = e^{-kt} \tag{10}$$

Because N_i is proportional to $A(t)$ (the amplitude of the internal component of the paramagnetic resonance spectrum of sodium ascorbate treated vesicles after exchange for an interval of time, t), eq 9 and 10 imply[5]

$$A(\infty) = \gamma_u A(0) \tag{11}$$

and

$$\frac{A(t) - \gamma_u A(0)}{A(0)(1 - \gamma_u)} = e^{-kt} \tag{12}$$

The left sides of eq 10 and 12 give the distance from equilibrium of the paramagnetic molecule distribution in a sodium ascorbate treated vesicle as a fraction of the initial distance from equilibrium.

The values of k in Table I were calculated by means of

[5] Equations 9 and 10 apply to single vesicles (N_i is the number of internal paramagnetic molecules in a single vesicle). Equations 11 and 12 apply to large vesicle populations ($A(t)$ is the amplitude of the paramagnetic resonance spectrum of an aliquot of a phospholipid dispersion). The transformation of eq 9 and 10 to eq 11 and 12 is valid for a homogeneous vesicle population. The transformation of eq 9 to eq 11 is valid for a heterogeneous vesicle population if eq 2 is taken as the definition of γ. The transformation of eq 10 to eq 12 is valid for a heterogeneous vesicle population if eq 2 is taken as the definition of γ and if the value of k is the same for every vesicle in the population. If the value of k is not the same for every vesicle in the population then eq 12 must be replaced by

$$\frac{A(t) - \gamma_u A(0)}{A(0)(1 - \gamma_u)} = \sum_n F_n e^{-k_n t}$$

where the index n denotes a class of vesicles which is characterized by a single value of k and F_n is the number of vesicles in class n as a fraction of the total number of vesicles in the heterogeneous population.

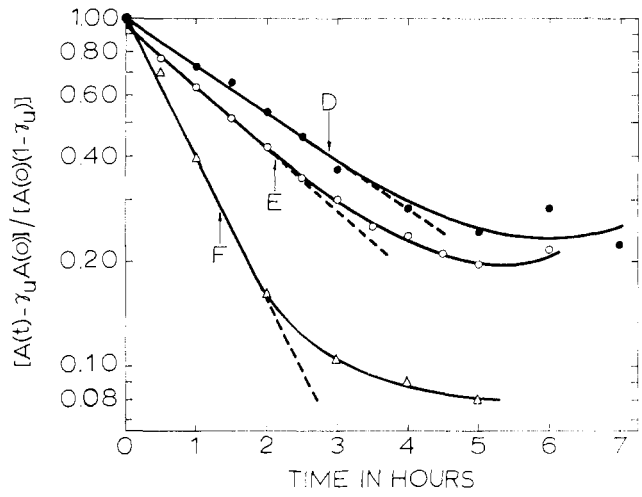

FIGURE 7: Time dependence of the internal spin-label paramagnetism of sodium ascorbate treated vesicles at 40°. The results of three separate experiments are recorded here. The procedures were as described in Figure 5 except that preparations D, E, and F were used and the Sephadex G-25 eluates were kept at 40°.

eq 12 from measurements on several different spin-labeled phosphatidylcholine vesicle preparations at various temperatures and on different days. The variation in the values of k (a random variation from preparation to preparation and systematic variation from day to day) was likely due to variable amounts of oxidized lipids in the vesicle preparations, larger amounts of oxidized lipids leading to larger values of k. (Johnson and Bangham (1969a) attributed an occasional very high value of the potassium ion permeability in 4% phosphatidic acid–phosphatidylcholine vesicles to oxidation of the lipids during sonication.) It is unlikely that the variation in the values of k was due to variable amounts of lysophospholipids in the vesicle preparations or a dependence of k on the vesicle concentration (k would be dependent on the vesicle concentration if collisions between vesicles enhanced the rate of the exchange process within the vesicle membrane), because no lysophospholipid was evident on silica thin-layer chromatograms of a vesicle preparation which had been kept for 4 hr at 40°, and simultaneous determinations on one vesicle preparation at two concentrations gave identical results (part of preparation F, 2 days old, was mixed, after sodium ascorbate treatment and Sephadex G-25 chromatography, with an equal volume of the chromatography buffer; the value of k at 30° for the undiluted part of the preparation was 0.266 hr^{-1} and the value of k at 30° for the diluted part of the preparation was 0.254 hr^{-1}).

The variation in the values of k, which we have attributed to lipid oxidation, obscures a much smaller systematic error which we also attribute to lipid oxidation. The integrated paramagnetic resonance absorption of sodium ascorbate treated vesicles increased during exchange experiments. In one of the worst cases, the increase restored 32% of the integrated absorption that sodium ascorbate treatment had abolished. We think that spin-labeled phosphatidylcholine was reduced to the nonparamagnetic hydroxylamine form by sodium ascorbate (just as 1-oxyl-2,2,6,6-tetramethyl-4-piperidinol is reduced to 1-hydroxyl-2,2,6,6-tetramethyl-4-piperidinol by ascorbic acid) and reoxidized to the paramagnetic nitroxide form by oxidizing agents in the vesicle preparation (many agents are known (Forrester *et al.*, 1968) to oxidize di-*t*-alkylhydroxylamines to nitroxides). The rate of the reoxidation process increased with the age of the vesicle preparation, which implicates oxidized lipids but does not, of course, exclude a contribution from traces of molecular oxygen and other oxidizing agents. The reoxidation process affects the results of the exchange experiment in two ways. (1) The internal spin-label paramagnetism of a sodium ascorbate treated vesicle does not decay to the theoretical (eq 9) equilibrium value. Reoxidation of sodium ascorbate reduced spin labels increases the total number of paramagnetic molecules in the vesicle and therefore increases the equilibrium number of internal paramagnetic molecules. (Equation 9 becomes $N_i^{eq} = \gamma_u (N_i^0 + \Delta)$, where Δ is the number of reoxidized spin labels.) Figure 7 shows the results of two experiments (plots D and E) in which the internal spin-label paramagnetism of sodium ascorbate treated vesicles decayed about 80% of the way to the theoretical equilibrium value. If the sum of oxidized and reduced spin labels in the internal monolayer is constant (which is the same as assuming that the probabilities per unit time of translocations of oxidized and reduced spin labels are equal), then all of the oxidized spin labels which are absent from the internal monolayer as a consequence of exchange have been replaced by reduced spin labels. It follows that reoxidation of 20% of the reduced spin labels limits the decay of the internal spin-label paramagnetism at 80% of the way to the theoretical equilibrium value. To the extent that the internal spin-label paramagnetism does decay to the theoretical equilibrium value, our supposition of no change in vesicle size or shape during the exchange experiment is confirmed. Plot F in Figure 7 resulted from an experiment in which the internal spin-label paramagnetism decayed more than 92% of the way to the theoretical equilibrium value. (2) The experimental value of k is smaller than the actual value. Each of the plots in Figure 7 is the sum of a decay of internal spin-label paramagnetism due to the exchange process and an increase of internal spin-label paramagnetism due to the reoxidation process. We conclude from the minimum values of the internal spin-label paramagnetism in these experiments that 20% or less of the reduced spin labels which replaced oxidized spin labels in the internal monolayer were reoxidized. So the value of k taken from a straight line in Figure 7 is at least 80% of the actual value of k. This error in k is small compared to the variation in values of k which we attributed to an accelerating effect of oxidized lipids. We therefore regard the values of k in Table I as upper limits on the values of k for a preparation free of oxidized lipids.

Due to the variation in values of k from day to day, only the results of simultaneous determinations could be applied to the calculation of an activation energy (E_a). Two or at most three simultaneous determinations were technically feasible. Each value of E_a in Table I was calculated from two values of k by means of the Arrhenius equation in the form[6]

$$k = fe^{-E_a/RT} \tag{13}$$

where f is the so-called frequency factor and R is the gas constant. The average of the many values of E_a in Table I is 19.4 kcal/mole. The corresponding value of f at 30°, calculated from the smallest value of k at 30° (preparation

[6] We assume that the activation energies for the inward and outward translocation processes were equal.

F, 0 days old), is 2.72×10^9 sec^{-1}. Random errors in k have an exaggerated effect on E_a because two values of k are involved in the calculation of a value of E_a, but six of the eight values of E_a in the table are within 20% of the average value. The systematic variation in the values of k which we have attributed to lipid oxidation was not reflected in the values of E_a (excluding the two extreme values of E_a, the average of the three values of E_a which correspond to the three largest values of k at 30° is 19.3 kcal/mole and the average of the three values of E_a which correspond to the three smallest values of k at 30° is 19.2 kcal/mole); that is to say, the accelerating effect of oxidized lipids on the exchange rate was not due to a decrease in activation energy (and must therefore have been due to an increase in frequency factor).

Equations 4, 6, and 8 lead[7] to

$$k_i = \gamma_u k \tag{14}$$

and

$$k_o = (1 - \gamma_u)k \tag{15}$$

Equations 14 and 15 contain, in experimentally accessible form, the same information as eq 4: the probability per unit time of a translocation event is greater for an internal than an external paramagnetic molecule in a vesicle at equilibrium which has fewer internal than external paramagnetic molecules. A trivial consequence of eq 15 is the possibility of determining the rates of penetration of reducing agents into phosphatidylcholine vesicles. The rate of decay of the internal component of the paramagnetic resonance spectrum of a mixture of spin-labeled phosphatidylcholine vesicles and reducing agent is the sum of the rate of outward translocation of spin-labeled molecules and the rate of inward penetration of reducing agent (provided that the reduction of spin labels by reducing agent is rapid compared to either process). We find for a mixture of spin-labeled phosphatidylcholine vesicles and sodium ascorbate at 30° (50 μl of preparation F, 4 days old, and 10 μl of 0.35 M pH 6.9 sodium ascorbate) that the rate of inward penetration of the reducing agent dominates the sum: the amplitude of the paramagnetic resonance spectrum of the mixture decays to zero with a half-time of about 10 min at 30° (following the initial very rapid reduction of the external component of the paramagnetic resonance spectrum); whereas the outward translocation process alone would lead to a half-time of about 4 hr at 30° (based on the value of k_o which we calculate from the value of k at 30° for preparation F, 2 days old).

Discussion

Many molecular mechanisms of phospholipid flip-flop are conceivable. One of them is depicted in Figure 8. The simultaneous flip-flop of two molecules opens space in one monolayer for a molecule from the opposite monolayer. Involvement of two (or possibly more) molecules in the process may account for the large activation energy (19.4 kcal/mole). That is, 19.4-kcal/mole activation energy for phospholipid flip-flop may be the consequence of 9.7-kcal/mole enthalpy change for each of two phospholipid molecules in an "activated complex."

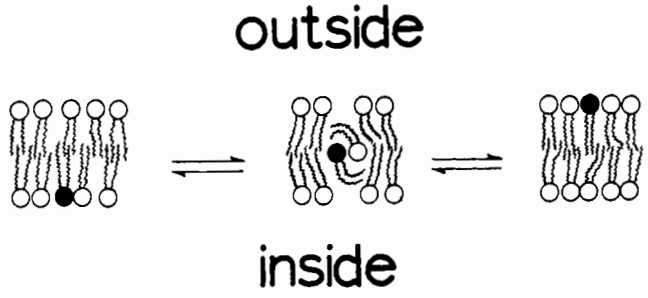

FIGURE 8: Schematic representation of one of the many conceivable mechanisms of phospholipid flip-flop. Part of the bilayer membrane of a spin-labeled vesicle is shown in cross section. The symbolism is explained in Figure 4.

Measurements of the rates of flip-flop of spin-labeled phospholipids with various charged and fatty acid substituents in solutions of various ionic compositions may reveal some details of the molecular basis of phospholipid flip-flop and its possible biological significance. If the "fluidity" of the hydrocarbon region of the phospholipid bilayer is a factor in the flip-flop rate, we anticipate an accelerating effect of unsaturation in the fatty acid substituents of the phospholipids. If ion movements are associated with phospholipid flip-flop, we anticipate a strong dependence of the flip-flop rate on the charged substituents of the phospholipids and the ionic composition of the aqueous phase.

The apparent sensitivity of the flip-flop rate to lipid oxidation could be due to a direct involvement of oxidized lipids in the translocation process. For example, the simultaneous flip-flop of two lipid molecules (Figure 8) might occur very much more rapidly if one of the two molecules were an oxidized lipid. Of course in our vesicle preparations the probability of a spin-labeled lipid having been adjacent to an oxidized lipid was very small because the fraction of oxidized lipids was very small (oxidized lipids were never evident on silica thin-layer chromatograms). So a direct role of oxidized lipids in phospholipid flip-flop would require translational diffusion of lipid molecules within each monolayer of a vesicle membrane (lateral diffusion). The results of preliminary unpublished experiments have enabled us to estimate the rate of lateral diffusion of lipid molecules in spin-labeled phosphatidylcholine vesicles. We have measured the broadening of the nuclear resonance spectra of the protons of phosphatidylcholine molecules in spin-labeled phosphatidylcholine vesicles, and tentatively concluded that the probability of a collision between a spin-labeled molecule and any other molecule in the same monolayer of a vesicle membrane is at least 1/sec at 35°. The largest value of the probability of spin-labeled phosphatidylcholine flip-flop which we have measured was 2×10^{-4}/sec at 40° (the value of k_0 a t40° for preparation G, 1 day old). Therefore the rate of spin-labeled phosphatidylcholine flip-flop by a mechanism involving oxidized lipids would not have been limited by the rate of lateral diffusion of lipid molecules (provided, of course, that there was at least one oxidized lipid molecule in each monolayer of every vesicle membrane).

Pagano and Thompson (1968) reported a unidirectional chloride ion flux of 34 $\mu\mu$moles/(cm^2 sec) for phosphatidylcholine vesicles of outside diameter about 4 mm in 0.1 M

[7] The transformations of eq 4, 6, and 8 to eq 14 and 15 apply to single vesicles. If the values of γ_u and k vary from one vesicle to another in a heterogeneous vesicle population (our preparations were heterogeneous by the criterion of Sepharose 4B chromatography) then, according to eq 14 and 15, the values of k_i and k_o must also vary from one vesicle to another.

NaCl at 30°. The largest value of k at 30° which we report here corresponds to the outward translocation[8] of 0.017 $\mu\mu$mole of phosphatidylcholine/(cm^2 sec) for vesicles of outside diameter about 250 Å in 0.1 M NaCl. If measurements on 4-mm vesicles and 250-Å vesicles can be compared, then our result excludes the possibility that the transverse motion of a phosphatidylcholine–chloride ion complex is responsible for most of the chloride ion flux. But the comparison is questionable because Pagano and Thompson reported a unidirectional sodium ion flux of about 0.4 $\mu\mu$mole/(cm^2 sec) for the 4-mm vesicles in 0.2 M NaCl at 30° whereas Johnson and Bangham (1969a) reported a unidirectional potassium ion flux of about 6×10^{-5} $\mu\mu$mole/(cm^2 sec) for 4% phosphatidic acid–phosphatidylcholine vesicles of outside diameter roughly 500 Å in 0.16 M KCl at 37°. This four orders of magnitude difference in cation fluxes is not likely due to an effect of phosphatidic acid (Papahadjopoulos and Watkins (1967) found that phosphatidic acid vesicles are more permeable to univalent cations than phosphatidylcholine vesicles) or a permselectivity for sodium ions (Papahadjopoulos and Watkins reported nearly equal sodium and potassium ion permeabilities for phosphatidylcholine vesicles), and might instead be attributable to the difference in vesicle sizes or the chloroform content of the 4-mm vesicles (the 4-mm vesicles were prepared from a solution of phosphatidylcholine and *n*-tetradecane in chloroform–methanol, and Johnson and Bangham (1969b) demonstrated an accelerating effect of chloroform on the potassium ion permeability). Since our preparations contained vesicles of outside diameter on the order of 500 Å and no chloroform, our result (the outward translocation of 0.017 $\mu\mu$mole of phosphatidylcholine/(cm^2 sec)) is better compared to the measurement of Johnson and Bangham (a unidirectional potassium ion flux of 6×10^{-5} $\mu\mu$mole/(cm^2 sec)). Papahadjopoulos and Watkins reported a chloride to potassium ion flux ratio of about 40 for briefly sonicated phosphatidylcholine dispersions, which together with the unidirectional potassium ion flux measurement might be taken to suggest a unidirectional chloride ion flux of 0.002 $\mu\mu$mole/(cm^2 sec). Our result would then seem consistent with the possibility that the transverse motion of a phosphatidylcholine–chloride ion complex is responsible for most of the chloride ion flux.

Acknowledgment

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[8] The calculation (involving, of course, eq 15) assumes equal rates of phosphatidylcholine and spin-labeled phosphatidylcholine flip-flop (phosphatidylcholine flip-flop is probably slower than spin-labeled phosphatidylcholine flip-flop because phosphatidylcholine is, according to the chromatographic evidence in Materials and Methods, more polar than spin-labeled phosphatidylcholine) and 71.7-Å^2 surface area per phospholipid molecule (Small, 1967) in the internal monolayer.

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