studies indicate that a similar thermodynamic explanation for heterodimer assembly also applies in the case of tropomyosin from *Rana temporaria* (Lehrer, Qian, and Hvidt, unpublished experiments).

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The Elasticity of Synthetic Phospholipid Vesicles Obtained by Photon Correlation Spectroscopy

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ABSTRACT: Osmotic-swelling experiments were conducted on a variety of preparations of "uniform" unilamellar vesicle systems. The synthetic lipid preparations included both vesicles produced by extrusion through polycarbonate ultrafiltration membranes and vesicles produced by the pH-adjustment method. The vesicles were monitored by photon correlation spectroscopy during swelling as the osmolarity of the external solution was decreased. Contrary to our previously reported results [Aurora, T. S., Li, W., Cummins, H. Z., & Haines, T. H. (1985) *Biochim. Biophys. Acta 820*, 250-258; Li, W., & Haines, T. H. (1986) *Biochemistry 25*, 7477-7483; Li, W., Aurora, T. S., Haines, T. H., & Cummins, H. Z. (1986) *Biochemistry 25*, 8220-8229; Haines, T. H., Li, W., Green, M., & Cummins, H. Z. (1987) *Biochemistry 26*, 5439-5447] large unilamellar vesicles produced from acidic lipids by the pH-adjustment technique were highly polydisperse and did not swell in a manner that permitted the computation of a Young's modulus, presumably due to the polydispersity. Also contrary to our previous reports, membranes derived from bovine submitochondrial particles did not produce evidence of swelling when subjected to similar protocols. Analysis of osmotic swelling of extruded unilamellar vesicles has allowed us to assign Young's moduli for bilayers of dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol, in the range (5-8) $\times 10^8$ and (3-6) $\times 10^8$ dyn/cm^2, respectively. The diameters and polydispersites obtained with electron microscopy and photon correlation spectroscopy were compared directly and with computer-modeling techniques. While excellent agreement was obtained for distributions with low polydispersity ($\lesssim$0.1), serious disagreement was found when the polydispersity exceeded $\sim$0.2.

Osmotic properties of unilamellar phospholipid vesicles have become increasingly important as our understanding of transmembrane proteins emerges. The exploration of mechanicoselective ion channels in the last five years as the patch-clamp technique was applied to biological membranes has shown that these channels are present in virtually all of the cells investigated (Morris, 1990). The data suggest that these channels

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Table I: Comparison of the Results of Current and Previous Experimental Studies[a]

author	membrane	solution	K_e (dyn/cm)	$d \times 10^8$ (cm)	$M_e \times 10^{-8}$ (dyn/cm^2)	max obsd $\delta A/A$	vesicle diam. (nm)
Wobschall	HDTAC-C	40 mM KCl	150–300	NA	NA	NA	NA
Alvarez and Latorre	GMO	1.0 M NaCl	NA	NA	1.4	NA	NA
Kwok and Evans	EPC	100 mM NaCl	140	40	3.5	2–3%[b]	10000
Sun et al.	DMPC	350 mM LiCl	660	34	19.4	2.8%	85.2
	DMPC	350 mM LiCl	305	34	8.7	6.4%	89.0
	DMPC	50 mM LiCl	68	34	1.9	9.7%	106
Hantz et al.	DOPC	150 mM NaCl	63	42	1.5	25%	160–200
	DMPC	150 mM NaCl	51	34	1.5	12.5%	160–200
Miyamoto et al.	brush border	200 mM mannitol with 0 mM glucose	150	NA	NA	5.5%	320–340
	MV	10 mM glucose	80	NA	NA	6.4%	320–340
this work	DOPC	150 mM KCl	340	42	8.2 ± 1.4	2.9%	116
	DOPC	250 mM sucrose	240	42	5.5 ± 0.7	4.6%	118.5
	DOPG	150 mM KCl	220	42	5.2 ± 0.5	4.5%	107.5
	DOPG	250 mM sucrose	165	42	3.9 ± 0.6	5.6%	108.7

[a] Elastic moduli, K_e (area compressibility modulus) and M_e (Young's modulus), and maximum surface area increase ($\delta A/A$). [b] In this case only the maximum expansion shown coincides with bursting of the vesicles.

may have a role in the regulation of osmotic gradients throughout the biosphere. Mechanisms involving ion permeability coupled to membrane tension have been proposed (Yang & Sachs, 1987, 1989; Morris, 1990), and the background against which such studies are considered is within lipid bilayers having specific lipid composition.

Activation of the glucose transport system has been attributed to changes in the elastic properties of brush border membranes of the intestine (Miyamoto et al., 1988). In systems such as erythrocytes, swelling (Mohandas et al., 1983) is especially complicated by the effects of the cytoskeletal (spectrin) network associated with the plasma membrane. Many of the reported expansions of the erythrocyte during osmotic stress involve the detachment of the membrane from this underlying framework without an increase of the surface area of the membrane. The distinct difference between membrane extension and surface area expansion of the erythrocyte was elucidated in micropipette aspiration studies of the erythrocyte (Evans et al., 1976; Evans & Waugh, 1980). Recent studies on rye plasma membrane (Lynch & Steponkus 1987, 1989) indicate that protoplasts derived from rye seedlings retain osmotic activity after freeze-induced osmotic contraction only after cold acclimation.

In addition to containing a mechanicoselective ion channel (Martinac et al., 1987) *Escherichia coli* contains at least three systems that respond to stress due to osmotic changes in the environment. The *kdp* operon alters the potassium ion concentration in the protoplasm in response to osmolarity changes in the external medium (Laimins et al., 1981; Epstein et al., 1990); the *omp* operon limits the size of transbilayer pores formed by the protein, porin, as a response to osmotic stress (Gehring & Nikaido, 1989), and the *mdo* operon adjusts the osmolarity of the periplasmic space between inner and outer membranes by the increased synthesis of polyanionic membrane-derived oligosaccharides (Kennedy, 1982; Miller et al., 1986). Another system operates in the eukaryotic organism *Dunaliella salina* in which an increase (via biosynthesis) in the cytoplasmic glycerol content (Ben-Amotz & Avron, 1983) counters an increase in the extracellular osmolarity. All of these responses to changes of osmolarity using different biochemical responses illustrate the significance of membrane elasticity in living cells.

Until 1986, approaches to the experimental quantitation of the elastic constants of bilayer membranes had been either capacitance measurements or micropipette aspiration [for a discussion, see Haines et al. (1987)]. A Young's modulus, M_e, of 3.5×10^5 dyn/cm^2 was found from capacitance measurements (Crowley, 1973; White, 1974). A reinvestigation of membrane capacitance (Alvarez & Latorre, 1978) produced a direct measurement of membrane capacitance as a function of voltage from which a Young's modulus (or thickness compressibility modulus) on the order of 10^8 dyn/cm^2 was inferred.

The standard method for determination of the bilayer elastic modulus had been the method of micropipette aspiration. The first measurements using this method were conducted on erythrocytes (Rand, 1964). Rand (1964) obtained Young's moduli for erythrocytes ranging from 7×10^6 to 3×10^8 dyn/cm^2. Waugh and Evans (1979) established that upon expansion of the membrane surface, an area compressibility modulus, K_e, of 450 dyn/cm or a Young's modulus of 9.0×10^8 dyn/cm^2 (assuming a 40-Å bilayer thickness) for erythrocytes was obtained. More recently this technique has been applied to giant liposomes (Kwok & Evans, 1981). The swelling of giant (10-μm) DOPC[1] vesicles (Kwok & Evans, 1981) produced an elastic area compressibility modulus of K_e = 140 dyn/cm or, with d = 40 Å, a Young's modulus of 3.5×10^8 dyn/cm^2. Here, the surface area expansion was limited to 2–3%.

These results have shown that the Young's modulus of biological membranes is essentialy that of phospholipid bilayers (see Table I). Presumably this is the Young's modulus of the material (bilayer) within which mechanicosensitive channels will be embedded; it is therefore useful to determine whether or not a variation of the modulus of bilayers occurs as the structure of the phospholipids is varied. All such measurements (of both membranes and synthetic phospholipid bilayers) have exhibited a fairly narrow range of Young's moduli (between 10^8 and 10^9 dyn/cm^2). The variation within this range may be attributed to the lipid component.

A third useful technique for examining the elastic constants of bilayers of phospholipids emerged in 1986. The development of photon correlation spectroscopy (PCS) (cf. Cummins & Pike, 1973; Berne & Pecora, 1976; Chu, 1974) provided a

[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-3-phosphatidylcholine; DOPG, 1,2-dioleoyl-*sn*-3-phosphatidylglycerol; EM, electron microscopy; EPC, egg phosphatidylcholine; EPC, egg phosphatidylcholine; FID, free induction decay; GMO, glycerol monooleate; HDTAC-C, hexadecyltrimethylammonium chloride–cholesterol; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; MV, membrane vesicle; NMR, nuclear magnetic resonance; PCS, photon correlation spectroscopy; rf, radio frequency SUV, small unilamellar vesicles; TEM, transmission electron microscopy Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

method by which submicron particles could be sized very precisely. This technique has been applied to the sizing and distributional analysis of unilamellar vesicles by Aurora et al. (1985), Li et al. (1986), Sun et al. (1986), Hantz et al. (1986), Li and Haines (1986), and Haines et al. (1987) and of biological membranes by Miyamoto et al. (1988); Miyamoto and Fujime (1988).

The photon correlation spectroscopy approaches to measuring bilayer elasticity in vesicles was developed by three groups, Sun et al. (1986), Hantz et al. (1986), and our group (Aurora et al., 1985; Li et al., 1986; Li & Haines, 1986; Haines et al. 1987). Sun et al. (1986) used vesicles that were fractionated by gel-exclusion chromatography; Hantz et al. (1986) employed ultrafiltration to restrict the size of their vesicles. Both groups gave brief theoretical calculations suggesting the relationship between swelling curves and elastic properties.

In the work from our group (Aurora et al., 1985) there was a claim of uniformity of the large unilamellar vesicles derived from the pH-adjustment procedure of Hauser et al. (1983). In that procedure Hauser et al. had not found uniformity in the fraction of large vesicles. Our report showing such uniformity has turned out not to be reproducible; indeed our work now confirms Hauser's finding that the fraction containing large vesicles does not exhibit uniformity. Additionally, our subsequent work based on that preparation (Li et al., 1986; Li & Haines, 1986; Haines et al., 1987) had drawn the following conclusions that have not been substantiated by repeating the experiments.[2]

In the publications subsequent to Aurora et al. (1985), we reported osmotic swelling following a smooth pattern of swelling and bursting of phospholipid vesicles produced by the pH-manipulation technique and submitochondrial particles (Li et al., 1986; Li & Haines, 1986; Haines et al., 1987).

With regard to this previous work we *retract* several previous conclusions based upon the spurious analyses of the experiments:

(1) The pH-adjustment method for obtaining a uniform preparation of vesicles does produce unilamellar vesicles; however, they are highly polydisperse. PCS measurements of this preparation during dilution of the solute do not permit the determination of a Young's modulus.

(2) We do not find a plateau region in our osmotic-swelling experiments, and we have no evidence for vesicle bursting. We therefore have no evidence regarding the elastic limit of vesicles.

(3) The elasticity of phospholipid bilayers in ionic solutions is not measureably different than it is in nonionic solutions. Thus the finding that the elastic properties of acidic phospholipid bilayer vesicles depend on the ionic strength of the solute is a spurious result.

(4) We have no evidence to indicate that the elastic properties of a phospholipid bilayer is affected by the presence of cholesterol.

(5) We have been unable to use the PCS method in connection with osmotic swelling on vesicles derived from natural membranes, namely submitochondrial particles.

In order to conduct a complete reinvestigation of the work reported in these papers we have chosen the vesicle preparations obtained by Hope et al. (1985) and Mayer et al. (1986) using repeated extrusion of the phospholipids through polycarbonate filters. This technique yields vesicle preparations that are usable for purposes of studying the Young's modulus of the phospholipid bilayer, although an improved method for obtaining monodisperse samples of large unilamellar vesicles may allow more precise determination of the Young's modulus.

In the course of this reinvestigation, we have developed improved methods for examining the uniformity of vesicle preparations. In order to make the "polydispersity" value of light-scattering spectroscopists useful to biochemists, we have estimated the conversion from polydispersity to normalized variance. Normalized variance may be converted to the more conventional term, normalized standard deviation, by taking the square root of the normalized variance. We have used a photographic scanning system to obtain an accurate determination of the size distribution of a sample of vesicles from an electron micrograph of a negatively stained vesicle preparation. The distributional information is then used as input to a computer program to synthesize a correlation function. This approach has enabled us to explore the relation between polydispersity and the variance of the size distribution. This technique has also enabled us to determine that the pH-manipulation technique of vesicle preparation (Aurora et al., 1985) produces an unreliable and severely polydisperse preparation of vesicles.

The present study investigates the osmotic swelling of vesicles composed of zwitterionic (DOPC) or of anionic (DOPG) phospholipids by using EM, NMR, and PCS. There are no cationic lipids in biological membranes, only zwitterionic, anionic, or uncharged. Our present data indicate expansion in the range of osmotic dilution we have imposed without an apparent "plateau region", which was previously purported to indicate vesicle bursting. This account of osmotic swelling is consistent with previous accounts of osmotic swelling of vesicles (Sun et al., 1986; Hantz et al., 1986). By fitting osmotic swelling data with a nonlinear least-squares algorithm and by using the equation we earlier developed (Li et al., 1986) for that purpose, we calculate the membrane Young's modulus of vesicles with an approximate diameter of 0.1 μm.

Materials and Methods

Preparation of Vesicles. Extruded vesicles were made from a vortexed dispersion of phospholipid. A solution of DOPC or DOPG (Avanti Polar Lipids, Pelham, AL) (10 mg/mL in chloroform) was rotary evaporated under vacuum so that a thin film covered the lower 3–4 cm of a (1.5 × 12.5 cm) screw cap test tube. The samples were placed in a vacuum desiccator and vacuum (0.25 mm Hg) was applied for an additional 3–4 h to assure complete removal of chloroform. The phospholipid film was dispersed in 3 mL of buffer at pH 7.55. We used either an ionic buffer; 0.150 M KCl, 0.002 M Tris, and 0.02% NaN_3, or a nonionic buffer, 0.250 M sucrose, 0.002 M Tris, and 0.02% NaN_3 [with nearly equivalent osmolality: 0.276 osm (0.150 M KCl) and 0.272 osm (0.250 M sucrose)]. The buffer solutions had been prefiltered through 0.1-μm polycarbonate filters (Nucleopore, Pleasanton, CA). The pH of the vortexed suspension of vesicles was checked and, if necessary, adjusted to pH 7.55 and vortexed for an additional minute. The resulting suspension was then subjected to the extrusion process.

Vesicles were made with a homemade apparatus by the extrusion technique described by Mayer et al. (1986) and Hope et al. (1985). Two 25-mm polycarbonate ultrafiltration membranes (Nuclopore, Pleasanton, CA) with pore size of 0.1 μm were stacked on a 25-mm polyethylene drain disc (Nucleopore) and clamped into a Millipore (New Bedford, MA)

[2] Experiments reported in these papers have been repeated by us and have been found to be nonreproducible. We (T.H.H. and H.Z.C.) apologize to the scientific community for the errors in the cited papers. The reporting errors were unrelated to the activities of Tarlock Aurora, Michael Green, Christopher Rutkowski, or Lloyd Williams.

25-mm stainless steel high-pressure filter holder. High-pressure (~300–400 psi) nitrogen was used to force the phospholipid dispersion through the filter. The turbid dispersion became opalescent on the first pass through the membrane. This procedure was repeated 10 times through the extruder. The resulting opalescent solution was then diluted five times with filtered (0.1 μm) buffer by using a Razel (Stamford, CT) (A-99) syringe pump. These vesicles were not fractionated by gel-exclusion chromatography as this does not produce a more uniform preparation (L. D. Mayer, personal communication). Furthermore, nonspecific adsorption of phospholipids to the gel matrix (Hauser & Gains, 1982; Sun et al., 1986) is a potentially perturbing influence on the bilayer structure produced in the vesicle isolation process. Since bilayers have a surface that is only two molecules thick, interpretation of elastic properties obtained after such exposure to surface adsorption is questionable.

The pH-adjustment procedure for making vesicles was that of Aurora et al. (1985) as modifed by Li et al. (1986).

Electron Microscopy. Vesicles were studied by TEM with a JEOL JEM-100CX at 60 kV. Formvar-coated grids were prepared with a 0.5% formvar solution in amyl acetate on uncoated 3-mm copper grids (Ladd Industries, Burlington, VT). The formvar grid was placed upon a drop of solution containing phospholipid vesicles. The solution was adsorbed with Whatman no. 1 filter paper after 15 s. The grid was placed upon a drop of uranyl acetate solution (1%); after 15 s, the excess was adsorbed with the filter paper. The grid was then allowed to dry in air for 10 min prior to examination with the EM. Electron micrographs were analyzed directly from negatives by utilizing a Microcomp Integrated Image Analysis System (Southern Micro Instruments, Atlanta, GA), a semiautomatic interactive program that measures vesicles sizes from video images of electron micrographs displayed on a computer monitor. Distributional information permitting us to obtain the mean size and the standard deviation of vesicles in a preparation was collected by using the Microcomp Planar Morphometry software.

Freeze–Fracture Electron Microscopy. Freeze–fracture electron microscopy was used to assess the size distribution and extent of unilamellarity of the vesicle preparations. No cryoprotectant was used. Upon being frozen in a freon slurry and fractured in a Balzers 301 freeze–fracture apparatus, the carbon-shadowed platinum replica was inspected in the JEOL JM 100CX electron microscope. The procedure used produced small fracture planes, which contained few vesicle images, making the technique unnsuitable for distributional analysis. However, it is noted that all vesicles images appear to be unilamellar, supporting the data from the NMR experiment. Analysis of the distribution of vesicle size was conducted solely on the results of negatively stained vesicle preparations.

Nuclear Magnetic Resonance. Phosphorus-31 NMR experiments were conducted to determine the extent of unilamellarity of the vesicles produced by extrusion (Hope et al., 1985) and by pH-adjustment (Hauser et al., 1983). Spectra corresponding to an initial 2000 transients were obtained, followed by an addition of an aliquot of $MnCl_2$ so as to make the final Mn^{2+} concentration 5 mM in the vesicle preparation. A second set of measurements was initiated, without disturbing the instrument settings, and this spectrum was compared with the initial spectrum. The addition of manganese broadens beyond detection the signal of phospholipid molecules that are in contact with the external medium. If the vesicles are unilamellar, 50% of the signal area is lost after the addition of Mn^{2+}. A retention of signal area greater than 50% of the initial value indicates multilamellarity in the vesicle preparations, since a fraction of phospholipids greater than 1/2 are protected from the external solvent and contribute to the phosphorus signal detected.

Extruded vesicles of DOPC and DOPG and pH-manipulated DOPG vesicles were prepared at a concentration of 80 mg/mL. Dr. Lawrence Mayer of the University of British Columbia, Vancouver, kindly performed a NMR analysis according to the procedure described by Hope et al. (1985). Both extruded and pH-manipulated preparations were found to be essentially unilamellar.

Photon Correlation Spectroscopy (PCS). The size of the vesicles was determined by photon correlation spectroscopy. The 4880-Å argon laser light scattered at 90° was detected with a Hamamatsu (Middlesex, NJ) (R464) photomultiplier tube. The photomultiplier signal was directed to a Langly-Ford model 1096 (Amherst, MA) digital autocorrelator. The sampling time was adjusted to obtain correlation functions spanning approximately one correlation time, which were then tranferred to a Plessy LSI-11/73 microcomputer (Plessy Peripheral Systems, Irvine, CA) and subsequently analyzed with a VAX 11/780 mainframe computer (Digital Equipment Corporation, Maynard, MA). Analysis of the correlation function gives the diffusion coefficient for the Browniann motion of the vesicles (Aurora et al., 1985). The vesicle diameter is related to the diffusion coefficient by the Stokes–Einstein equation: $D = \frac{k_b T}{3\pi\eta d}$ where k_b is the Boltzmann constant, T is the temperature, and η is the viscosity. After sample introduction, 5 min was allowed for thermal equilibration. Count rates of 30 000–50 000 cps were used. A total of 10 or 11 runs of 180-s duration were performed for each sample.

The average diffusion coefficient and the standard deviation were calculated by averaging 10 or 11 successive autocorrelation functions, each with the same bin (sampling) time and experimental duration. In addition to the diffusion coefficient, the analysis yields an index of polydispersity of the sample that was determined by the cumulants analysis method (Koppel, 1972). In order to determine the diffusion constant and the radius from the measured correlation function, it is necessary to accurately determine both the refractive index and the viscosity of the buffer solutions. The refractive indices of diluted sucrose and KCl solutions were directly determined by using a Bausch and Lomb (Model 799) refractometer (Rochester, NY) with a fluorescent light source filtered with a Kodak (Rochester, NY) Wrattan 45A filter. Distilled water was used as a standard. The viscosity, η, of sucrose and KCl solutions was calculated from equations in the literature (Stokes & Mills, 1965; Barber, 1966) and compared to the experimental values of Aurora et al. (1985).

Dilution of Vesicle Suspensions. The dilution technique used produced samples with the same final volume and concentration of vesicles (~1×10^{12}/mL) in each sample. By designing the swelling experiment so that there was a constant number of vesicles per unit volume, we obtained equivalent total photon counts and equivalent error statistics for all samples. Thus each aliquot of the vesicle solution was diluted with a fixed volume of diluent. A total of 11 1-mL aliquots of vesicles in 0.250 M sucrose or 0.150 M KCl were diluted each with 1 mL of buffer of decreasing concentration (in 10 steps) ultimately to 0.125 and 0.075 M, respectively. The diluent was injected at the rate of 0.05 mL/min with the Razel syringe pump. The initial sample, diluted with full-concentration buffer and therefore containing unswollen vesicles, was

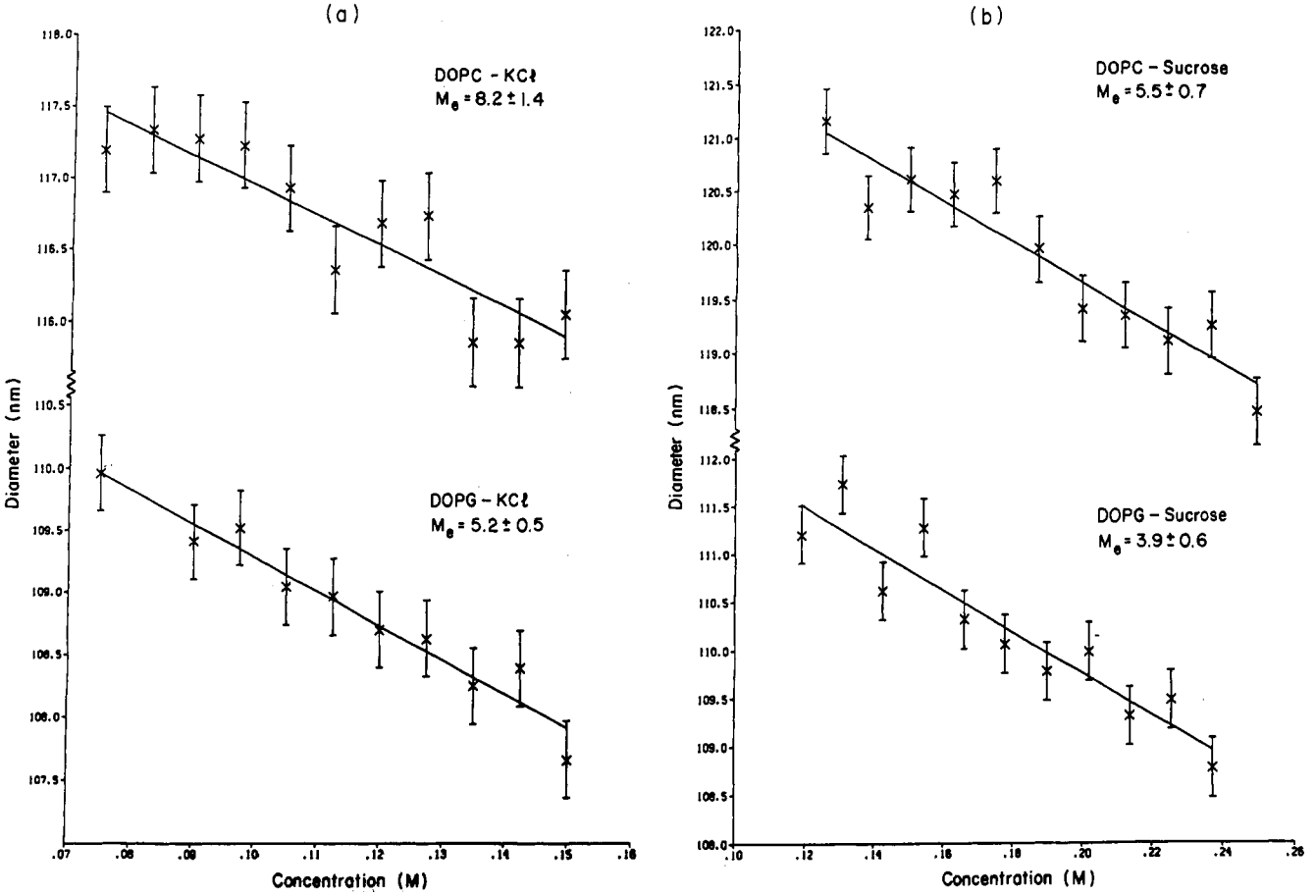

FIGURE 1: (a) Osmotic swelling of DOPC and DOPG in 0.150 M KCl. (b) Osmotic swelling of DOPC and DOPG in 0.250 M sucrose. The Young's moduli given are $\times 10^{-8}$ dyn/cm^2, and the uncertainty shown represents a 90% confidence level. The error bars are the standard deviation of 11 separate correlations divided by square root of 11.

retained and monitored throughout the experiment in order to evaluate possible changes in the samples as a function of time.

The pH-manipulated vesicle suspensions were filtered through 1.0-μm pore size filter membrane (Nucleopore, Pleasanton, CA) prior to dilution. This step was not required for extruded vesicles because they are produced by filtration at the level of 0.1 μm, as are the buffer solutions.

Results

Reinvestigated Experiments. The experiments described in Aurora et al. (1985) reporting the procedure for making pH-adjustment vesicles were reinvestigated, since we could not reproduce the previous results. Additionally, we repeated the experiments reporting swelling on samples of submitochondrial particles obtained from Dr. Harvey Penefsky as reported in Li et al. (1986) using the procedure previously described for isolating the particles (Penefsky et al. 1960). We now find that these submitochondrial particles were in the size range of 220–230 nm with a polydispersity of 35%. Repeated attempts to produce swelling curves as cited in Aurora et al. (1985), Li et al. (1986), Li and Haines (1986), and Haines et al. (1987) did not yield the results reported in these papers. A new investigation using extruded vesicles was therefore undertaken.

Photon Correlation Spectroscopy. The size of the phospholipid vesicles prepared by the extrusion technique varied with respect to their mean diameter from sample to sample in the range of 105–120 nm as determined by PCS. These measurements were made to an accuracy of 0.3% in a mean of 10 measurements. The polydispersity of the vesicles varied by preparation between 7 and 12%; vesicles extruded in sucrose solutions are slightly more uniform than those extruded in KCl solutions. Swelling of DOPC and DOPG vesicles produced by extrusion was monitored. A consistent pattern of swelling was noted for both lipids (Figure 1) in ionic as well as nonionic solutions. The vesicle size as a function of time was also monitored. The observed increase in size was not a time-dependent characteristic for the duration of the experiments.

For each experiment the Young's modulus was obtained by fitting the osmotic-swelling curves to the following elasticity equation [eq 8 of Li et al. (1986)]. Upon rearrangement this equation may be rewritten

$$(d - d_0) = \frac{d_0^2 K}{8tM_e}[C_0(d_0/d)^3 - C] \qquad (1)$$

Where d_0 is the initial vesicle diameter, C_0 is the initial solute concentration inside of the vesicle, t is the membrane thickness, and K is an experimentally determined proportionality constant relating the concentration difference to the pressure difference across the bilayer. The data were analyzed by a two-parameter nonlinear least-squares fit in which the initial diameter, d_0, and the membrane Young's modulus, M_e, were the fitting parameters. The moduli so extracted are given in Table I. The error shown in the table indicates a 90% confidence limit on M_e. We also computed increases in the surface area of the vesicles (Table I).

Electron Microscopy. We examined the size distribution of the extruded vesicle preparations using the Microcomp Integrated Image Analysis System on the negatively stained electron micrographs. This yielded a histogram that is shown in Figure 2.

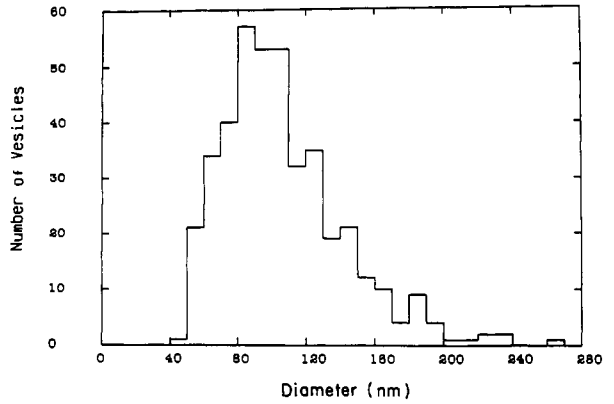

FIGURE 2: Histogram of vesicle sizes of 0.1-μm extruded DOPC vesicles measured by negative-staining TEM.

Computer Modeling. When PCS is used for measuring vesicle diameters, the correlation function of the scattered light always shows deviation from simple exponential behavior. An absolutely monodisperse scattering sample would have an intensity correlation function $[g^{(2)}(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle [I(t)]^2\rangle}]$ of the form

$$g^{(2)}(\tau) = 1 + \beta e^{-2\Gamma\tau} \tag{2}$$

Where I is the intensity of the scattered light, β is an experimentally determined parameter ~ 0.7, τ is the delay time, and Γ is the decay rate of $g^{(2)}(\tau)$, which is determined by the size of the scatterers. As all vesicle-preparation methods produce vesicles with some distribution of diameters, there is always deviation of the correlation function from eq 2. The most common method of analysis (Koppel, 1972) fits $g^{(2)}(\tau)$ to

$$g^{(2)}(\tau) = 1 + \beta e^{-2\Gamma\tau + \mu_2\tau^2} \tag{3}$$

A polydispersity index is then defined as $Q = \mu_2/\Gamma^2$. This index characterizes the degree of polydispersity in the system.

Here we use the distribution of the vesicles sizes obtained from the TEM measurements to synthesize a correlation function. From this synthesized correlation function, we extract a mean diameter and polydispersity index using the same analysis used for the experimentally obtained data. We were then able to compare the diameter and polydispersity index obtained from light scattering to that derived from electron microscopy.

If the size distribution $N(d)$ is given, the correlation function is given by

$$g^{(2)}(\tau) = 1 + \beta|g^{(1)}(\tau)|^2 \tag{4a}$$

where

$$g^{(1)}(\tau) = \frac{\sum_{|d_i|} e^{-\Gamma(d_i)\tau} N(d_i) I(d_i)}{\sum_{|d_i|} N(d_i) I(d_i)} \tag{4b}$$

where $|d_i|$ indicates the set of diameters measured in the EM experiment and $I(d_i)$ is proportional to the intensity of the light scattered by a vesicle of diameter d_i and is given by the appropriate Mie function.

When used a solution to the Mie problem of a hollow sphere recently found by Aragon and Elwenspoek (1982) in the approximation of a thin wall that gives the intensity as

$$I(d_i) \propto d^4 \left[j_0(x) - \frac{(m^2-1)}{m^2} j_1(x)/x \right]^2 \tag{5}$$

Table II: Comparison of Electron Microscopy, PCS, and Computer-Modeling Determination of the Mean Size and Normalized Variance of Extruded Vesicles[a]

	mean diameter (nm)	normalized variance (v) or polydispersity (p)
electron microscopy	106.2	0.11 (v)
photon correlation spectroscopy	110.0	0.09 (p)
computer modeling	111.0	0.09 (p)

[a] Vesicles were sized by PCS to obtain the mean diameter and polydispersity. The vesicles were then examined by TEM to obtain their mean size and variance. The size distribution obtained from TEM was used to generate synthetic correlation functions that were subsequently analyzed in a manner identical within the experimental light-scattering data to obtain a mean diameter and a polydispersity. The close correspondence of both the mean sizes and the polydispersities suggests that the distributional information obtained from TEM is a good representation of the actual size distribution of the vesicles in solution.

where j_0 and j_1 are Bessel functions, $m = \frac{n_1}{n_2}$ (n_1 and n_2 are the refractive indices of the bilayer and surrounding solution, respectively) and $x = \frac{2\pi n_2 d}{\lambda} \sin(\theta/2)$ (λ is the wavelength of the light, and θ is the scattering angle).

The procedure for computer modeling of a particular sample was as follows. With the size distribution $N(d_i)$ obtained from the EM measurements, a correlation function was generated by utilizing eq 4 and 5. This correlation function was then analyzed as though it were experimental data by fitting to eq 3. This enabled us to extract both a diameter and a polydispersity.

The values given by the computer modeling were then compared with the light-scattering and EM values, for both the extruded an pH-manipulated vesicles. For the extruded vesicles, agreement was found for the diameters for all three results, and the polydispersity found from the computer modeling agreed with the PCS result, as shown in Table II. This was not the case for the pH-adjusted samples. The severe inhomogeneity of the sample produced a serious disagreement between the PCS and EM results.

Finally we have utilized this same procedure to determine the relationship between the polydispersity index and the normalized variance for Gaussian distributions of vesicles. A Gaussian distribution of diameters

$$N(d) = N_0 e^{-(d-\bar{d})^2/2\sigma^2} \tag{6}$$

was inserted in eq 4, and a synthetic data file $g^{(2)}(\tau)$ was generated. This data file was then analyzed to find the polydispersity index Q. In Figure 3 we plot Q against the normalized variance of the vesicle sizes $\sigma_n^2 = \sigma^2/\bar{d}^2$. For small values of σ_n^2, Q and σ_n^2 are nearly equal. For $\sigma_n^2 \gtrsim 0.40$, there are large oscilations in Q, which eventually saturates at $Q \sim 0.20$, so that Q is not very sensitive to σ_n^2 for $\sigma_n^2 \gtrsim 0.40$. Thus, a PCS result for the polydispersity of between 0 and 10% indicates a normalized size variance of 0–20%. These results also illustrate the limitations of the PCS technique for samples with polydispersities $\gtrsim 0.2$.

Discussion

Our results confirm that osmotic swelling of uniform preparations of vesicles measured with PCS can be used to obtain a Young's modulus for the bilayers of the vesicles. In contrast to Miyamoto et al. (1988), who have applied PCS measurements to isolated brush border membranes and chromaffin granules under osmotic stress, we have been unable to obtain useful measurements of the Young's modulus for

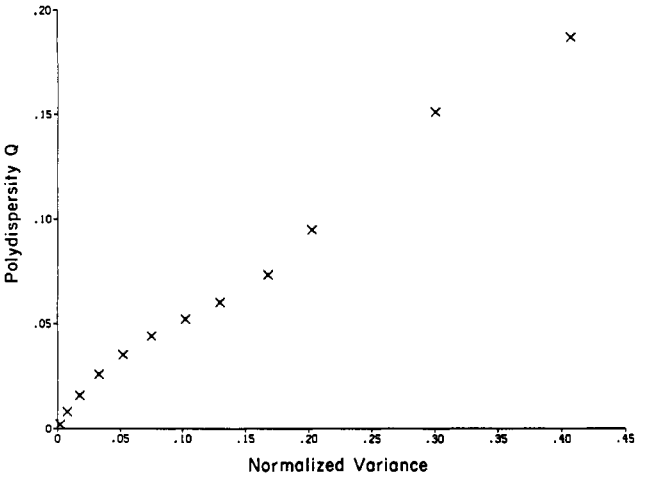

FIGURE 3: Polydispersity (Q) from cumulants analysis of synthetic PCS data vs variance of Gaussian size distribution used to generate the data. This permits an approximate conversion of polydispersity as determined in light-scattering experiments to the normalized variance of the sample size distribution.

submitochondrial membrane bilayers. We note that our submitochondrial preparation was highly polydisperse and that the measured mean diameter was time dependent.

Two methods were used for vesicle preparation in this study. The first, "the pH-adjustment method", we used by us earlier to prepare vesicles for measuring the Young's modulus of the bilayers. This method yielded vesicle preparations with diameters that have large standard deviations and from which it was not possible to obtain a modulus. The second method for vesicle preparation was that of Cullis and co-workers (Mayer et al., 1986, Hope et al., 1985) employing high-pressure filtration of lipids through 0.1-μm filters. Osmotic swelling experiments on vesicles prepared with this procedure gave results (Table I) for the moduli measurements that are consistent with the results previously obtained by others in the literature.

Our experiments have shown that vesicle preparations with large polydispersity do not display reproducible swelling behavior in osmotic swelling experiments. A plausible (though untested) explanation is that the very large vesicles, which initially dominate the high-scattering intensity, swell fastest and burst first, leaving a vesicle distribution with a decreased apparent mean diameter. Indeed, we have found such an apparent decrease in mean diameter with increasing osmotic stress in our PCS studies of vesicles prepared by the pH-manipulation method.

Preparation Uniformity and Computer Modeling. Because uniformity of the vesicle size is so important for making measurements of this type, we synthesized a correlation function (eq 4 and 5) by computer starting from vesicle sizes measured directly with TEM. It was possible to extract both a diameter and a polydispersity index from such a correlation function using eq 3. Comparing this computer-produced result to the actual diameter and polydispersity results on preparations with a variety of polydispersities, we could assess the usefulness of PCS in obtaining a meaningful diameter and hence a Young's modulus. As a result of these studies, it became clear that a large polydispersity, such as that found in the pH-adjustment-derived vesicles, precluded the possibility of determining a modulus. This is due to the fact that the few larger vesicles present ($\sim$500 nm) scattered disproportionately, causing a large uncertainty in the measured average diameter. PCS alone as a measurement technique for vesicle diameter for severely polydisperse samples was often deceptive because small vesicles produce less scattering than large ones (see eq 5). TEM grids with pH-manipulated samples yielding mean diameters of 80 nm were found to have mean diameters of 250 nm by PCS. Where there was good agreement between the two techniques for measuring the diameters, the swelling curves yielded reproducible values of the Young's modulus. Where the two techniques showed severe disagreement for the mean diameter, the dilution experiment yielded erratic results. We conclude that PCS cannot reliably detect swelling with vesicle preparations displaying a polydispersity of greater than $\sim$0.2.

Measurements of the Young's Modulus. We have reported Young's moduli for DOPC and DOPG in ionic and nonionic solutions of equal osmolarity. The Young's modulus of dioleoylphospholipids is on the order of 5×10^8 dyn/cm^2. This corresponds to an area compressibility modulus of 200 dyn/cm, assuming a bilayer thickness of 40 Å. The moduli obtained from vesicles produced by this technique are similar to those determined by alternate methods such as micropipette aspiration. In our study, we observed no significant difference in the moduli obtained from DOPC and DOPG vesicles. Hantz et al. (1986) have noted equivalent moduli in DOPC and DMPC systems.

We have noted a small increase in the degree of swelling of both DOPC and DOPG vesicles in sucrose buffer relative to KCl buffer. However, the observed variation in the Young's modulus from system to system is of the order of the error associated with each measurement. Since this effect is of minimal significance, the assertion that the elasticity of unilamellar vesicles of acidic phospholipids during osmotic swelling is dominated by the ionic strength of the media (Li 1987; Haines et al., 1987) has no basis.

The membrane Young's moduli are in good agreement with the micropipette aspiration studies of Kwok and Evans (1981), upon conversion of K_e to M_e (see Li et al., 1986), as well as with those found in PCS measurements of osmotically swollen vesicles (Sun et al., 1986). In the osmotic-swelling study performed by Sun et al., an order of magnitude decrease in the elastic modulus was noted as the initial diameter increased by $\sim$20%. Our moduli agree with the moduli Sun et al. obtained from vesicles in the 85–91-nm range. However, our moduli are higher than the those obtained from his 97–106-nm vesicles, even though our vesicles are in the range of 108–120 nm. We do note that the lower moduli provided by Sun et al. are derived from a small number of data points: 3 as opposed to 6–7 values for their higher moduli or 11 as in our measurements. Hantz et al. (1986) reports a smaller elastic modulus for DOPC vesicles. These vesicles are 160–200 nm in size. In attempting to reconcile the different values of Young's moduli obtained in these experiments, we note that each group utilizes a different vesicle preparation technique. This may result in vesicles of differing size, polydispersity, and, potentially, degree of unilamellarity or content of residual solvent.

Methods of Uniform Vesicle Preparation Used for PCS Experiments. Sun et al. (1986) utilized the ether injection method of Kremer et al. (1977). In order to remove the solvent used in the synthesis of the vesicle dispersion, the preparation was subjected to extensive dialysis and gel filtration. Nevertheless, residual solvent in the bilayer may affect the elasticity of the membrane. The polydispersity of the vesicle preparation was not quantitated, although it is described as "nearly as mondisperse as latex polystyrene spheres".

Hantz et al. (1986) utilized the reverse-phase evaporation technique of Szoka and Papahadjopoulos (1980), which yielded large unilamellar DOPC and DMPC vesicles; this procedure

produced a polydispersity of 0.15–0.25 after ultracentrifugation and filtration. In this work, osmotic swelling was noted only in preparations made by the extrusion method (Hope et al., 1985), which produced LUV of 105–120-nm diameter. We found that the extrusion technique produced a vesicle polydispersity of 0.07–0.12. Jamshaid (1988) reported a polydispersity of 0.05–0.07 in 70–90-nm vesicles produced by cholate dialysis.

Bilayer Expansion and Bursting. The maximum surface expansion that we measured upon dilution exceeds the burst limit associated with micropipette aspiration studies, yet it is within the bounds of other experiments coupling osmotic swelling with PCS measurements. In the studies of Sun et al. (1986), Hantz et al. (1986), and the present work, no evidence of bursting was noted. In our work, we observe surface expansion of up to 6%, although we have no evidence that this is a limit to the bilayer surface expansion. Kwok and Evans (1981) report a maximum surface expansion of 2–3% before bursting. Surface-area increases on the other of 2.8–11.0% were noted by Sun et al. (1986). Hantz et al. (1986) indicated bilayer surface expansion of up to 25% for DOPC vesicles in 150 mM KCl. Miyamoto et al. (1988) noted a 3.6–10% surface area increase in brush border membranes. The bilayer expansion reported by Miyamoto et al. may be considered to be even larger if the concept of the swell–burst–reseal effect (Li et al., 1986) is discounted as it might well be. We note that the evidence for bursting in the reports by Miyamoto et al. is developed from trends in subsets of the data points. We have noted similar trends in our data but found them to be nonreproducible. We now interpret these trends in our data as scatter in the data rather than deviation in the size caused by bursting and resealing.

We note that simulation studies (Rivers & Williams, 1990) of impermeant (mannitol) leakage from brush border membrane vesicles (Miyamoto et al., 1988) led to questions concerning the general applicability of the osmotic swelling method for the determination of bilayer Young's moduli. Impermeant (KCl) leakage is several orders of magnitude lower in pure phospholipid vesicles (Hauser et al., 1972; Papahadjopoulos & Kimelberg, 1974) than in vesicular preparations of biological origin, supporting our contention that the solutes used are essentially impermeant. Furthermore, we note that, in our experiments, swelling was observed 24–48 h after osmotic dilution, and the degree of swelling was, within experimental error, equivalent to that obtained 1 h after dilution. Thus Young's moduli may be measured for phospholipid vesicles at least 24 h after the initial swelling. These results do not demonstrate a complete investigation of the temporal appearance or disappearance of osmotic swelling; they are noted as a response to the objections posited by Rivers and Williams (1990).

We have demonstrated that extruded vesicles of 0.1-μm size fulfill the requisite condition of monomodality. Furthermore, there is excellent agreement between the average size obtained from TEM and the average size obtained from PCS. Comparison of the polydispersity index obtained from our vesicle preparation and the normalized variance of sizes obtained from TEM with the computer-modeled polydispersity and normalized variance of a true Gaussian distribution of vesicles shows good agreement. Although the distribution of sizes produced in the extrusion technique is not Gaussian, as one would expect from a technique that utilizes a size barrier (the ultrafiltration membrane) as the primary size determinant, the good agreement between the computer-modeling, TEM, and light-scattering results clearly demonstrates a uniform monomodal preparation of vesicles. Presently, the preparation of vesicles with monomodal distribution of sizes appears to be the crucial issue in the utilization of osmotic-swelling/PCS experiments. Until efficient strategies are developed for the production of narrow size ranges of subcellular preparations similar to those obtainable for synthetic phospholipid vesicles, size data produced from PCS techniques may not be reliable or reflect the true mean size of the distribution of vesicle sizes measured. PCS sizing of these preparations with polydispersities over 0.1 should be confirmed with other techniques (TEM, freeze–fracture EM) so as to account for any potential outlying set of large vesicles.

With preparation procedures that minimize the polydispersity of the samples under investigation, the coupled osmotic-swelling/light-scattering experiment may provide the most rapid and reliable means of determining the Young's modulus in submicron liposome systems.

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Spontaneous Phospholipid Transfer: Development of a Quantitative Model†

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ABSTRACT: The effects of lipid structure on the kinetics of spontaneous transfer of a series of phosphatidylcholines have been determined. Donors, which were model-reassembled high-density lipoproteins composed of apo A-I, 1-palmitoyl-2-oleoylphosphatidylcholine, and a trace of a radiolabeled lipid, were mixed with acceptors, which were human low-density lipoproteins. Within a series of phosphatidylcholines, the addition of double bonds and methylene units, respectively, increased and decreased the rate of transfer in a predictable way. An equation that predicts the rates of transfer of a large number of diacylglycerides and phosphoglycerides from any lipoprotein has been empirically derived from these data. The transfers of phosphatidylcholines that contain superpolyunsaturated fatty acids (four or more double bonds) do not obey the derived equation, probably due to limitations on the number of conformational degrees of freedom in these lipids. The range of measured transfer halftimes extends from less than 2 h to more than 12 days. Thus, the spontaneous transfer halftimes of some (but not all) lipids are short compared to the lifetime of lipoproteins in plasma. These results suggest that some lipids transfer among lipoproteins and cells via a spontaneous mechanism while others require specific transfer factors or hydrolysis to achieve this within a physiologically significant time frame.

PCs[1] transfer between lipid surfaces by both spontaneous and protein-mediated processes (Roseman & Thompson, 1980; Massey et al., 1981, 1982, 1984; Nichols & Pagano, 1981; Wirtz et al., 1972; McLean & Phillips, 1981). The former process is highly sensitive to the structure of the transferring species and its microenvironment. Additional double bonds or methylene units in the acyl chains increase or decrease,

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[1] Abbreviations: Phosphatidylcholine, PC; 1-lauroyl-2-oleoyl-PC, LOPC; 1-myristoyl-2-oleoyl-PC, MOPC; 1-palmitoyl-2-oleoyl-PC, POPC; 1-palmitoyl-2-stearoyl-PC, PSPC; 1-palmitoyl-2-linoleoyl-PC, PLnPC; 1-palmitoyl-2-linolenoyl-PC, PLinPC, 1-palmitoyl-2-arachidonyl-PC, PAPC; 1-palmitoyl-2-eicosapentaenoyl-PC, PEPC, 1-palmitoyl-1-docosahexaenoyl-PC, PDhPC; 1,2-dioleoyl-PC, DOPC; low-density lipoproteins, LDL; high-density lipoproteins, HDL; model-reassembled HDL, R-HDL.

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