VARIATIONS OF SIZE AND DISTRIBUTION IN SUSPENSIONS OF SONICATED PHOSPHOLIPID BILAYERS

S.B. Andrews*, Robert M. Hoffman and Adam Borison

Section of Cytology, Yale University School of Medicine, New Haven, Connecticut 06510

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SUMMARY

Agarose gel partition chromatography conditions are reported which allow the separation of a range of unilaminar particles from a sonicated suspension of phosphatidyl choline. Column fractionation, radiolabeling, NMR and electron microscopy show that the unilaminar population is heterogeneous in size. The distribution is unimodal, but somewhat asymmetrical, with a broader range to larger particle diameters. The distribution is estimated to encompass vesicles differing in radius by at least 100 Å. It is also shown that populations enriched in vesicles of a chosen size range may be prepared by column fractionation methods. These results are reconciled with the previous literature on vesicle size distributions.

Studies of small, unilaminar, phospholipid dispersions, i.e., vesicles, continue to provide insight into the role of the lipid bilayer in membranous structures. However, a fundamental disagreement exists concerning the characterization of such sonicated preparations. Huang, Thompson and coworkers have presented evidence that their vesicle preparations are homogeneous with respect to size (1-4); others have supported this conclusion (5). Nevertheless, some workers have reported their results while remaining noncommital on this point (6,7), while others are clearly skeptical (8,9). The possible existence of a size distribution in such dispersions is an important matter, since the interpretation of potentially revealing experiments, such as those demonstrating a distributional asymmetry in mixed-lipid systems (5-7,10), is dependent upon the extent to which a size distribution might be present.

The present report concerns a series of straightforward experiments which clearly demonstrate a substantial size inhomogeneity for certain unimodal, unfractionated, phosphatidyl choline vesicle preparations, and we attempt to reconcile these data with reports that would imply otherwise.

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*To whom correspondence should be addressed.
Abbreviation used: NMR, nuclear magnetic resonance

METHODS AND MATERIALS -- Egg yolk phosphatidyl choline was prepared from fresh eggs by the method of Papahadjopoulos & Miller (11), or purchased from Koch-Light (cat. no. 3431t) and rechromatographed on silicic acid. The experiments reported below were carried out on a preparation from fresh eggs. [N-methyl-^{14}C]-phosphatidyl choline was synthesized according to Stoffel (12). Careful thin-layer analysis (before and after sonication) indicated that all preparations were substantially pure, although traces of lysolecithin and possibly phosphatidic acid were always detectable. Sonicated phospholipid dispersions were obtained as previously described (13), as were NMR spectra in the presence of Pr^{+3} ion. Analytical gel partition chromatography was performed on a Sepharose 2B column (2.6 x 60.0 cm), pre-equilibrated with phospholipid, using upward flow and a peristaltic pump to afford constant flow. Column eluent was monitored with a null-detecting differential refractometer (Waters R-403). The response of this detector was shown to be precisely proportional to the concentration of lipid phosphorus. Column void (96.3 ml) and total (318.0 ml) volumes were determined by elution of Dextran Blue 2000 and multilayered liposomes, and deuterium oxide, respectively. These values were reproducible to $\pm$3 ml (0.03 K_d units), as were elution volumes for standard proteins, viruses and vesicle suspensions. Elution times and fraction numbers were of similar precision. The buffer utilized throughout was 0.15 M KCl, 2 mM Tris·HCl, pH 7.2, or the D_2O equivalent. Concentration and dialysis of vesicle aliquots was performed with an Amicon ultrafiltration apparatus (UM-10 membrane).

RESULTS -- Fig. 1A illustrates the refractive index trace of a normal, unfractionated vesicle preparation. The long leading edge of the profile is invariably present, and the vesicles detected here are not contaminated with multilayered structures, which are separated to the baseline from this population by ca. 80 ml. Fig. 2A is a similar trace, but of four times the amplitude. Fractions to the left of the left arrow in Fig. 2A were pooled, concentrated and rechromatographed to give the left trace of Fig. 2B; an analogous procedure indexed at the right arrow of Fig. 2A gave the right profile of Fig. 2B. The vesicle populations present in these two fractions are clearly of significantly different size distribution. Further manipulations support this conclusion. The summation of the two traces in Fig. 2B gives Fig. 2C; concentration and admixture of the effluent from both runs depicted in 2B yields curve 2D. Figs. 2C and 2D are indistinguishable. Further, recombination of all of the original components by addition of the intermediate fractions of 2A (between the arrows) to the total lipid effluent of 2D returns the original monotonic profile, since 1B is similar to 1A (or 2A).

The heterogeneity of these preparations may be demonstrated in another way. Two similar preparations, one of which was radiolabeled using [N-methyl-^{14}C]-phosphatidyl choline, gave identical traces, Fig. 1A. The radioactive effluent was pooled to the right of the right arrowhead (1A); conversely, the unlabeled effluent was pooled to the left of the left arrowhead. Partition of a mixture of these dispersions gives the refractometer trace shown in Fig. 1C (solid line), which is not markedly different from either original profile (1A). However, the radioactivity profile of 1C is narrower and shifted to the right (1C, dotted line). Indeed, it is indistinguishable from the refractive index and

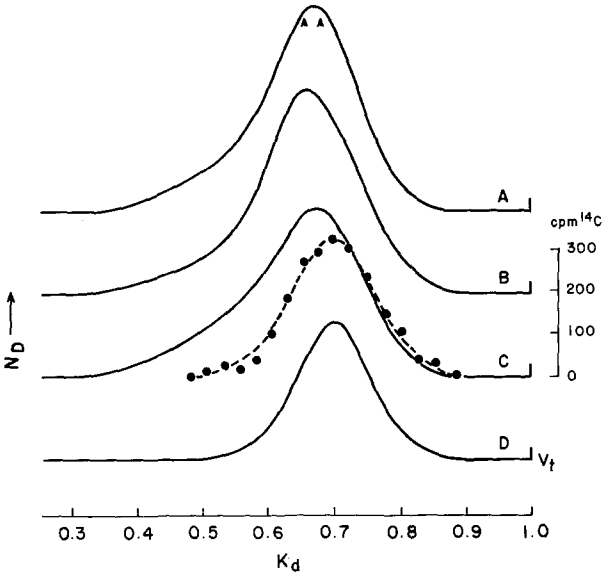

Fig. 1. Sepharose 2B gel partition profiles; change in refractive index (arbitrary units) vs. partition coefficient. (A) Typical unfractionated dispersion. (B) Result of the fractionation of Fig. 2A into three portions at the arrows, and recombination. (C) Solid line - result of the fractionation of a normal preparation at the left arrowhead in Fig. 1A, and of a radioactive dispersion at the right arrowhead, and recombination; dotted line - radioactivity profile of the same mixture. (D) Chromatogram of a portion of the radioactive suspension cut at the right arrowhead. The radioactivity and refractive index traces were coincident. Arrowheads represent the points of fractionation to obtain Figs. 1C & 1D.

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radioactivity trace of the initially pooled radiolabeled fractions, Fig. 1D.

NMR spectroscopy provides corroboration of the size differential implied by the shifts of gel partition maxima. Fig. 3 illustrates the resolved choline methyl resonances of topologically outer and inner phospholipids for suspensions similar to those in Fig. 2B. The resolution was obtained through the agency of praesiodymium ion (13,14). The integrated areas of inside and outside choline resonances for the large vesicle population are substantially different from those of the smaller population. The measured outer/inner ratio is 1.43 for Fig. 3A and 2.33 for Fig. 3B.

DISCUSSION -- The results of the column fractionation experiments are inconsistent with a homogeneous vesicle population, for had the distribution been homogeneous, the manipulations carried out would have produced consistently the same trace. In fact, the profiles are very different, indicating substantial heterogeneity. The mean size disparity between fractions shown in

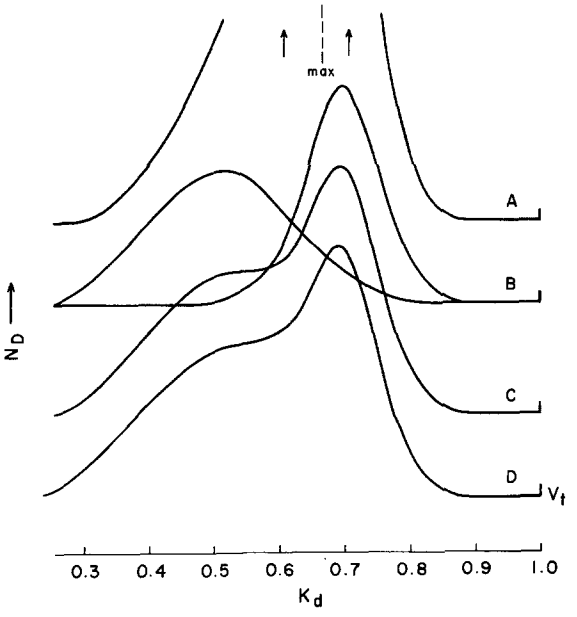

Fig. 2. Cf. legend to Fig. 1. (A) Typical unfractionated preparation, amplitude 4X. (B) Left - profile of portion fractionated and pooled at the left arrow in 2A; right - profile of aliquot obtained by cutting and pooling at the right arrow. (C) Calculated sum of the traces in Fig. 2B. (D) Experimental trace of the mixture of the two populations represented in Fig. 2B. Arrows indicate the points of fractionation. The maximum of the unfractionated dispersion is shown.

Fig. 2B has been verified by electron microscopy, using phosphotungstic acid negative stain (not shown). The smaller fraction shows a relatively narrow distribution, with a maximum frequency at ca. 225 Å radius, while the larger vesicle fraction evidences a much broader distribution with a poorly defined frequency maximum of 150-200 Å radius. NMR experiments further support this interpretation. The spectra in Fig. 3 are shown primarily to illustrate the large differences in area ratios possible in extreme cases. In systems such as bilayers, where the lines are very broad for high-resolution spectra and considerable overlap occurs, integration is to some extent a subjective technique, because it is dependent upon where the baseline is chosen. This introduces some uncertainty into the values reported by different laboratories, and also makes small differences between adjacent fractions of questionable significance. Nevertheless, the spectra shown in Fig. 3 are clearly very different, and integration of them gives an indication of the large mean size difference between the populations they represent. Assuming a bilayer thickness of 36 Å

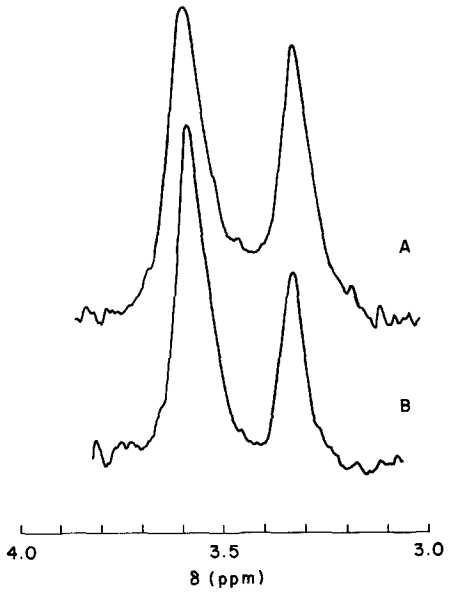

Fig. 3. ^1H NMR spectra of the N-methyl choline region in the presence of ~1 mM Pr^{+3} ion (13). The downfield and upfield resonances correspond to the outer and inner phospholipids, respectively. (A) Spectrum from a population similar to that in Fig. 2B, left. (B) Spectrum corresponding to Fig. 2B, right.

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(15), the calculated average radii of the populations in Figs. 3A & 3B are 220 Å and 105 Å, respectively. Other factors, in addition to integration errors, render this method imprecise; in particular, the calculation is biased in favor of larger vesicles. Therefore, the values given should be considered to be estimates of the upper limit of the true number average.

Two factors were crucial to the success of these experiments. Firstly, the use of Sepharose 2B allowed the entire population to be sieved within the useful range of the gel; specifically, larger components of the distribution were well-separated from multilayers. Previous fractionation experiments have usually employed Sepharose 4B (1,5,8), which either restricts workers to the small vesicle portion or results in contamination with multilayered liposomes. (Cf. Ref. 17 for an exception.) Secondly, the vesicle stability required to perform these experiments did not occur in all cases. Five different lecithin preparations were used in these experiments. Both lipid preparations from fresh eggs and one commercial preparation gave results as indicated. However, two commercial preparations (Koch-Light) failed to behave as described, but rather always gave fractions which chromatographed at K_d=0.67 regardless of where original cuts were made. We have not been able to ascertain any chemical

difference between these preparations: All were slightly contaminated (∿1%) as indicated in METHODS.

It is necessary to discuss the results of these experiments in the light of previously published data on vesicle size distributions. Our experiments are not directly incompatible with the previous literature. Most of the data implying homogeneity come from experiments performed on fractionated vesicle preparations. In particular, zone-broadening analysis of sedimentation velocity experiments (10) convincingly supports the conclusion that the distribution is narrow in these cases. The fractionated preparations utilized by Huang, Thompson and coworkers should be similar to that shown in Fig. 2B, right. By careful column calibration and diffusion-broadening analysis[†] we also find that this pool of vesicles must have a very narrow size distribution. The experiments of Berden, et al. (5) are difficult to compare with ours since these authors give few parameters for their column conditions. However, some of their data (Fig. 4, Ref. 5) show trends consistent with our results. Finally, it should be noted that certain criteria taken to imply homogeneity may be interpreted in other ways. Measurements of P_i/turbidity ratios (2) are likely to be insensitive measures of distribution for small vesicles, since the wavelength of scattered light used (∿3000 Å) is greater than ten times the diameter of the particles (∿220 Å). Further, observation of a Gaussian Schlieren pattern in sedimentation experiments may not necessarily imply homogeneity if the vesicle distribution itself approximates Gaussian. Indeed, Johnson (Fig. 2, Ref. 16) has reported Schlieren photographs from vesicle sedimentation experiments which, with their marked leading edges, are strikingly similar to our comparable gel profiles (Fig. 1A). Hauser and Irons have previously reported evidence for inhomogeneity in somewhat different vesicle dispersions (8). These authors used exhaustively purified lipid, and sonicated for short times to avoid degradation. While in qualitative agreement, it is difficult to extrapolate their conclusions to vesicle suspensions such as ours, since longer sonication times and minor impurities are known to change the characteristics of these preparations. Most workers find it impossibly inconvenient to work with analytically pure lipids; further, samples are usually sonicated "to completion." Thus, we suspect that our preparations more closely resemble the usual situation.

The above considerations lead to some important conclusions concerning the interpretation of data obtained from vesicle systems. Quantitative analysis of experimental data which reveal an asymmetry of bilayer components (5-7,10) must consider the breadth of the size distribution. However, the assumption of a homogeneous population does not invalidate the qualitative conclusion that sig-

[†]S.B. Andrews and J.W. Faller, to be published.

nificant asymmetry exists in mixed-lipid systems studied to date. Similarly, studies of lipid-lipid interactions as a function of vesicle size change are potentially more revealing if the distributions involved are considered (17). It is unfortunate that such considerations are required, since no straightforward method is available to assess the extent of inhomogeneity. Further, it will likely prove difficult to isolate fractions with distributions sufficiently narrow to assume homogeneity, since in the agarose fractionation method employed diffusion broadening is severe. The Huang-Thompson preparations (and other populations of very small mean size) may be the only exception to this generalization; this may reflect the high energy increment required to increase the radius of curvature when the vesicles are very small.

A potentially useful implication of this study is the possibility of exploring compositionally identical structures of differing sizes and distributions. The expectation that certain fundamental properties of bilayers, e.g., rates of translational diffusion or ionic permeabilities, may be a function of curvature could conceivably be approached in this way. We have found† that it is possible to derive a description of the distribution of unfractionated, monotonic vesicle preparations, and to predict the results of fractionation experiments, by computer modeling methods. Refinement of these techniques should allow experiments conducted on heterogeneous but defined populations to be treated quantitatively.

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