Biochimica et Biophysica Acta, 468 (1977) 451—460

BBA 77767

BINDING OF URANYL TO PHOSPHATIDYLCHOLINE LIPOSOMES

LIPOSOME AGGREGATION EFFECT ON SURFACE AREA

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(Received January 25th, 1977)

Summary

The binding of uranyl ion, UO_2^{2+}, to egg phosphatidylcholine liposomes was studied as a potential method for the determination of liposome surface areas. Unbound uranyl was determined spectrophotometrically as the Arsenazo III complex with centrifuge supernatant. There is an apparent positive cooperativity in uranyl binding at phosphatidylcholine concentrations above approx. 0.1 mM. The binding capacity per mol increases upon liposome dilution. The data are consistent with liposomes existing in a highly aggregated state. The binding constant in the limit of low concentration of bound uranyl was $(9 \pm 3) \cdot 10^6$ M^{-1} in 0.1 M NaCl, pH 4.1. At saturation about four uranyl ions are bound per 100 phosphatidylcholine molecules. Relative surface areas of different dispersions may be calculated from intercepts of extrapolated binding isotherms, and absolute surface areas may be calculated if a value for the uranyl-phosphatidylcholine stoichiometry is assumed.

Introduction

As models of biological membranes, liposomes have the major disadvantage that it is apparently impossible to form dispersions with reproducible particle size distributions or surface areas [1] and no convenient method is available for determining either relative or absolute surface areas of dispersions. Furthermore, we have found that even using the largest-pore gel filtration media commercially available, liposomes cannot be separated into different size fractions (Chen, C.-Y., unpublished observations). Huang [2] has shown that gel filtration of sonicated liposomes yields a homogeneous fraction of small, single bilayered vesicles. Such preparations have subsequently been intensively stud-

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ied by many groups. Also a preparative procedure not involving sonication and its danger of degradation [3] has been developed by Batzri and Korn [4]. Although some studies [5,6] indicate there are no important differences between the vesicle and liposome bilayers, there is also considerable evidence [7—11] that the small radius of curvature in the vesicles ($d \simeq 30$ nm) results in molecular packing that is significantly different than in the more nearly planar liposome membranes.

Since the membranes of the multilayered liposomes may be more realistic models of cell membranes and since the liposomes are much simpler to prepare, we have tried to develop procedures for overcoming the disadvantages of the liposome system. The purpose of this study was to develop a convenient method for the determination of the surface area of a liposome dispersion. Our approach was to determine the binding isotherm for the binding of uranyl (UO_2^{2+}) to phosphatidylcholine liposomes and thereby the amount of uranyl needed to saturate the surface of the liposome dispersion.

The only other published method for determining the surface area of liposomes was developed in Bangham's laboratory [12] and also utilized the strong binding of uranyl to phosphatidylcholine. This method, which is perhaps too tedious for routine use as an adjunct to liposome experiments, has only been used by Bangham and co-workers [12—14].

Materials and Methods

Hen egg chosphatidylcholine was purified by the method of Singleton et al. [15]. Uranyl nitrate was Fisher Scientific Co., A.C.S. Certified grade, lot 725176. Arsenazo III was purchased from Aldrich Chemical Co. and used without further purification.

Absorbance measurements were made in a Hitachi Model 191 spectrophotometer using 0.5, 1.0, 4.0, and 10.0 cm path cells.

Centrifugation was done in 50-ml plastic tubes in Sorvall SS-1 Superspeed Angle Centrifuges in a cold room at 4° C.

Preparation of liposomes. Liposomes were prepared in large test tubes by drying the chloroform/methanol solvent under a stream of nitrogen while rotating the tube so as to yield a thin shell of phosphatidylcholine. The phosphatidylcholine was dispersed by adding 0.1 M NaCl (pH 4.1, unbuffered) and mixing at high speed for 1 min on a vortex mixer. Liposomes containing stearylamine were formed by adding a methanol solution of stearylamine hydrochloride to the phosphatidylcholine before the nitrogen evaporation step. All liposomes were initially prepared as 1.6 mM phosphatidylcholine and then diluted as necessary.

Phosphatidylcholine uranyl mixtures were prepared by adding 1.00 or 0.10 mM uranyl nitrate (in 0.1 M NaCl, pH 4.1) to 5.0 ml of liposomes and bringing to a total volume of 6.0 ml with 0.1 M NaCl, pH 4.1. The mixtures were centrifuged at 4° C and 13 500 $\times g$ for 1 h. 1- or 2-ml aliquots of the supernatant were withdrawn for spectrophotometric determination of the free uranyl. It was necessary to do the centrifugation in the cold since the liposomes in the presence of uranyl would not satisfactorily centrifuge down at room temperature. All experiments were performed on the same day the liposomes were

prepared. The amount of uranyl bound to the liposomes was independent of time for up to at least 23 h. This was true both if the liposomes were aged and then mixed with uranyl or if the liposome-uranyl mixture was aged.

Spectrophotometric determination of uranyl. The free uranyl was determined spectrophotometrically as the complex with Arsenazo III (*o*-(1,8-dihydroxy-3, 6-disulfonaphthalene-2,7-bisazo)-bisbenzene arsonic acid) [16]. 1 or 2 ml of centrifuge supernatant were added to 0.5—2.0 ml of $2.5 \cdot 10^{-4}$ M Arsenazo III in 0.2 M HSO_4^- buffer at pH 1.9. A molar excess of Arsenazo III was maintained. The absorbance at 652 nm was read against a blank prepared from liposomes with no added uranyl. Standard solutions gave linear Beers Law plots up to 40 μM uranyl with a molar absorptivity of $5.1 \cdot 10^4$ $1 \cdot cm^{-1} \cdot mol^{-1}$. Interference of Ca^{2+} at a concentration equimolar to the uranyl was shown to be negligible.

Results and Discussion

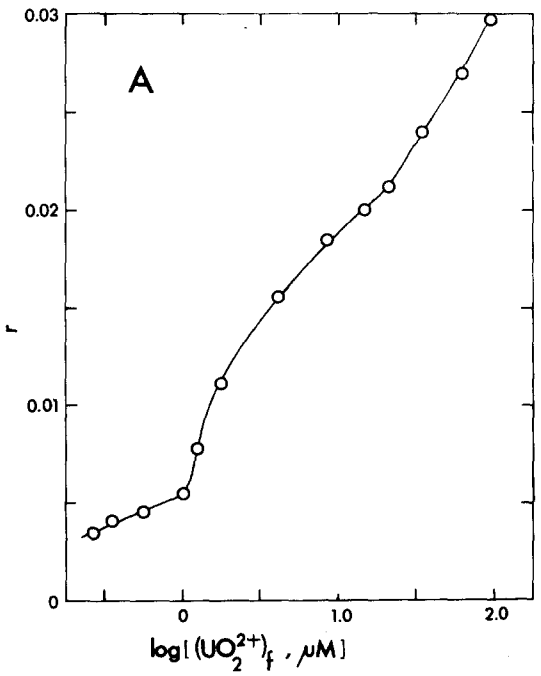
The complex binding isotherm at millimolar phosphatidylcholine

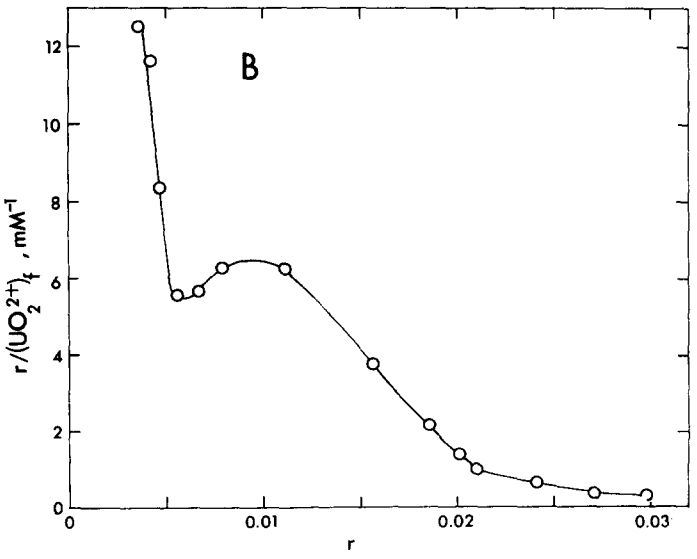
Since the binding isotherm for uranyl in 1.34 mM phosphatidylcholine liposomes is rather complex, it is plotted in three different ways in Fig. 1. The binding ratio, r, is the molar ratio of bound uranyl to total phosphatidylcholine in the dispersion. This differs from the fractional saturation by an unknown factor since we know neither the binding stoichiometry nor the fraction of phosphatidylcholine molecules which are on the outermost surface of the liposomes and available for binding. The subscripts f and b on (UO_2^{2+}) indicate free and bound uranyl concentrations, respectively. Fig. 1A shows the standard saturation binding curve [17]. The sigmoidal portion of the plot suggests cooperative binding or multiple binding sites [18]. The complex nature of the binding is clearly shown and probably most readily interpreted by Fig. 1B where the data are plotted according to Scatchard [19]. In a Scatchard plot, a straight line with negative slope indicates simple binding to one class of independent binding sites; a maximum, or convex upward plot indicates positive cooperativity; a hyperbolic curve convex toward the origin is consistent with either negative cooperativity or multiple classes of binding sites [20]. It appears then that uranyl binds to phosphatidylcholine liposomes initially (low r) as though there were single independent binding sites. This is followed by a region of apparent positive cooperative binding which is in turn followed by a region of either negatively cooperative binding or binding to an additional class of binding site. An initial linear region is expected even for interacting sites since at low enough uranyl concentration occupied sites would be too widely separated to interact. Also negative cooperativity at high uranyl is expected due to increasing charge repulsion. Fig. 1C shows the data plotted according to the Langmuir adsorption isotherm. For a single class of independent sites a positive-sloped straight line is predicted. This plot emphasizes the apparent simple binding to effectively independent sites in the limit of low uranyl concentration.

Effect of dilution on the binding isotherm

The Scatchard plots in Fig. 2 show that the region of positive cooperativity

disappears upon dilution of the liposomes. Furthermore the curves are not superimposed whcih suggests either a direct dilution effect on the liposomes, non-binding impurities interfering with the spectrophotometric analysis, or an activity coefficient effect [21]. The latter possibility can probably be ruled out since all solutions were a constant 0.1 M NaCl. In regards to the second possibility, we showed that one of the most likely impurities, Ca^{2+}, did not interfere under our conditions. Also the same shift in the binding curve was ob-





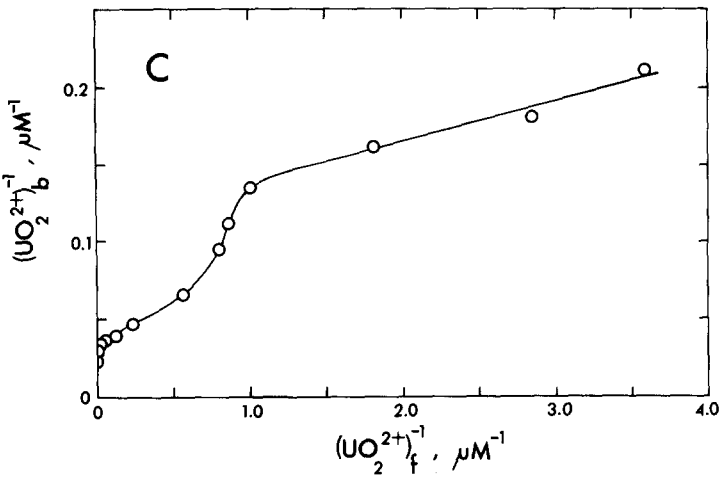


Fig. 1. Binding isotherm for uranyl to 1.34 mM phosphatidylcholine plotted in three different ways. Symbols are defined and experimental details are given in the text. (A) Binding ratio versus $\log(UO_2^{2+})_f$. (B) Scatchard plot. (C) Langmuir plot.

served when the rhodamine B analysis for uranyl was used; the rhodamine B analysis has been shown to be insensitive to a large number of inorganic cations [22].

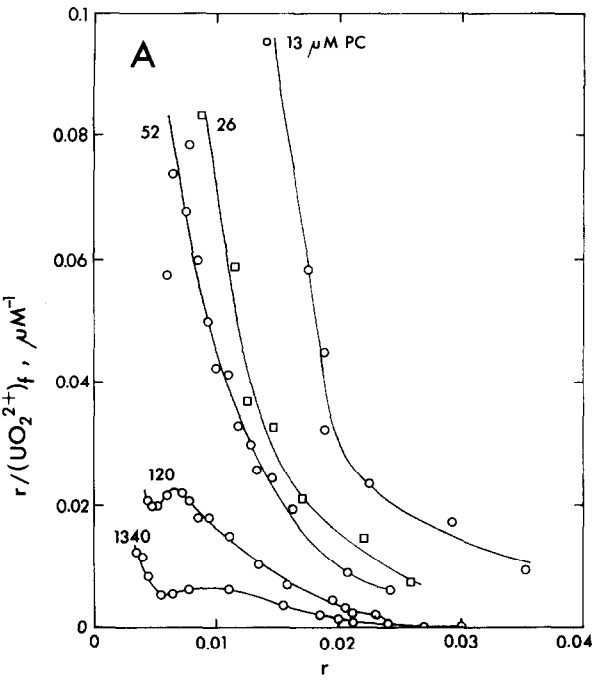
We interpret these data as follows. The binding curve shift upon dilution is due to the existence of aggregates of liposomes which disaggregate upon dilution thereby exposing more surface to binding. The region of apparent positive cooperativity at high phosphatidylcholine concentration is due to disaggregation caused by charge repulsion as the exposed surfaces of the aggregates become increasingly charged with uranyl. As the aggregates break up, new surface is made available for binding. The continued shift in the binding isotherm indicates that significant aggregation of the liposomes exists down to at least 13 μM phosphatidylcholine.

An attempt was made to test the aggregation hypothesis by incorporating stearylamine · HCl into the liposomes to give liposomes with an initial net positive charge. When the stearylamine concentration was greater than 4 mol % the liposomes could not be centrifugated down. Liposomes containing 4 mol % stearylamine yielded a Scatchard plot similar to Fig. 1B except the initial downward sloping portion was missing. Apparently the surface charge due to 4% stearylamine, while not sufficient to disrupt aggregates, destablizes the aggregates enough so that disaggregation commences upon binding of the first uranyl ions.

Determination of the binding constant

Langmuir binding isotherms for 1.34 and 0.052 mM phosphatidylcholine in the limit of low uranyl are shown in Fig. 3. The linear portions confirm that simple binding occurs in the low uranyl limit.

The binding constant was determined to be $1.1 \cdot 10^7$ M^{-1} and $7.4 \cdot 10^6$ M^{-1} in 1.34 and 0.052 mM phosphatidylcholine, respectively. These values are judged to be within experimental error of each other and indicate that the



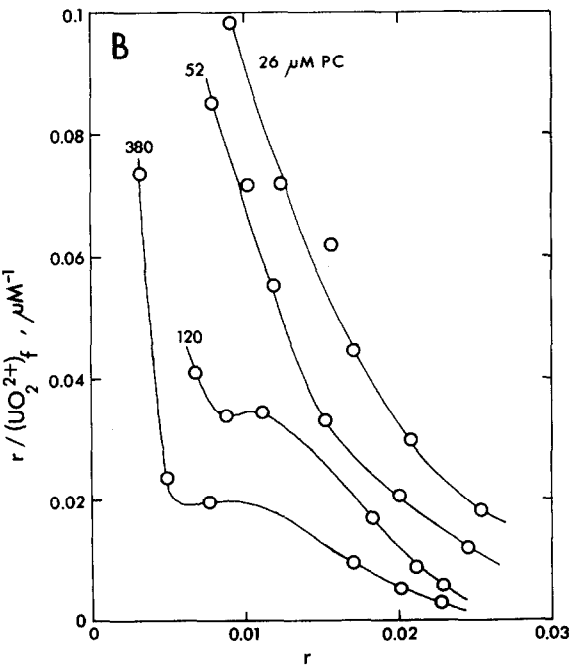


Fig. 2. Scatchard plots at various phosphatidylcholine concentrations. The phosphatidylcholine concentration in micromolarity is shown next to each curve. (A) Samples prepared from dilutions of different 1.6 mM dispersions. (B) Samples prepared as dilutions of the same 1.6 mM dispersion and ran on the same day.

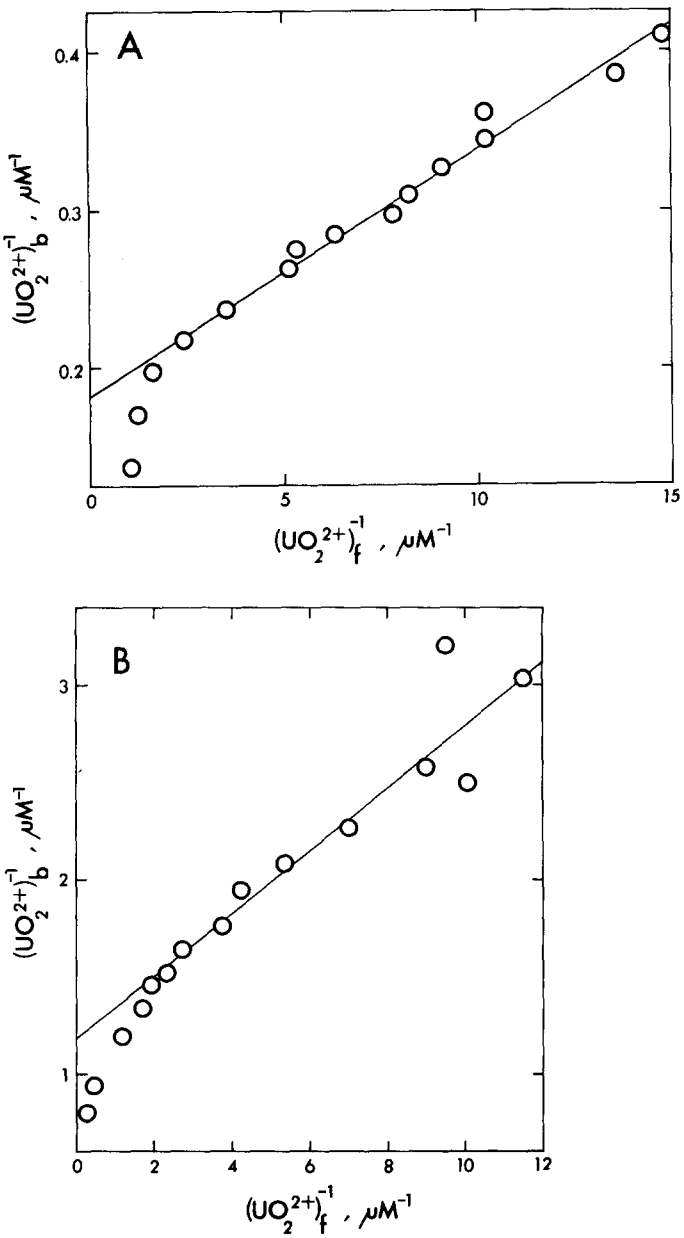


Fig. 3. Langmuir plots for determination of the binding constant and relative effective surface areas in the limit of low uranyl concentration. (A) 1.34 mM phosphatidylcholine (B) 0.052 mM phosphatidylcholine.

nature of the initial uranyl binding is the same in both aggregated and disaggregated liposomes.

Calculation of relative surface areas

The y-intercept of the linear portion of the Langmuir plots in Fig. 3 predicts the amount of uranyl that would be needed to saturate the surface, $(UO_2^{2+})_{sat}$

if liposome disaggregation, binding site interaction, and any other complications did not set in. The ratio of $(UO_2^{2+})_{sat}$ to total phosphatidylcholine concentration can be called r_{sat} and is related by an (unknown) stoichiometric factor to the fraction of phosphatidylcholine molecules initially exposed to binding. Values of r_{sat} were 0.0042 and 0.016 for 1.34 and 0.052 mM phosphatidylcholine, respectively. We interpret this difference as due to a factor of 3.8 greater effective surface area (per mol of phosphatidylcholine) in the less aggregated 0.052 mM dispersion.

The x-intercept of a Scatchard plot is r_{sat} in the limit of high uranyl concentration and complete liposome disaggregation. The results shown in Fig. 4 indicate that saturation occurs with about four uranyl ions bound per 100 phosphatidylcholine molecules, or $r_{sat} = 0.04$. A separate plot of these data (not shown) as r vs. $\log(UO_2^{2+})_f$ also indicates saturation at $r \simeq 0.04$ both by a plateau at high (UO_2^{2+}) and by an inflection at $r \simeq 0.02$ (ref. 23). Comparing the values of r_{sat} from Figs. 3 and 4 suggests that about 60% and 90% of the liposome surface area in 0.052 and 1.34 mM phosphatidylcholine dispersions, respectively, is hidden by aggregation.

Calculation of absolute surface areas

The surface area of a dispersion can be calculated from r_{sat} if the stoichiometry of the uranyl-phosphatidylcholine interaction is known. No published value is available, however other workers [24,25] have assumed 1 : 1 stoichiometry in studying a variety of other metal ion-phospholipid complexes. 1 : 1 stoichiometry has been established for Ca^{2+}, Mg^{2+} and Ce^{3+} complexes with phosphatidylcholine in anhydrous methanol [26]. Levine et al. [27] using

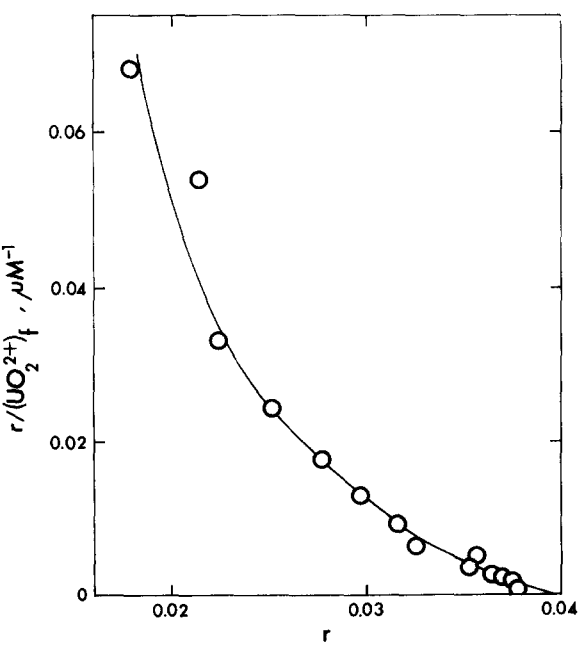


Fig. 4. Scatchard plot for determination of saturating uranyl concentration. Phosphatidylcholine concentration is 0.052 mM.

NMR have calculated there is a minimum of 7.5 phosphatidylcholine molecules per binding site for Eu^{3+} or Nd^{3+} binding to sonicated dipalmitoylphosphatidylcholine vesicles, but observed that uranyl has a greater affinity for phosphatidylcholine than either Eu^{3+} or Nd^{3+}.

If we assume 1 : 1 stoichiometry and use an area of 71.7 $Å^2$ per phosphatidylcholine molecule [28] the following surface areas are calculated for the three dispersions in Figs. 3 and 4: 18 and 69 $cm^2/\mu mol$ for the effective surface area of 1.34 and 0.052 mM phosphatidylcholine, respectively, 172 $cm^2/\mu mol$ total surface area of 0.052 mM phosphatidylcholine after complete disaggregation.

The only numbers available for comparison are the data of Bangham et al. [12]. For liposomes of pure egg yolk phosphatidylcholine in 0.145 M KCl a range of 156—276 $cm^2/\mu mol$ was reported. Since the uranyl and phosphatidylcholine concentrations were not given, we cannot estimate the extent of aggregation expected under their experimental conditions. We do note however, that the assumption of Bangham et al. [14] that the amount of free uranyl is negligible does not seem to be justified.

Other evidence for aggregation in liposome systems

That liposomes undergo extensive aggregation should not be surprising in light of the common observation that liposome dispersions are not stable indefinitely but precipitate upon standing for periods varying from hours to days. Liposome aggregation is evident by both light [29] and electron microscopy [30,31] and has been invoked to explain results from temperature jump [32] and turbidity [33] studies.

Since other laboratories have reported using a Coulter Counter to determine particle size distributions in liposomes [34,35] we attempted to verify the aggregation phenomenon by this method. Using a 70 μm aperture no significant differences in particle size distribution were indicated by the instrument even when differences in aggregation were clearly apparent to the eye. We conclude that the aggregates, which are probably about the size of the aperture, are broken up by the shear forces upon passing through the aperture.

Conclusions

The binding data presented here are consistent with extensive aggregation in phosphatidylcholine dispersions. Since the extent of aggregation is sensitive to perturbations such as ion binding, incorporation of charged amphipaths, and dilution, experiments in which it is important for the liposome surface area to remain constant must be designed carefully so as not to perturb the aggregation. Relative effective surface areas of (aggregated) phosphatidylcholine liposome dispersions can be determined by suitable extrapolation of the uranyl binding isotherms. The reliable determination of absolute surface areas by this method awaits a definite evaluation of the uranyl-phosphatidylcholine stoichiometry, although reasonable values of surface areas are obtained when 1 : 1 stoichiometry is assumed.

Acknowledgements

Support from U.S. National Science Foundation Grant EPP-75-04639 and a Research Corporation Cottrell Research Grant is gratefully acknowledged.

Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the partial support of this research. We thank Dr. Norman Ho, Pharmacy Department, University of Michigan for his kind assistance in use of the Coulter Counter.

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