A SPIN-LABEL STUDY OF FRACTIONATED EGG PHOSPHATIDYL CHOLINE VESICLES

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SUMMARY

The 5-keto stearic acid spin label has been used to investigate differences in phospholipid bilayer organization of the various vesicular structures separated by fractionation of sonicated egg phosphatidyl choline suspensions. The vesicles have been characterised at a macroscopic level using ultracentrifugation and electron microscopy. The order parameters of the homogeneous, single bilayer vesicle fraction is found to be smaller than for the larger, multi-lamellar vesicles, corresponding to a more fluid bilayer environment. Systematic variations throughout the fractions are attributed to varying admixtures of the inhomogeneous vesicles. Similar effects are found with vesicles whose molecular order has been perturbed by addition of small quantities of cholesterol or lysolecithin.

Sonicated phosphatidyl choline (PC) dispersions have been intensively studied as models for the phospholipid bilayer component of biological membranes (1). To this end, Huang (2) has been able to separate the sonication products of aqueous egg PC dispersions by gel filtration chromatography. Characterisation of the fractionated material by analytical gel filtration, ultracentrifugation and electron microscopy (2) has shown two major components (a) large, multilamellar vesicles of varying sizes and (b) a homogeneous fraction of small, single-bilayer vesicles with a diameter of about 250Å. However, little evidence is available for differences at a molecular level between the various vesicular structures.

This work presents the results of a spin label study on fractionated egg PC vesicles undertaken in an attempt to discover any differences in the phospholipid bilayer environment between the various fractions. The nitroxide oxazolidine derivative of 5-keto stearic acid was used in this study because it has previously been shown to be sensitively dependent on bilayer structure

(3, 4). Preparations have also been investigated which contain small quantities of cholesterol and of lyso phosphatidyl choline, which are expected to perturb (5, 6) the structure of phosphatidyl choline bilayers. The fractions investigated have been characterised by sedimentation velocity ultracentrifugation and freeze etch and negative staining electron microscopy as a check on vesicle size, structure and homogeneity.

MATERIALS AND METHOD

P.C. was isolated from egg yolk by column chromatography (2) and stored as a chloroform solution in sealed ampoules at -20°C. Immediately before use the P.C. was rechromatographed on a silicic acid column to eliminate any storage degradation products.

The spin label, 5-spiro [2'-(N-oxyl-4'-4'-dimethyloxazolidine)] stearic acid was obtained from Syva Co., Palo Alto, Calif. Cholesterol (B.D.H., Poole, U.K.) was twice recrystallized from methanol. Lysolecithin was obtained from Lipid Products, Epsom, U.K. and found to be pure by thin layer chromatography on silica gel. Approximately 150 mgs of rechromatographed P.C. was mixed with 1% by weight of spin label in chloroform solution. Lysolecithin or cholesterol, if included, were added at this point. Sonication and Sepharose 4B column chromatography of a buffered dispersion of the lipid was performed according to the scheme of Huang and Charlton (7) except that the column eluate was monitored at 257nm on an LKB Uvicord instead of at 300nm. Appropriate fractions (see FIGURE 1) were combined and vacuum dialysed to a concentration of approximately 20mM as determined by phosphate assay (8). The same samples were used for electron paramagnetic resonance (epr) study as for ultracentrifuge and electron microscopic investigation. E.p.r. spectra were run at 20.0°C on a Decca X1 9 GH_z spectrometer the magnetic field sweep of which had been calibrated using a proton resonance probe. The sedimentation velocity experiments were carried out at 20.0°C in a Beckman Model E analytical ultracentrifuge equipped with schlieren optics and using a synthetic boundary cell. Samples for electron microscopic examination were

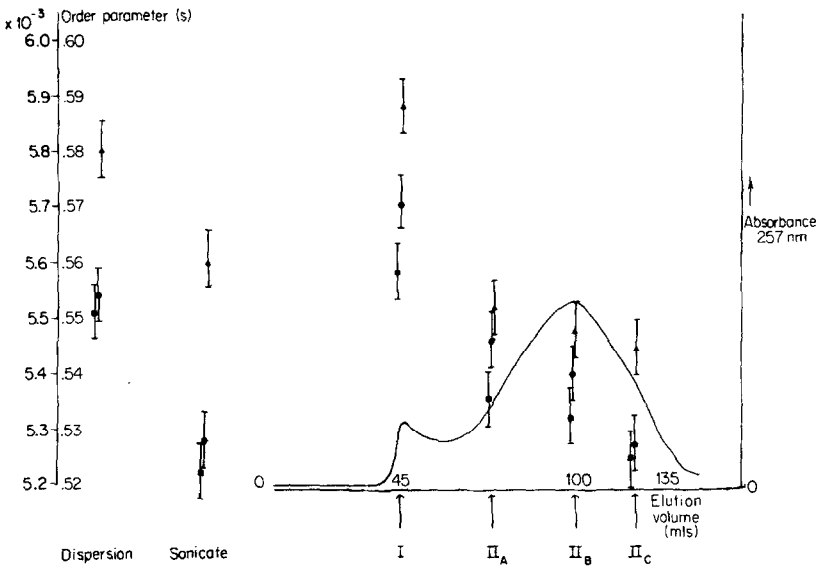

FIGURE 1

Typical elution diagram of sonicated PC dispersion. The spin label order parameters of the various fractions are indicated on the same elution coordinate.

□ PC ● PC + 4% lyso PC Δ PC + 10% cholesterol

negatively stained with 2% potassium phosphotungstate solution buffered with ammonium acetate to pH 6.5. The grids were examined immediately after drying. Freeze etch specimens were cleaved after rapid freezing to -160°C and subsequently etched for 20 seconds.

RESULTS AND DISCUSSION

A typical elution diagram for a phosphatidyl choline sonicate fractionated on Sepharose-4B is given in FIGURE 1, and is similar to that reported by Huang (2). The elution pattern is composed of two main fractions (Fraction I and II), corresponding to the large multilamellar structures and the smaller diameter vesicles respectively. The results of Huang et al. (2, 7) indicate that the fractions beyond the maximum of peak II show a linear relation between absorbance at 300nm and inorganic phosphate concentration. These are the homogeneous suspensions of single bilayer vesicles.

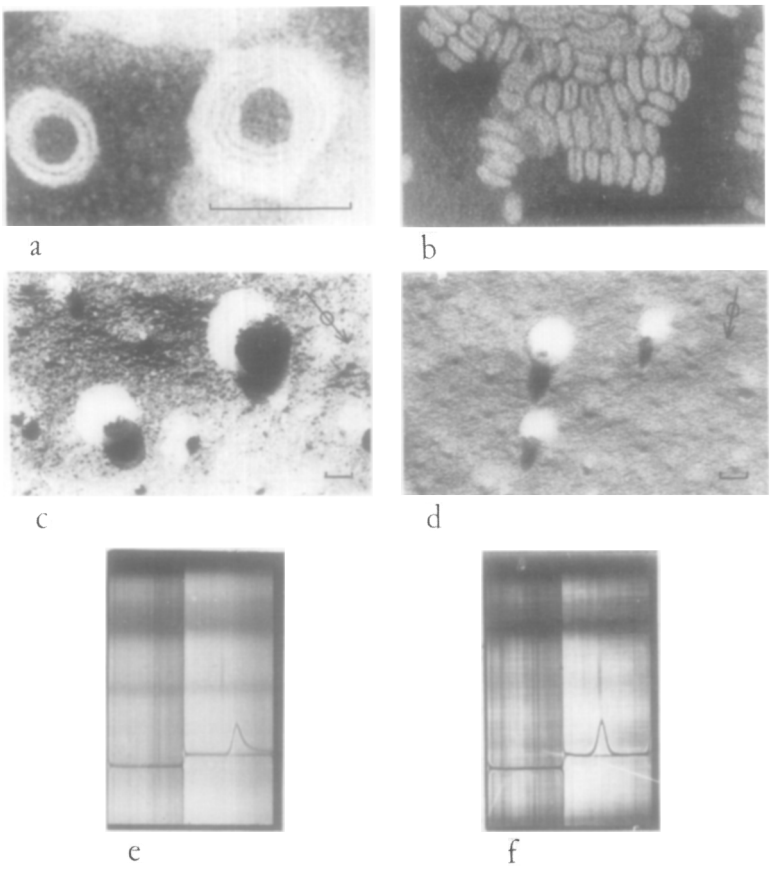

FIGURE 2

Electron microscopic and ultracentrifuge characterisation of the vesicle fractions. Dimension markers represent 500Å.

(a) and (b) Electron micrographs from negatively stained samples of Fraction I and IIb.

(c) and (d) Freeze etched electron micrographs of Fractions I and IIb.

(e) and (f) Sedimentation velocity ultracentrifuge schlieren peaks from Fraction I and IIb.

(Speed 48,000 exposures taken after 28 minutes).

FIGURE 2 shows some of the results of the electron microscope and ultracentrifuge characterisation of the vesicle fractions. FIGURE 2a, b shows the electron micrographs of negatively stained samples from the peaks of fractions I and II respectively. FIGURE 2a reveals multilamellar vesicles

and various other structures, of a highly inhomogeneous nature. FIGURE 2b shows the presence of much smaller single bilayer vesicles, and indicates a much more homogeneous distribution than FIGURE 2a. The vesicles are flattened in appearance which may be due to sample preparation (9). FIGURES 2c, d show the freeze etch electron micrographs from fraction I and IIB respectively. Fraction I contains mainly multilamellar species of varying size, the smallest being approximately 500Å diameter. Fraction II is essentially homogeneous and is composed of spherical vesicles, diameter 250Å. FIGURES 2e, f are typical ultracentrifuge schlieren peaks from sedimentation velocity experiments on Fractions I and IIB respectively. The asymmetry of the peak in FIGURE 2e qualitatively shows Fraction I to be inhomogeneous. An $S^{o}_{20,w}$ of 6.4 S was determined for Fraction I. By contrast the peak in FIGURE 2f is symmetrical showing that Fraction IIB is essentially homogeneous. An $S^{o}_{20,w}$ of 2.8 S was determined for this fraction which is in reasonable agreement with the value of 2.1 S reported by Huang (2).

The above evidence confirms that the present sonication and gel filtration procedure has produced fractionated vesicles similar to those described by Huang (2). The Fractions I, II_A, II_B, II_C investigated by epr are indicated in FIGURE 1. A typical epr spectrum is shown in FIGURE 3.

A convenient measure of the amplitude of anisotropic motion (and hence degree of orientation) is afforded by the spin probe order parameter (3, 4):

$$S = \frac{A_{11} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \qquad (1)$$

A_{11}, $A_{\perp}$ are the hyperfine splittings parallel and perpendicular to the phospholipid bilayer normal, and are measured as indicated in FIGURE 3.

A_{zz}, $\frac{1}{2}(A_{xx} + A_{yy})$ are the values of A_{11}, and $A_{\perp}$ corresponding to complete order within the bilayer, and are deduced from the single crystal splittings of 2-Doxylpropane (10). Polarity and $A_{\perp}$ corrections have been made according to reference 3.

The S parameters of the various fractions are plotted relative to the

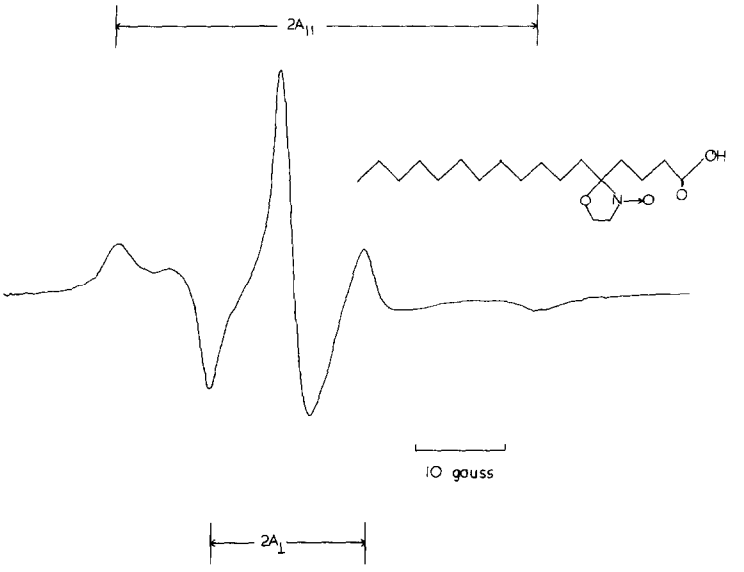

FIGURE 3

E.p.r. spectrum from 5-keto acid spin label in the homogeneous single bilayer vesicles (Fraction II C).

elution deagram in FIGURE 1. Also included are the order parameters of the crude phospholipid dispersion before sonication and the crude sonicate before fractionation, as well as the parallel data for preparations containing lysolecithin and cholesterol.

Significant differences in order parameter are found on fractionation. The order parameter of Fraction I is considerably higher than Fraction II, and small differences are found within the broad peak of Fraction II which show a systematic trend with elution volume. The results are reproducable with identical sonication conditions. Lower sonication powers produce a greater proportion of Fraction I (as shown by the absorbance at 257nm) and larger S-parameters for the earlier parts of Fraction II. The small differences within the peak of Fraction II can thus be interpreted as contamination by Fraction I. The S-parameter of Fraction II_C is found to be relatively insensitive to sonication conditions which is consistent with the homogeneity of this fraction.

A real difference between the molecular environments of the phospholipid bilayer in homogeneous single bilayer vesicles and in the larger multibilayer vesicles is revealed by these experiments. This difference corresponds to a more fluid environment in the single bilayer with larger amplitude motion of the spin probe. According to the restricted random walk model of Jost et al (10), the change in S-parameter between Fractions I and II corresponds to changes of approximately 2° - 3° in the amplitude of anisotropic motion. These are relatively small changes, as might be expected since the difference in bilayers occurs at a macroscopic rather than molecular level.

There are two possible explanations for the difference observed at the molecular level. The first involves interbilayer interactions in the multilamellar vesicles of Fraction I and the second involves a packing effect caused by the tight radius of curvature of the single bilayer vesicles of Fraction II. The first explanation seems less likely since it would involve interactions between headgroups of adjacent bilayers across an aqueous phase. Packing of the terminal ends of the phospholipid chains could require a more open structure in the outer surface of the single bilayer vesicles than in the larger vesicles of Fraction I. This would explain the larger motional amplitude observed for the single bilayer vesicles, since the outer half-bilayer accounts for 70% of the total phospholipid (250Å diam.). Clearly care must be taken in the interpretation of small changes in molecular motion in unfractionated phospholipid dispersions since this depends on the state and size of the phospholipid vesicles which in turn depends on sonication conditions.

Addition of 10% cholesterol causes a pronounced increase in S-parameter and of 4% lysoPC an increase which lies almost within experimental error. In both cases the trends in S-parameter on sonication and fractionation are the same as for pure egg PC. The ordering effect of cholesterol reported here is consistent with results obtained on other bilayer systems. The lysoPC results suggest that the marked changes in bilayer conductivity caused by small

additions of this phospholipid (B) are not paralleled by increased fatty acid side chain mobility as monitored by the spin label probe used. This conclusion is considered to be significant since the experiments of Huang & Charlton (7) which show that no detectable lysoPC is produced from PC during vesicle preparations have been confirmed in our laboratory (11).

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