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PROPERTIES OF SONICATED VESICLES OF THREE SYNTHETIC PHOSPHOLIPIDS

M.A. ROSEMAN, B.R. LENTZ, B. SEARS, D. GIBBES and T.E. THOMPSON

Department of Biochemistry, University of Virginia, Charlottesville, Va. 22901, USA

Received August 5, 1977 accepted November 9, 1977

The following synthetic phospholipids were prepared, and the structures that were formed by ultrasonic irradiation in aqueous solution were studied: 1,2-di(10-bromo stearoyl)-3-*sn*-phosphatidylcholine (DBrPC), 1,2-di(10-methyl stearoyl)-3-*sn*-phosphatidylcholine (DMePC), and 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (POPC). Uniform populations of small, unilamellar vesicles were obtained in all cases by gel filtration on Sepharose 4B. Hydrodynamic and trapped volume measurements show that POPC is nearly identical in size and shape to vesicles of egg phosphatidylcholine whereas DBrPC and DMePC appear to have a non-spherical shape. Fluorescence depolarization measurements show that vesicles from all three lipids are in the liquid crystalline state between 5 and 50°C.

The partial specific volume of DMePC is larger than that of egg PC, whereas the partial specific volume of DBrPC is considerably lower; these lipids should therefore be useful in studies requiring the separation of vesicle populations. POPC, being virtually identical in size, shape and bilayer fluidity to egg PC, should be an excellent model of a 'natural' lecithin with a defined fatty acid composition.

I. Introduction

We report here the synthesis of three phospholipids and the characterization of unilamellar vesicles formed from them by ultrasonic irradiation. Two of these, 1,2-di(10-bromo stearoyl)-3-*sn*-phosphatidylcholine (DBrPC) and 1,2,-di(10-methyl stearoyl)-3-*sn*-phosphatidylcholine (DMePC), were synthesized with a practical purpose in mind. For the past several years there has been an increasing interest in the exchange or transfer of membrane components between different membranes as well as with the plasma lipoproteins. In order to study these processes in model systems, it would be extremely useful to have available a population of phospholipid vesicles which can be readily separated from a population of vesicles composed of the more customary lipids. We have found that vesicles of DMePC have a somewhat lower density than egg PC vesicles, whereas vesicles of DBrPC have a very much greater density. Separation of populations can therefore be achieved by centrifugation. The third phospholipid, 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (POPC) is of greater fundamental importance. In order to understand the details of the physical properties of phospholipid bilayers and their interactions with membrane components such as cholesterol, proteins and other lipids, it is necessary to study

synthetic phospholipids. However, nearly all studies of synthetic phospholipid vesicles to date have utilized phospholipids containing identical fatty acid chains on the 1 and 2 positions of the glycerol. The information obtained with those lipids is frequently of limited value since the phospholipids of natural membranes usually contain two different fatty acids on each molecule, as in the case with 1-palmitoyl-2-oleyl phosphatidylcholine. Our results with POPC indicate that vesicles formed from this lipid would in fact be ideal for the kind of studies mentioned above.

II. Syntheses of lipids

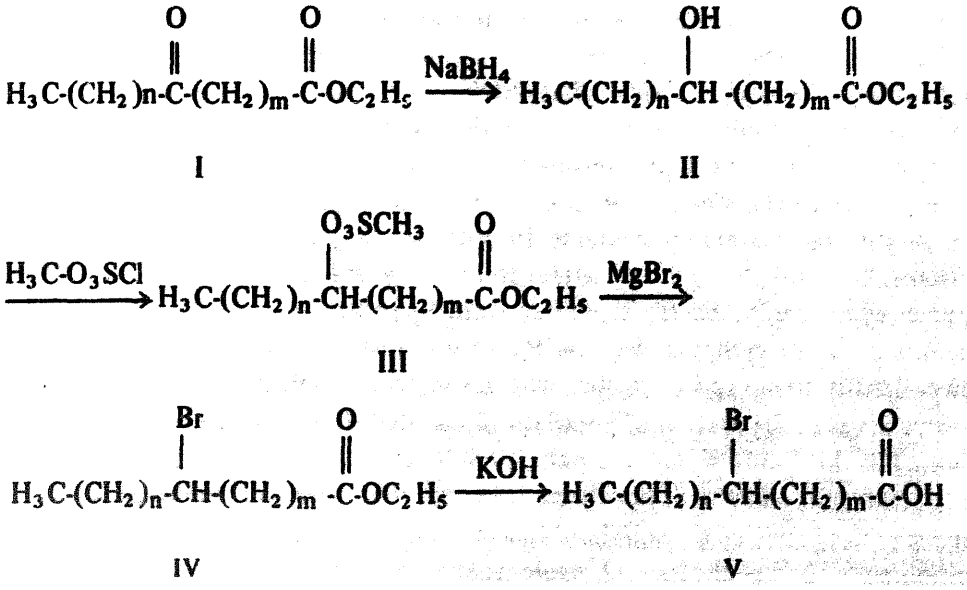
DBrPC and DMePC were synthesized from the appropriate fatty acids by the procedure of Cubero Robles and Van den Berg [1]. POPC was prepared by a modification of this method. We first describe the syntheses of the brominated and methylated fatty acids and then briefly describe the procedure used to incorporate these into phosphatidylcholine.

A. Synthesis of ethyl 10-ketostearate

Long chain keto esters of the general formula I may be prepared by the procedure outlined by Chasin and Perkins [2]. In this case, monoethyl sebacate (m = 8) was prepared from sebacic acid and diethyl sebacate by the method of Jones [3]. 10-Ketostearate was obtained from this as described by Cason [4].

B. Synthesis of 10-bromostearic acid

10-Bromostearic acid was prepared by the following synthetic route:



The reduction of I to 10-OH ethyl stearate (II; m=8, n=7) was accomplished by reaction with an equimolar quantity of sodium borohydride. The sodium borohydride was added slowly and with stirring to an ethanolic solution of I (∿ 4% w/v) at room temperature. The reaction mixture was stirred at ambient temperature for about 1.5 hr, or until the evolution of hydrogen gas had ceased. At the end of this period any unreacted sodium borohydride was destroyed by adjusting the pH of the mixture to neutrality with acetic acid, then stirring until the evolution of hydrogen gas had ceased. The solvent was removed under vacuum on a rotary evaporator and the resulting solid residue recrystallized from 30% H_2O–MeOH (v/v) to yield a white crystalline solid with an uncorrected melting point of 45–45.5°C. The product (94% yield) was judged to be better than 98% pure by silica gel TLC, using hexane–diethyl ether–acetic acid (30: 70: 1, v/v/v) as the eluting solvent. The infrared spectrum showed a broad band at 3200–3600 cm^{-1} (–OH) and a sharp band at 1745 cm^{-1} (ester – C=O).

Synthesis of ethyl 10-mesylstearate (III) was carried out by the procedure of Baumann and Mangold [5] for the preparation of mesylates of long chain alcohols, with the modifications suggested by Baumann et al. [6]. A 20–23% solution (w/v) of II in pyridine (dried over KOH) was purged with N_2 and cooled on an ice bath before adding dropwise, and with stirring, a 50% molar excess of methanesulfonyl chloride. The reaction mixture was stirred at ambient temperature for 14–16 hr. A volume of ice-cooled water approximately equal to that of the reaction mixture was added, followed by ether extraction as described by Baumann and Mangold [5]. The combined ether phases were dried over anhydrous Na_2SO_4, and the solvent was removed on a rotary evaporator. The resulting yellow-orange residue was twice recrystallized at –70°C from absolute methanol (8–10% solution, w/v) to give, in 71% yield, a slightly yellow liquid that was judged to be about 95% pure by silica gel TLC, eluting with hexane–ether (70:30, v/v). The infrared spectrum showed bands at 1735 cm^{-1} (ester C=O), 1375 cm^{-1} (sulfone), and 1175 cm^{-1} (sulfone).

The conversion of III to ethyl 10-bromostearate (IV) was carried out with minor modification as described by Baumann et al. [6] for the synthesis of *cis*-9-octodecenyl bromide. The reaction mixture (containing 0.2 mol $MgBr_2$ and 0.1 mol VI) was stirred continuously for 22 hr at room temperature, after which about one-half of the solvent was removed on a rotary evaporator under vacuum at a temperature of 30–35°C. Five hundred ml of ice-cold water were added to the reaction mixture followed by the series of extractions described by Bauman et al. [6]. The combined organic phases were dried over $MgSO_4$, after which the solvent was removed on a rotary evaporator. The resulting pale yellow liquid was judged by TLC (solvent: hexane–diethyl ether, 7:3) to be better than 95% pure, containing only a trace impurity with the same R_f value as the impurity found in compound III.

The saponification of IV to 10-bromostearic acid was performed under conditions that result in little or no hydrolysis of the C–Br bond. The ethyl 10-bromostearate (IV, 0.1 mol) was dissolved in 900 ml methanol to which 100 ml of 5 N KOH were

added. The mixture was stirred at 38°C for 3 hr by which time all the ethyl ester had gone into solution. Three hundred ml H_2O were then added so that the final composition was MeOH–H_2O (9:4) and the mixture extracted twice with 250 ml portions of hexane. The resulting MeOH–H_2O phase was isolated, acidified with 5 N HCl to pH ≃ 1 and again extracted twice with 250 ml portions of hexane. The final two hexane phases, containing extracted fatty acids, were evaporated under vacuum to yield a white amorphous solid with an uncorrected melting point of 41.5–42.5°C (the reported melting point of a mixture of 9,10-bromostearic acid is 43–44°C, Fox et al. [7]. TLC chromatography utilizing 20:70:1 hexane–diethyl ether–acetic acid indicated a trace impurity with an R_f corresponding to that of 10-OH stearic acid. In order to remove this trace impurity, the product was recrystallized at −70°C from 9:1 MeOH–H_2O (v/v). The resulting white amorphous solid (84% yield relative to VI) was judged uniform by TLC and had an uncorrected melting point of 43–44°C. The infrared spectrum is consistent with the proposed structure, showing bands at 1710 cm^{-1} (acid C=O) and 510–540 cm^{-1} (–C–Br). The mass spectrum showed a principal peak with $^m/_e$ = 283, which is consistent with M^+/e – 79.9 (for loss of a bromine atom). Gas chromatographic analysis on a Shimadzu GC-4B instrument using 10% Silar 10C on Gas Chrome Q (Allteck Associates, Arlington Heights) was also attempted (column temperature, 170°C; = injection point, 220°C; detection, 220°C). However, the methyl ester of the product appeared to decompose during the chromatographic run, giving two principle peaks. The first of these had a retention time identical to that of methyl oleate and trailed significantly into the second and broader peak. The column effluent collected from either of these peaks gave, when reapplied to the column, a single peak having the retention time of methyl oleate.

C. Synthesis of 10-methylstearic acid

The synthesis of 10-methylstearic acid, from ethyl-10-ketostearate, was carried out with modification according to the procedure described by Chasin and Perkins [2].

D. Synthesis of 1,2-diacyl-3-sn-phosphatidylcholines

The synthesis of the three phosphatidylcholines used in this study were carried out with modifications according to the procedure of Cubero Robles and Van den Berg [1]. Specific details that differed from the standard procedure are reported below. Fatty acids were converted to anhydrides using dicyclohexylcarbodiimide (Swarz-Mann, ultrapure) according to the procedure of Selinger and Lapidot [8]. L-α-glycerophosphorylcholine (GPC).$CdCl_2$ was derived from chicken egg yolk phosphatidylcholine as described by Chadha [9] and this was converted to free L-α-GPC by passage through a mixed bed ion exchange resin (Mallinkrodt Amberlite IRC50 and IR45 in a 12-fold excess to the equivalents of $CdCl_2$ to be removed). Fatty acid anhydride, the potassium salt of the fatty acid, and GPC (in a molar

ratio of about 3:2:1) were mixed in a 50 ml pear-shaped flask, which, after flushing with argon, was evacuated, and rotated in a heated oil bath. The temperature of the oil bath and the time of reaction were varied according to the particular acyl chains being esterified to GPC (see below).

DMePC was synthesized from 10-methylstearic anhydride by the Cubero Robles and Van den Berg procedure with the following modifications. The ratio of reactants for the acylation was 25:2:1 (anhydride–potassium 10-methyl stearate–GPC). The reaction vessel was rotated on an oil bath for 24 hr at 60°C. The resulting mixture was shaken with $CHCl_3$–MeOH–H_2O in the ratio 8:4:3 (Folch et al. [10]) and the lower phase collected. The solvent was removed on a rotary evaporator and the residue dried in vacuo over P_2O_5. The dried residue was dissolved in chloroform and applied to a silicic acid column (Mallinckrodt Silicar CC-4, 60–200 mesh). The column was eluted stepwise with $CHCl_3$–MeOH mixtures of increasing polarity, with the phosphatidylcholine being recovered in the 4:6 $CHCl_3$–MeOH fraction. TLC on silica gel G plates (250 μm; solvent, 65:25:4, $CHCl_3$–MeOH–H_2O) indicated a single spot on staining with iodine. The yield was 52% based on phosphorus determination by the procedure of Bartlett [11].

In the case of DBrPC, it was found that the low reaction temperature used for synthesis of DMePC resulted in very low yields of about 15–20%. For this reason, the DBrPC reaction mixture was heated for 3.7 hr at 85–87°C. The reaction mix was taken up to 400 ml 2:1 (v/v) $CHCl_3$–MeOH and this solution was shaken with 100 ml water. The upper MeOH–H_2O phase was acidified with HCl and the two phases were again mixed. The lower phase was dried over $MgSO_4$, evaporated under vacuum on a rotary evaporator, and the residue taken up in CCl_3. The $CHCl_3$ solution was applied to a batch column (40% load w/w) of Mallinckrodt Silicar/CC-7 silicic acid and eluted stepwise with $CHCl_3$–MeOH mixtures of increasing polarity. All of the excess acid and anhydride were removed in this way, along with much of the other impurities. The crude yield at this point was 65%. In order to remove all the breakdown products caused by the high reaction temperature, two subsequent chromatographic separations on silicic acid columns were necessary (1–2% loading, w/w). The resulting DBrPC (2.5 mmol, 34% yield relative to L-α GPC) was judged to be uniform by TLC, utilizing 65:25:4, $CHCl_3$–MeOH–H_2O, as the eluting solvent.

The 10-bromostearic acid recovered from the initial few fractions of the first silicic acid column was recrystallized from methanol, with the filtrate saved for analysis. The gas chromatograph of the methyl ester of the material recovered from the filtrate revealed that the breakdown peak, with the retention time of methyl oleate, was considerably larger than in the chromatograph of methylated pure 10-bromostearic acid which had been run under identical conditions and with a similar load. This analysis suggested that about 2.5% of the 10-bromostearic acid had decomposed during the acylation. In order to check the fatty acid composition of the purified DBrPC, a 10 μmol sample was hydrolyzed and the released fatty acids were isolated and methylated. TLC (500 μm thick layer containing 10% $AgNO_3$ and silica gel G; solvent, 9:1 hexane–ether) gave R_f values of 0.45, 0.29, 0.34, and

0.35 for methyl stearate, methyl oleate, methyl 10-bromostearate, and the methyl ester of the hydrolysate. It was impossible to detect any contamination in the hydrolyzed fatty acid. On the basis of these analyses, we estimate that the breakdown of 10-bromostearic acid to unsaturated derivatives was less than 3% during the synthesis, purification and subsequent storage of DBrPC. DBrPC and 10-bromostearic acid were at all times stored at −20°C in brown glass containers.

E. Synthesis of 1-palmitoyl-1-oleyl phosphatidylcholine

POPC was synthesized from oleic and palmitic acids (Sigma, 99% pure) by a modification of the Cubero Robles and Van den Berg method. The crude *lyso*-PC was dissolved in 1:9 MeOH–$CHCl_3$ and purified by silicic acid column chromatography.

Acylation of the *lyso*-PC with oleic anhydride (prepared by the DCC method) was performed by a procedure similar to that used by Cubero Robles and Van den Berg to prepare the diacyl phosphatidylcholines. Two mmol *lyso*-PC were evaporated to dryness in a 50 ml pear-shaped flask, washed with CCl_3, and dried overnight under vacuum over P_2O_5. Five mmol oleic anhydride in CCl_4 (prepared by the DCC method) were added and the CCl_4 removed by evaporation under vacuum. Finally, 4 mmol potassium oleate containing a small excess (%) oleic acid were added. The flask was flushed with argon, evacuated, and rotated for 24 hr at 60°C in an oil bath The product was worked up in the usual way and purified by silicic acid column chromatography. This procedure differs from that for the diacyl lecithins in the order of addition of the reactants. The usual procedure for diacyl lecithins calls for evaporating to dryness a methanolic solution of GPC and fatty acid salts, drying overnight, then adding anhydride. When we followed this procedure (using *lyso*-PC instead of GPC), the final product of the acylation was exclusively dioleyl PC. We presume that methanolysis of *lyso*-PC to GPC occurred in the first step. This could occur if the K^+-oleate (prepared by titration of oleic acid with methanolic KOH) contains even a slight excess of methanolic KOH. Therefore, to avoid hydrolysis, the anhydride was added before the salt. Also, the fatty acid 'salt' contained a 10% excess of free fatty acid. Analysis of the lecithin showed equimolar amounts of oleic and palmitic acid, and > 97% palmitic acid in the 1-position.

III. Experimental

A. Preparation of phosphatidylcholine vesicles

Small vesicles of the synthetic phosphatidylcholines were prepared by the method of Huang [12]. Lyophilized phosphatidylcholine was suspended in buffer and subjected to ultrasonic irradiation in a Branson W-350 sonifier, at 0°C under N_2 until a clear dispersion was obtained. After removal of undispersed lipid and titanium particles by centrifugation, the clear dispersion was subjected to molecular sieve

chromatography on fresh Sepharose 4B at 4°C. A typical elution profile, showing the absorbance at 300 nm, concentration of lipid phosphorous, and trapped glucose, is shown for DBrPC in fig. 3A. The trapped volume determinations will be discussed in detail later. The vesicle fractions obtained from the descending portion of the included peak showed a constant ratio of 300 nm absorbance to lipid phosphorus, and were used for the hydrodynamic measurements.

B. Sedimentation velocity measurements

Sedimentation velocity measurements were carried out at 20 ± 0.05°C in a Spinco Model E ultracentrifuge equipped with an RTIC temperature-control unit and a schlieren optical system fitted with a phase plate Kodak metallographic plates were used to photograph the schlieren patterns which were then read on a two-dimensional microcomparator. A double sector capillary-type synthetic boundary cell with a 12 mm optical path was used in all experiments. The rotational speeds were maintained at velocities between 42,040 and 60,000 rpm. Since the schlieren peaks were highly symmetrical, sedimentation coefficients were calculated from plots of time versus log r determined from measurements of the positions of the maximum ordinate.

C. Diffusion measurements

Diffusion studies were carried out in the analytical ultracentrifuge under conditions identical with those employed for sedimentation velocity studies except that the experiments were performed at a lower speed of approximately 10,000 rpm for 3–4 hr. At this speed, the boundary did not move appreciably. The apparent diffusion coefficient, D_{app}, was calculated by the maximum ordinate-area method from the following relationship (Ehrenberg [13]):

$$D_{app} = \frac{1}{4 \P t} \left(\frac{A}{kH_{max}}\right)^2$$

where A is the area under the gradient curve as measured on the photographic plate at time t, H_{max} is the maximum height of the curve, and k is the magnification factor along the radial coordinate. Apparent diffusion coefficients obtained by this method were reduced to values in water at 20°C, $D_{20,w}$ (Svensson and Thompson [14]).

D. Determination of apparent partial specific volumes

Apparent partial specific volumes were calculated from density measurements made at 20 ± 0.01°C in an Anton Paar Model 02C magnetic densitometer. The apparent partial specific volumes were calculated from density data using the following relationship (Cassassa and Esenberg [15]):

$$\Phi^1 = \left(\frac{1}{\rho_0}\right)\left[1 - \frac{100(\rho - \rho_0)}{c}\right]$$

where ρ_0 is the density of the buffer, ρ is the density of the phosphatidylcholine vesicle solution, and c is the concentration of lipid.

E. Trapped volume determinations

The volumes of the internal, solute-available, aqueous compartments of the three vesicles species considered here were determined by 'trapping' potassium ferricyanide ($K_3Fe(CN)_6$) or [^{14}C]glucose (Schwarz/Mann, lot XR-2337). Vesicles containing trapped marker were prepared by sonication of the lipid in a buffer containing the chemical marker followed by gel filtration on Sepharose 4B (Bio Rad) as described above. The chromatography step served not only to size the vesicles, but also to remove non-trapped marker from the vesicles. In the case of [^{14}C] glucose trapping, it was found that about 10% of the glucose leaked out of the vesicles during the 4 hr required to elute the homogeneous vesicle fraction from the Sepharose 4B column. For this reason, columns were eluted with a buffer identical to the buffer used in sonication, except that it contained 'cold' glucose instead of [^{14}C]glucose. Buffers contained, in addition to glucose, 0.01 M KCl (Heico, ultrapure grade) and 0.001 M Tris (Schwarz-Mann, enzyme grade) at pH 7.5. Trapped $K_3Fe(CN)_6$ was determined by first disrupting the chromatographed vesicles with 1-propanol (to a final concentration of 25:75 propanol–water (v/v)) and then measuring the absorbance of the sample at 420 nm (Newman and Huang [16]). The trapped aqueous volume was calculated as:

$$\bar{V}_t = \frac{M_i/M_0}{P}$$

where $\bar{V}_t$ is expressed as μl of trapped volume per μmol of vesicle phospholipid, M_i and M_0 are the concentrations of trapped solute in the chromatographed vesicle sample and in the sonication buffer, respectively, and P is the molar concentration of phospholipid in the vesicle sample determined as inorganic phosphate (Bartlett [11]). For $K_3Fe(CN)_6$ trapping, M_i and M_o were proportional to the absorbance at 420 nm (with appropriate corrections for dilution). The radiopurity of the [^{14}C]-glucose used in these measurements was checked by TLC on Eastman microporous cellulose plates developed in butanol–pyridine–H_2O (6:4:3, v/v/v) and visualized with aniline diphenylamine (Sigma Chemical). One spot, with R_f corresponding to a glucose standard, contained 99.1% of the radioactivity found on the plate and no other spots were detected.

F. Nuclear magnetic resonance determination of vesicle monolayer mass ratio

As an additional check on the size of the vesicles prepared here, measurement

was made of the ratio of *N*-methyl protons on the outside monolayer relative to the inside monolayer of the vesicle bilayer. Vesicle samples were dialyzed into D_2O buffer (99.9% D_2O), containing 0.01 M KCl. $PrCl_3$ was added to a ratio of 0.05–0.06 $PrCl_3$/phospholipid in order to split the inner and outer *N*-methyl proton peaks (Huang et al. [17]). Proton magnetic resonance spectra and integrated peak intensities were recorded on a JEOL FT100 instrument at a pulse rate of 2.1 sec. The ratio of the number of phosphatidylcholine molecules in the outer monolayer of the vesicle to the number in the inner monolayer was calculated simply as the ratio of the downfield $N-CH_3$ peak intensity (i.e., shifted downfield by the presence of Pr^{3+} ion) to the unshifted $N-CH_3$ peak intensity.

G. Bilayer microviscosity measurements

Estimates of the bilayer microviscosity were obtained from measurement of the fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Shinitzky et al. [19], Shinitzky and Barenholz [20]) as previously described (Lentz et al. [21]). For all measurements reported, light-scattering corrections were less than 1% of total fluorescence intensity.

IV. Results

A. Hydrodynamic and trapped volume measurements

The values of the hydrodynamic parameters for vesicles of the three synthetic phospholipids, along with the values of the trapped volumes and monolayer mass ratios, are summarized in table 1. The hydrodynamic parameters were obtained in a straightforward manner by the methods that have been used previously for the characterization of egg PC vesicles. By contrast, the trapped volume determinations were complicated by the finding that this number varies with K_3FeCN_6 concentration.

The dependence of trapped volume estimates on the concentration of $K_3Fe(CN)_6$ or glucose in the trapped buffer is presented in fig. 1 for egg yolk phosphatidylcholine vesicles. Trapped volumes obtained from [^{14}C]glucose experiments were essentially independent of glucose concentration over the range studied with an average value of 0.170 μl/μmol phospholipid resulting from the data in fig. 1. Data obtained from $K_3Fe(CN)_6$ trapping, on the other hand, gave very large values for the volume of the internal solute-available aqueous compartment at low $K_3Fe(CN)_6$ concentrations, but approached the [^{14}C] glucose values at high concentrations of the salt. That this variation in trapped volume was not due to ferricyanide-induced changes in the size of the phosphatidylcholine vesicles was demonstrated by the constancy of the outer monolayer to inner monolayer mass ratio, as determined by proton magnetic resonance (see fig. 1). Since variation in vesicle structure has been ruled out, the shape of the ferricyanide trapped volume curve was taken to suggest

Table 1

Hydrodynamic parameters, trapped volumes, and monolayer mass ratios for synthetic phosphatidylcholine vesicles as compared to egg phosphatidylcholine vesicles.

	$S^{\circ}_{20,w}$ (Swedbergs)	$\Phi'20$ ($\frac{cm^3}{9}$)	D^{20}_w ($\frac{cm^2}{sec}$)	Stokes' radius A	Packing[a] density (moles/(A^3))	Nb	V°_t ($\mu l/\mu mol$)	Monolayer mass ratio (out/in)
Egg yolk	2.63[c]	0.9848	2.03×10^{-7} [c]	106	7.93×10^{-4}	2400	0.17	2.40
	± 0.23	± 0.0005	± 0.04×10^{-7}	± 2	± 0.004×10^{-4}	± 365	± 0.01	± 0.12
1,2-di(10-bromostearoyl)	34.5	0.884	1.90×10^{-7}	115	7.19×10^{-4}	4142	0.269	1.83
	± 1.5	±0.002	± 0.08×10^{-7}	± 5	± 0.02×10^{-4}	± 621	± 0.04	± 0.18
1,2-di(10-methylstearoyl)	−0.536	1.005	1.55×10^{-7}	118	7.32×10^{-4}	2766	0.203	1.91
	± 0.02	± 0.002	± 0.06×10^{-7}	± 5	± 0.01×10^{-4}	± 415	± 0.03	± 0.20
1-palmitoyl-2-oleyl	2.59	0.9853	1.86×10^{-7}	106	8.042×10^{-4}	2500	0.193	1.75
	± 0.25	± 0.0005	± 0.09×10^{-7}	± 5	± 0.004×10^{-4}	± 375	± 0.02	± 0.20

a Packing density - $0.6023/\Phi^1 \times$ mol. wt. lipid.

b N = number of molecules per vesicle.

c From C. Huang and L. Lee, J. Am. Chem. Soc. 95 (1973) 234.

d Errors are reported as one standard deviation as determined by statistical analysis or by estimate where insufficient data was available for analysis.

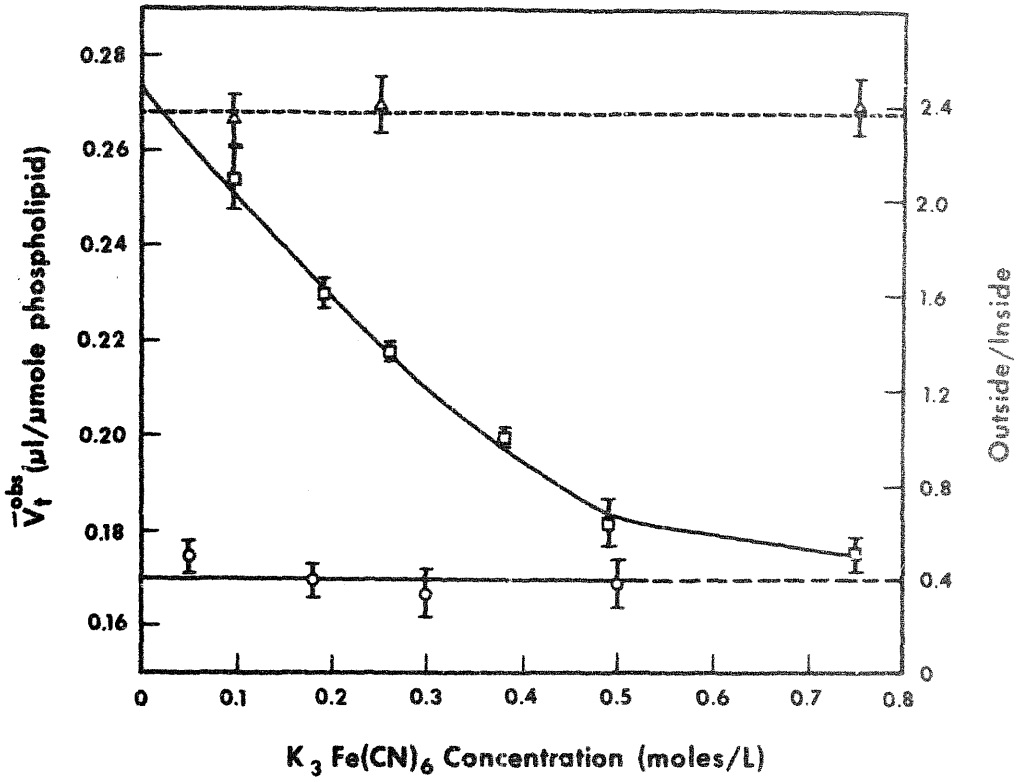


Fig. 1. Left ordinate: the dependence of trapped volume estimates for egg PC vesicles on the concentrations of $K_3Fe(CN)_6$, (□), and glucose (○) in the trapped buffer. Right ordinate: outer monolayer to inner monolayer mass ratio, as determined by proton magnetic resonance, (△)

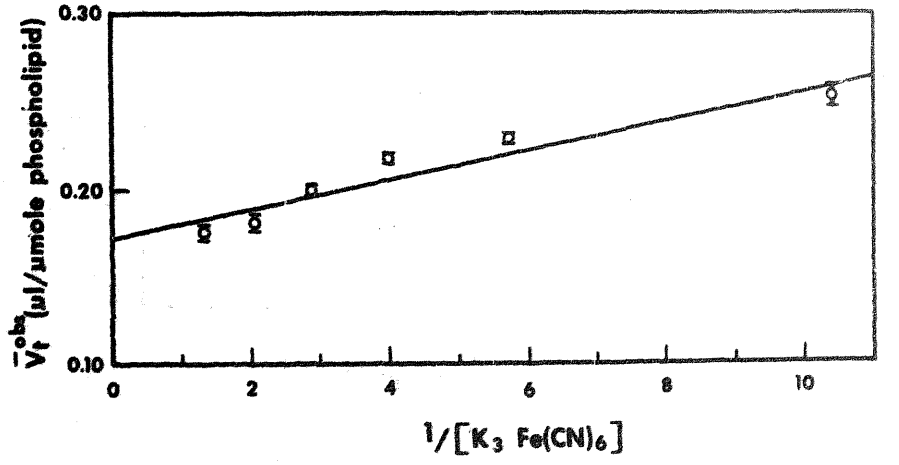


Fig. 2. Evidence for the binding of $Fe(CN_6)^{3-}$ to egg PC vesicles, according to the relation: $V_t^{obs} = V_t^0 + K/[Fe(CN)_6^{3-}]$.

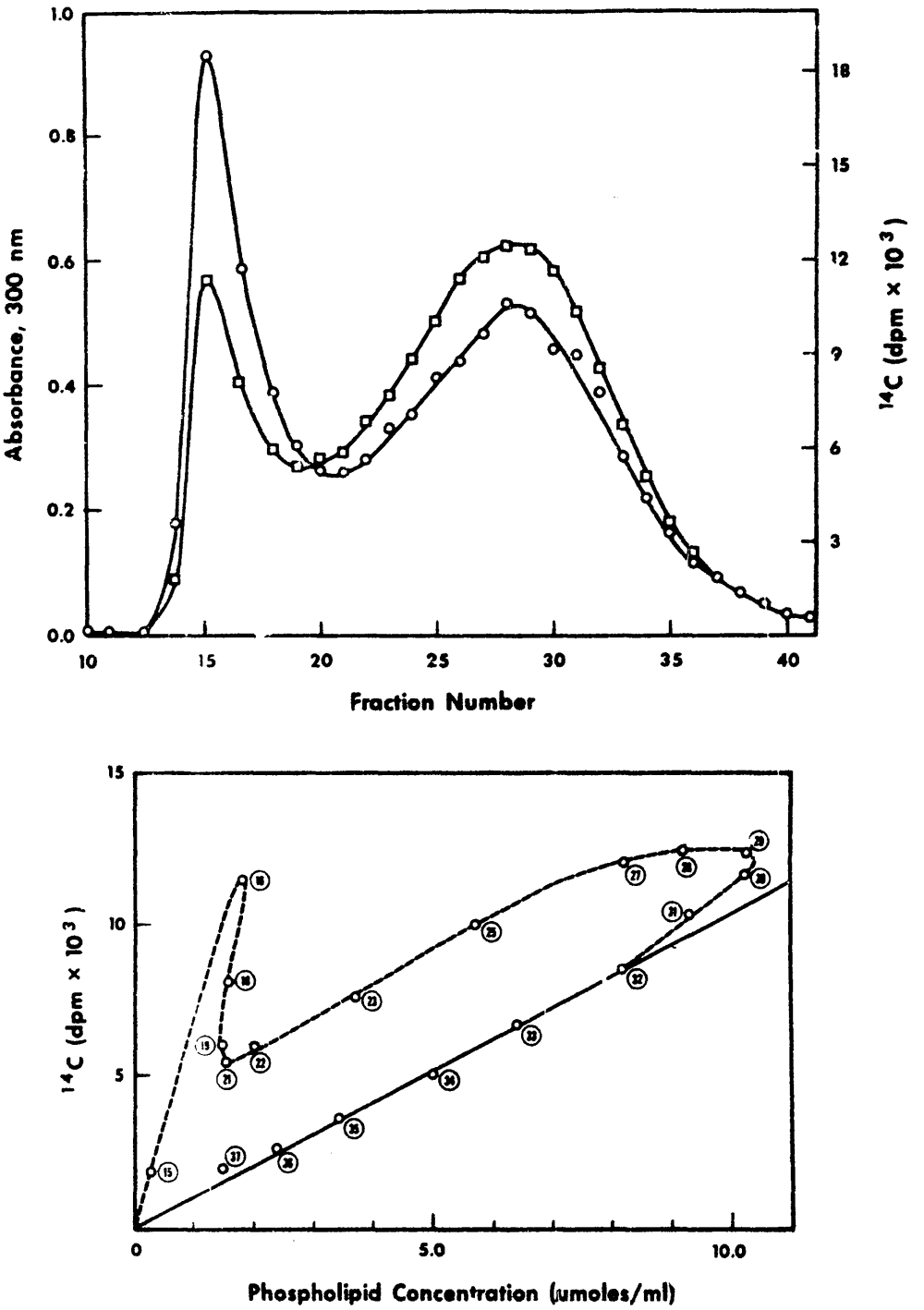


Fig. 3. A: elution profile of DBrPC vesicles from a Sepharose 4B column. Vesicles were prepared in buffer containing [^{14}C]glucose. Absorbance at 300 nm, (○); [^{14}C]glucose, (□). B: plot of [^{14}C]glucose versus phospholipid concentration.

that the ferricyanide ion might be binding to the egg phosphatidylcholine vesicle according to the equation:

$$[Fe(CN)_6^{3-}]_{bound} = K[\text{phospholipid}] \quad (3)$$

Such binding, independent of ferricyanide concentration, results in values for the trapped, solute-available volume that vary with ferricyanide concentration according to:

$$\bar{V}_t^{obs} = V_t^0 + K/[Fe(CN)_6^{3-}] \quad (4)$$

where $\bar{V}_t^{obs}$ is the observed trapped volume, and $\bar{V}_t^o$ is the trapped volume that would be obtained in the absence of binding (i.e., 0.170 μl/μmol, as obtained in the glucose trapping experiments). $\bar{V}_t^{obs}$ is plotted against reciprocal ferricyanide concentration in fig. 2, and the data is seen to fit reasonably to a straight line. Linear regression analysis gave a slope, K, of 0.85 mol ferricyanide bound/mol phospholipid and an intercept ($\bar{V}_t^o$) of 0.172 μl/μmol, in excellent agreement with $\bar{V}_t^o$ obtained from glucose trapping.

The fit of the data to eq. 4 and the reasonable value of $\bar{V}_t^o$ obtained strongly suggest that a substantial number of ferricyanide ions bind to the phosphatidylcholine vesicles. Thus, ferricyanide trapping would seem an inappropriate means of determining the solute-available volume of phospholipid vesicles unless a curve such as in fig. 2 were derived for every vesicle species studied. For this reason, glucose trapping was used to measure the trapped volumes of the synthetic phosphatidylcholine vesicles.

In fig. 3A, the elution profile of DBrPC vesicles from a Sepharose 4B column is plotted using turbidity at 300 nm and ^{14}C dpm to monitor the column fractions. The shape of the elution profile is very similar to that obtained with egg phosphatidylcholine (Huang [12]). In fig. 3B, the trapped glucose concentration (as ^{14}C dpm) in each fraction is replotted versus the phospholipid concentration of the fraction. In this plot, a straight line, which passes through the origin, has been drawn through the points corresponding to fractions in that portion of the elution profile containing vesicles of uniform size. The slope of this line is directly related to the trapped volume.

B. Bilayer microviscosity measurements

Arrhenius plots of the temperature dependence of bilayer microviscosity within DBrPC and DMePC vesicle bilayers are shown in fig. 4A and B, respectively. By way of comparison, fig. 4C contains a similar plot for egg yolk phosphatidylcholine. The fact that these curves are essentially linear over the major portion of the temperature range studied indicates that no gel–liquid crystalline phase transition takes place in these vesicles in the temperature range of 5–50°C (Lentz et al. [21]). In addition, the data in table 2 compare the microviscosities of the phosphatidylcholine vesicles under study here with those in two phosphatidylcholine vesicles that

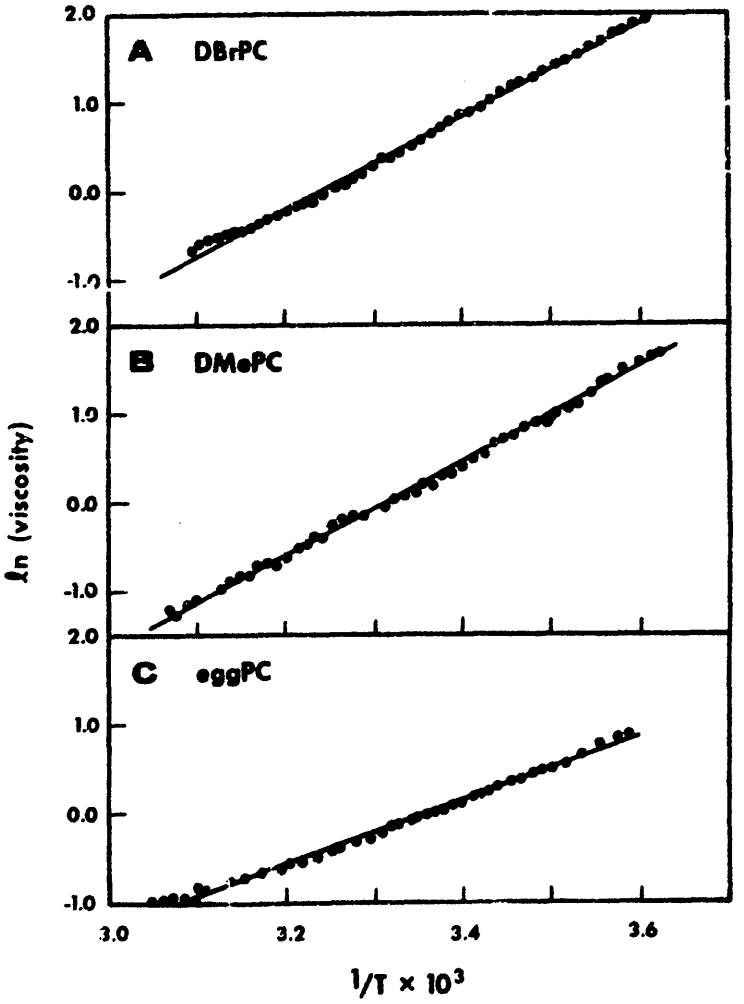


Fig. 4. Arrhenius plots of bilayer microviscosity, as determined by depolarization of fluorescence of diphenylhexatriene for vesicles of DBrPC, A; DMePC, B; egg PC, C.

are known to undergo phase transitions in this temperature range, dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine (Lentz et al. [21]). From the values of the microviscosity at low temperature and from the activation energy (ΔH^*) of the microviscosity, it should be clear that small, single-walled vesicles of DBrPC and DMePC are below the gel–liquid crystalline phase transition temperature in the range of 5–50°C. An earlier study has obtained a microviscosity Arrhenius plot for small POPC vesicles which was linear down to 4°C (Lentz et al. [21]), but this study also suggested that a phase transition might occur at slightly lower temperatures. In the range of 5–50°C, however, all three synthetic phosphatidylcholines under consideration here formed small, single-walled vesicles with fluid, liquid-crystalline bilayers, as did egg phosphatidylcholine.

Table 2
Microviscosities within the bilayers of several synthetic phosphatidylcholine vesicles as compared to egg phosphatidylcholine vesicles.

Phosphatidylcholine	Microviscosity (7°C) (poise)	Microviscosity (45°C) (poise)	ΔH^* viscosity (Kcal/mol)
Egg yolk	2.3	0.47	7.5 ± 0.1
1,2-di(10-bromostearoyl)	6.0	0.64	10.5 ± 0.1
1,2-di(10-methylstearoyl)	4.2	0.41	10.2 ± 0.1
1-palmitoyl-2-oleyl[a]	2.9	0.35	9.6 ± 0.1
1,2-dipalmitoyl[a]	44.0	0.69	13.3 ± 0.4[b]
			3.0 ± 0.6[c]
1,2-dimyristoyl[a]	42.1	0.38	13.1 ± 0.2[b]
			1.8 ± 1.8[c]

[a] From the data of Lentz et al. [21].
[b] Above the gel–liquid crystalline phase transition.
[c] Below the gel–liquid crystalline phase transition.

C Separation of DBrPC and DMePC vesicles

As noted in the Introduction, one motivation for this work was to develop similar, synthetic phosphatidylcholine vesicle systems which, despite their similarity, could be separated one from the other. The data in table 1 suggests that separation of DBrPC and DMePC vesicles could indeed be accomplished by centrifugation techniques. In order to test this, we prepared two populations of vesicles, one containing DBrPC with 2 mol% [^{3}H] egg phosphatidylcholine (prepared after Aneja and Chadha [22] from methyl [^{3}H] choline chloride obtained from Amersham-Searle, batch 13), and the other containing DMePC with 1.6 mol % methyl [^{13}C] egg phosphatidylcholine in DMePC. The two vesicle populations were prepared as described above, but were not chromatographed before use, and, for this reason, each contained a heterogeneous mixture of small vesicles and some larger species. Equal molar quantities of these two vesicle populations were mixed into a total volume of 5.1 ml of D_2O–H_2O (69:32, mol/mol) buffer containing 0.05 M KCl. This mixture was centrifuged for 17.5 hr at 40,000 rpm in a Beckman SW50 rotor. Fractions were collected from the top using a 20-gauge syringe needle and a peristaltic pump. The 11 equal fractions were counted in a Beckman LS-233 liquid scintillation counter using the cocktail described above. The ^{3}H and ^{14}C activities of each sample were determined by standard ratio-method quenching correction techniques using the fixed Beckman ^{14}C, ^{3}H, and 1/2 ^{3}H windows. The results are shown in fig. 5 as concentration of DBrPC and DMePC in the various fractions removed from the centrifuge tube versus fraction number. This figure clearly demonstrates that vesicles prepared from these two synthetic lipids can be easily separated from each other.

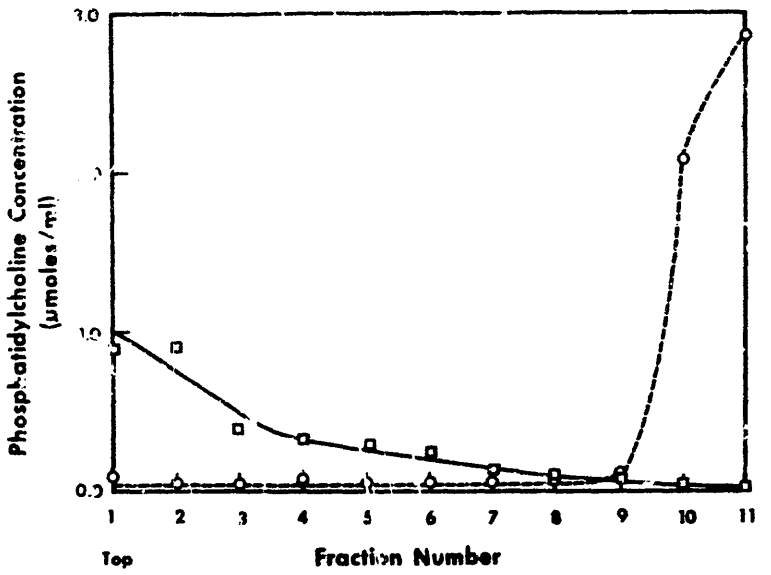


Fig. 5. Separation of DBrPC and DMePC vesicles by centrifugation. DBrPC, (○); DMePC, (□).

The smearing of the DMePC peak near the top of the centrifuge tube was probably caused by mixing of the first few fractions during the unloading process.

V. Discussion

As indicated in the Introduction, it would be extremely useful to have available vesicles of phospholipid which differ in density from those of the more customary phospholipids. We have shown that DBrPC does indeed form a population of small, stable, vesicles of uniform size having a density approximately 10% greater than vesicles of egg PC; The $S_{w,20}$ value for DBrPC is approximately 15 times that of egg PC. Another important finding is that the lipid is in a fluid state between 5 and 50°C so that the physical properties of the bilayer should be reasonably similar to that of natural phospholipids. DMePC similarly forms a population of small, stable vesicles of uniform size, having a fluid hydrocarbon region between 5 and 50°C. The density of these vesicles is slightly less than that of water so that flotation, rather than sedimentation, occurs upon centrifugation.

We may predict several potential uses for these vesicles. The DBrPC vesicles would be valuable in studying intervesicle exchange phenomena, or as a reference phase from which the partitioning of various water insoluble membrane components, such as cholesterol, between vesicles of naturally occuring lipids could be determined. Finally, DBrPC vesicles might be fused with plasma membranes of cells, enabling them to be fractionated more readily from other cellular membranes. The DMePC could also be made useful in nuclear magnetic resonance studies by

incorporating C^{13} methyl groups into the 10 position.

It should be pointed out, however, that although the physical properties of the vesicles formed from these lipids are similar to those of the more customary lipids, the hydrodynamic data in table 1 indicate that differences do exist, as shown by the following treatment. We know that the molecular weight (or number of molecules per vesicle), and trapped volume of the well-characterized egg PC vesicles are most consistent with a spherical particle having an anhydrous bilayer thickness of 46 Å and 3 Å layer of hydration on each side of the bilayer. This can be calculated by simple geometry. The volume of the anhydrous bilayer, V_b, is given by $4/3 \P (r_0^3 - r_1^3)$; the number of lipid molecules per vesicle is then V_b times the packing density of the lipid, and the trapped volume per vesicle, in A^3, is given by $4/3 \P (r_i - 3)^3$. If we now consider vesicles of DBrPC, it is clear that the experimentally determined values of the Stokes' radius, number of molecules per vesicle, N, and trapped volume do not correspond to a spherical vesicle having the bilayer thickness and/or hydration of egg PC. Specifically, V_t and N are much too large for a spherical vesicle with a Stokes radius of 115 Å. The discrepancies are well beyond experimental error; in order to accommodate about 4000 lipid molecules, a vesicle would have to have a Stokes radius of approximately 135 Å and a trapped volume of 0.362. Furthermore, it is not possible to fit both V_t and N to a spherical particle by assuming different values for the bilayer thickness and hydration. Therefore, we must conclude that vesicles of DBrPC are not spherical. Similar calculations show that the vesicles cannot be ellipsoidal either. Although we have not tried all possible shapes, it is possible to fit the results to a cylindrical particle. The dimensions of one such particle are as follows: height, 125 Å: radius, 156 Å: anhydrous bilayer thickness, 35 Å. A vesicle of these dimensions would contain 3775 lipid molecules and have a trapped volume of 0.257. It is not unreasonable to expect that molecules of DBrPC would have to pack in a rather unusual way to accommodate the bulky bromine groups which are located at uniform depth throughout the bilayer.

Vesicles of DMePC also appear to be non-spherical, although the deviation is not as large as that found for vesicles of DBrPC. The radius and molecular weight are consistent with a sphere, but the trapped volume is low. The results are consistent with a prolate ellipsoid having major and minor axes of 146 and 106 Å and an anhydrous bilayer thickness of 46 Å. Of course, other structures are possible.

The results with POPC show clearly that vesicles of this lipid are nearly identical to those of egg PC with respect to size, shape, and bilayer fluidity. We feel that this lipid is an excellent model for naturally occurring phospholipids. The advantage of using POPC instead of lecithins isolated from a natural source is that vesicles of POPC contain only a single phospholipid component. This will enable rigorous studies of the fine structure and physical properties of the bilayer to be made. In addition, the interaction of a natural phospholipid with other lipids and membrane components can be studied in a simple and well-defined system.

References

[1] E. Cubero Robles and D. Van den Berg, Biochim. Biophys. Acta 187 (1969) 520
[2] D.G. Chasin and E.G. Perkins, Chem. Phys. Lipids 6 (1971) 8
[3] R.G. Jones, J. Am. Chem. Soc. 69 (1947) 2350
[4] J. Cason, Chem. Rev. 40 (1947) 15
[5] W.J. Baumann and H.K. Mangold, J. Org. Chem. 29 (1964) 3055
[6] W.J. Baumann, R.D. Gee, T.H. Madson and H.K. Mangold, Chem. Phys. Lipids 9 (1972) 87
[7] C.F. Fox, J.H. Law, N. Tsukagoshi and G. Wilson, Proc. Nat. Acad. Sci. USA 67 (1970) 598
[8] Z. Selinger and Y. Lapidot, J. Lipid Res. 7 (1966) 174
[9] J.S. Chadha, Chem. Phys. Lipids 4 (1970) 104
[10] J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 497
[11] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466
[12] C. Huang, Biochemistry 8 (1969) 344
[13] A. Ehrenberg, Acta Chem. Scand. 11 (1957) 1257
[14] H. Svensson and T.E. Thompson, in: Analytical Methods of Protein Chemistry, Vol. 3, P. Alexander and R.J. Block (Eds.), Pergamon, New York, 1961, p. 58
[15] E.F. Cassassa and H. Eisenberg, J. Phys. Chem. 65 (1961) 427
[16] G.C. Newman and C. Huang, Biochemistry 14 (1975) 3163
[17] C. Huang, J.P. Sipe, S.T. Chow and R.B. Martin, Proc. Nat. Acad. Sci. USA 71 (1974) 359
[18] D. Chapman and A. Morrison, J. Biol. Chem. 241 (1966) 5044
[19] M. Shinitzky, A.C. Dianoux, C. Gitler and G. Weber, Biochemistry 10 (1971) 2106
[20] M. Shinitzky and Y. Barenholz, J. Biol. Chem. 249 (1974) 2625
[21] B.R. Lentz, Y. Barenholz and T.E. Thompson, Biochemistry 15 (1976) 2441
[22] R. Aneja and J.S. Chadha, Biochim. Biophys. Acta 248 (1971) 455