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Studies on the Phase Transition in the Single Lamellar Liposomes I. Preparation of Uniformly Sized Phosphatidylcholine Liposomes in Different Molecular Weight Regions

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SUMMARY

Uniformly sized phosphatidylcholine liposome dispersions are prepared in different molecular weights ranging from 2×10^6 to 3×10^7 daltons by means of molecular sieve chromatography on a Sepharose 2B gel. A linear relationship between their partition coefficients on the gel, K_{av}, and log M_w determined by sedimentation equilibrium has been obtained. The relationship was found to hold both in the L-α-dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylcholine (egg PC) liposomes. In connection with the molecular weight determination, the partial specific volume of DPPC in the liposome dispersion was obtained as a function of temperature around its transition point, 25°C-48°C.

INTRODUCTION

Phospholipid molecules have quite low critical concentrations in aqueous phase to form liposomes, a typical lyotropic liquid crystal composed of spherical multi or single lipid bilayers. Because of their significance in

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connection with the structure and function of biological membranes, the nature of the physical state of phospholipids has been extensively studied. A particularly interesting feature in the bilayer systems is the thermotropic fluid to solid phase transition with regards to the configuration of the acyl chains of lipid molecule. The phase transition in the multilamellar liposomes has been characterized by a highly cooperative transition.[1,2] However, a change of a phospholipid from multilamellar to single lamellar structure results in the alternation of a number of properties such as decrease in the enthalpy change associated with the transition by a factor of 0.5[3] and decrease in the cooperativity of the transition.[4] These differences between the multi and single lamellar structures might be interpreted in terms of the interbilayer interactions within a liposome, which are expected only in the multilamellar liposomes. Therefore, for the purpose of the investigation of the phase transition dependent properties of biological membranes, the single lamellar liposomes are preferable to the multilamellar liposomes as a model.

As will be shown in part II of this series, the transition behaviour of the single lamellar liposomes strongly depends on the liposome size. Although similar size effects have been suggested on verious properties of the single lamellar liposomes,[3,5,6,7,8] the quantitative investigation has never been carried out, mainly because the preparation of well defined liposomes in size was not established over wide range of the size.

In this paper, we describe the preparation of homogeneously sized liposomes of phosphatidylcholine in different molecular weights, as a preliminary report of our present work on the phase transition in the single lamellar liposomes. For the convenience of the molecular weight determination of the liposomes, a relationship between the molecular weight and K_{av} on Sepharose 2B gel are presented.

EXPERIMENTAL

Materials

L-α-dipalmitoylphosphatidylcholine (DPPC) purchased from Calbiochem Co., tris(hydroxymethyl)aminomethane and KCl from Wako Pure Chemicals Ltd. were used without further purification. Sepharose 2B and Sephadex G-50 molecular sieves were purchased from Pharmacia Fine Chemicals. Egg phosphatidylcholine (egg PC) was extracted from hen egg yolk and purified by the method of Pangborn.[9]

Preparation of phosphatidylcholine liposome dispersions

The liposome dispersions were prepared by sonication[10] and by injection of ethanolic phosphatidylcholine (PC) solution into a buffered solution accord-

ing to Batzri and Korn.[11,12] The preparation procedures of the liposomes are described mainly for DPPC, since it is essentially applicable to form the egg PC liposomes except temperature (mentioned in parentheses) and other minor alternations. (a) Sonication: Lyophilized DPPC (egg PC) in a buffered solution (50 mM KCl, 5mM tris-Cl at pH = 8.0) was sonicated at 20 kHz and at 45° C (4° C) under Ar atmosphere for 10 minutes or more upto 60 minutes. The dispersion thus obtained was centrifuged at 7,000 g for 10 minutes to remove undispersed phospholipids. 5 ml of the resulting supernatant was applied to molecular sieve chromatography on Sepharose 2B column (1.5 cm × 40 cm) at 40° C (4° C). The effluent was collected in 3 ml fractions and stored at 45° C (4° C) prior to use. (b) Ethanol method: An ethanol solution of 1.5 ml containing 100 mg DPPC was injected drop by drop into 50 ml of the buffered solution under vigorous stirring at 60° C, while for egg PC, 2 ml ethanol solution of 100 mg PC was injected into 50 ml of the buffered solution at 20° C. Undispersed phospholipids were removed by the centrifugation and the supernatant was concentrated to 5 ml by ultrafiltration (Amicon XM 100A membrane) with a gentle stirring under N_2 pressure at 45° C (4° C). In order to remove ethanol from the dispersion, it was applied to a Sephadex G-50 column (1.5 cm × 30 cm) which had been equilibrated with the buffered solution. 5 ml of the column effluent with highest liposome concentration was collected and applied to the Sepharose 2B chromatography in the same manner as case a.

Rechromatograph on Sepharose 2B

Several fractions of the first Sepharose 2B column effluent were rechromatographed on the same gel (1.5 cm × 40 cm) at 40° C and at flow rate of 10 ml/cm^2 hr. for the DPPC and egg PC liposomes. The elution profile was monitored by the optical density at 300 nm. The effluent was collected in 3 ml fractions.

Sedimentation equilibrium

The experiments of sedimentation equilibrium for DPPC (egg PC) liposomes were performed in the following procedures. A freshly obtained peak fraction of each second Sepharose 2B chromatograph was concentrated to a final concentration of about 2.5 mmole of lipid/liter of buffered solution at 45° C (4° C) and applied to sedimentation equilibrium experiments. The sedimentation equilibrium was carried out in a Hitachi ultracentrifuge (model UCA-1A type) using multichannel cell at 35° C (20° C) controlled by the RTIC unit. The rotor (RA60H type) was operated at 1,500-9,500 rpm (2,500-8,000 rpm) for

12–30 hrs to get equilibrium state, depending on the molecular weight of the samples. In the both cases, the initial PC concentration in the sample solutions was determined by the sedimentation velocity patterns obtained as a function of time.

Partial specific volume

Partial specific volume of DPPC suspended in the same buffered solution as used in the sedimentation equilibrium was measured using density meter (Paar DMA O2D) over the temperature range of 25-48° C with an accuracy of ±0.05° C.

RESULTS AND DISCUSSION

In Figure 1, typical examples of the first Sepharose 2B gel filtration profiles of DPPC liposomes prepared by the sonication and ethanol method are shown and compared with the rechromatographic profiles obtained for several fractions in the first filtration.

The size distribution of the "sonicated" liposomes mainly depends on the sonication period, while that of the "ethanol" liposomes depends on the injection temperature, the concentration of PC in ethanol and on the volume ratio of ethanol to the buffered solution. In our preparations of the "ethanol liposomes", particularly in the case of egg PC, it was essential to avoid the formation of a large amount of the multilayer liposomes that the concentration of PC in ethanol and the final volume ratio of ethanol to the buffered solution were withheld less than 0.1 mmol/ml and 0.04, respectively. The optimal injection temperature was found to be 60° C for DPPC and 20° C for egg PC. It seemed to us that ethanol method is suitable for the preparation of larger size liposomes, and the sonication method is rather suitable for smaller size liposomes.

As shown in Figure 1 and Figure 2, when the fractions eluted from the first column were directly applied to rechromatograph on Sepharose 2B at 40° C, the highly symmetric elution profiles were obtained whether it is DPPC or egg PC, unless the effluent of the parent fraction was not very close to the void volume of the column. This result indicates that the liposomes in each fraction of the first gel filtration were homogeneous in size.

In the case of DPPC, when the fractions from the first column were stored at 45° C for given periods and applied to the rechromatograph, change in the elution profile was not detectable within 3 hrs incubation, but it became detectable after the period, as the absorption at the void volume in the rechromatograph slightly increased with the incubation time. Apart from the

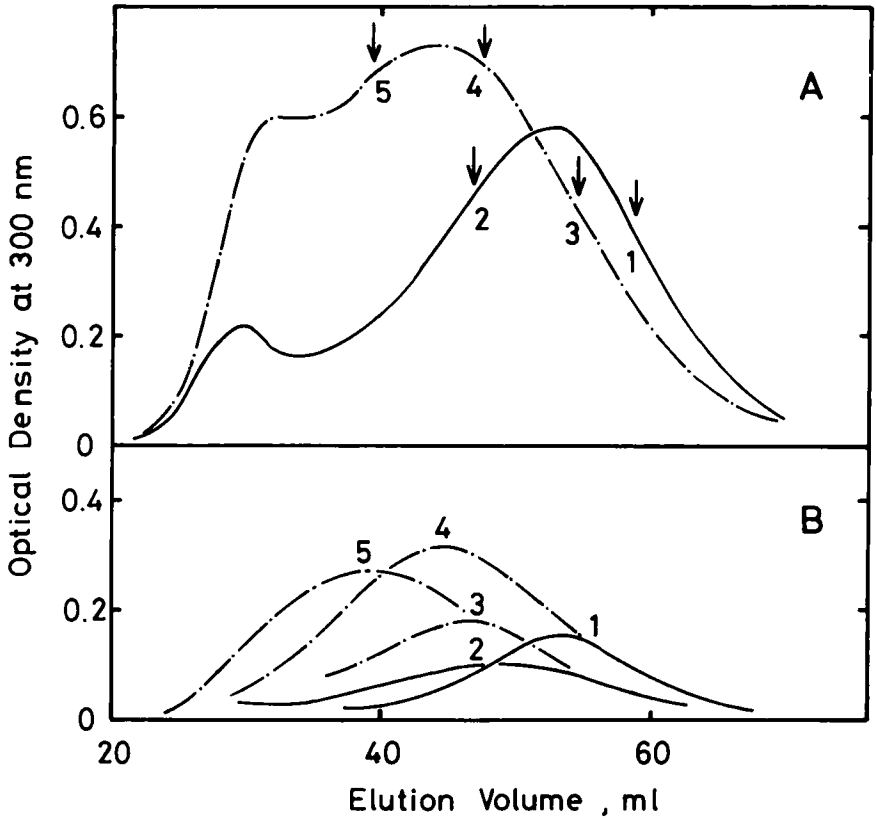

FIGURE 1 Elution profiles of the DPPC liposomes on Sepharose 2B column. A) First chromatograph ———; "sonicated" liposomes prepared by sonication for 10 min at 45°C, — — —; "ethanol" liposomes. The arrows designate the elution volume of the fractions submitted to rechromatograph. B) Rechromatograph Notations correspond to shown in A.

absorption at the void volume, the symmetric profile around the peak was maintained and the peak position did not shift. These results suggest that the aggregation of the liposomes during the incubation over 3 hrs results in the formation of large multilamellar liposome eluted in the void volume of the column. While, in the case of egg PC liposomes, no change in the elution profile was detectable up to 2 days, provided that the fractions of the first column were incubated at 4° C under Ar atmosphere.

The partition coefficients for the liposomes on the gel, K_{av}, defined by[13]

$$K_{av} = (V_e - V_o)/(V_t - V_o) \quad (1)$$

were determined for the peak fractions of the several second chromatographes. Here, V_t and V_o are the total volume and void volume of

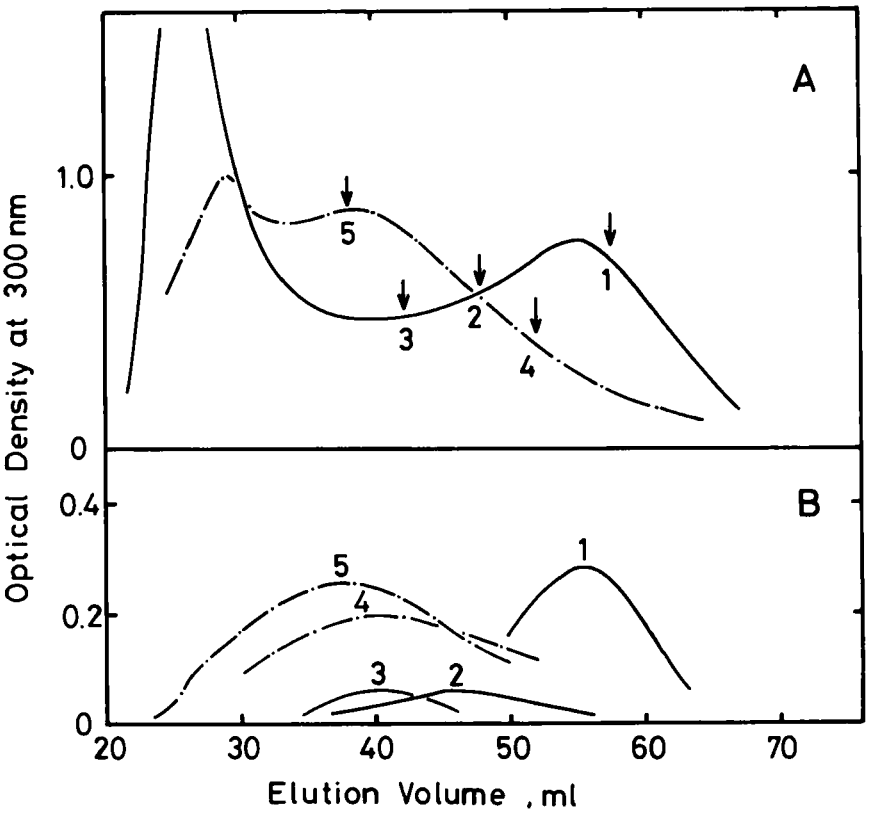

FIGURE 2 Elution profiles of the egg PC liposomes on Sepharose 2B column. A) First chromatograph ———; "sonicated" liposomes prepared by sonication for 50 min. at 4°C, — — —; "ethanol" liposomes. The arrows designate the elution volume of the fractions submitted to rechromatograph. B) Rechromatograph Notations correspond to shown in A.

the column and V_e is the elution volume of the peak fraction. It should be mentioned here that the elution volume of the peak in the second chromatograph slightly shifted toward the void volume compared with that of each parent fraction in the first chromatograph.

The values of K_{av} thus obtained for egg PC liposomes were confirmed to be independent of temperature (4°C-40°C) and ionic strength of the buffered solution.

Weight averaged molecular weight of the liposomes in each peak fraction of the second chromatograph was determined by using the following equations applicable to multicomponent system[14] under the equilibrium condition:

$$M_{app} = \frac{2(C_b - C_m)RT}{C_o(r_b^2 - r_m^2)(1 - \phi'\rho)\omega^2} \tag{2}$$

$$M_{app}^{-1} = \bar{M}_w^{-1} + BC_o \tag{3}$$

where subscript b and m represent bottom and meniscus of the solution and C_o is initial concentration.

The effective partial specific volume of the lipids in the buffered solution, ϕ', was calculated from equation 4.[15]

$$\phi' = \frac{1}{w_2}\left(\frac{1}{d} - \frac{w_1}{d_o}\right) \tag{4}$$

where w_1 and w_2 are weight fractions of the buffered solution and lipid, respectively. d and d_o are the densities of the buffered solution with and without lipids, respectively. In Figure 3, ϕ' of DPPC in the buffered solution obtained in the present work are plotted against temperature and compared with the results reported by Nagle et al.[16] These results agree with each other within the experimental error.

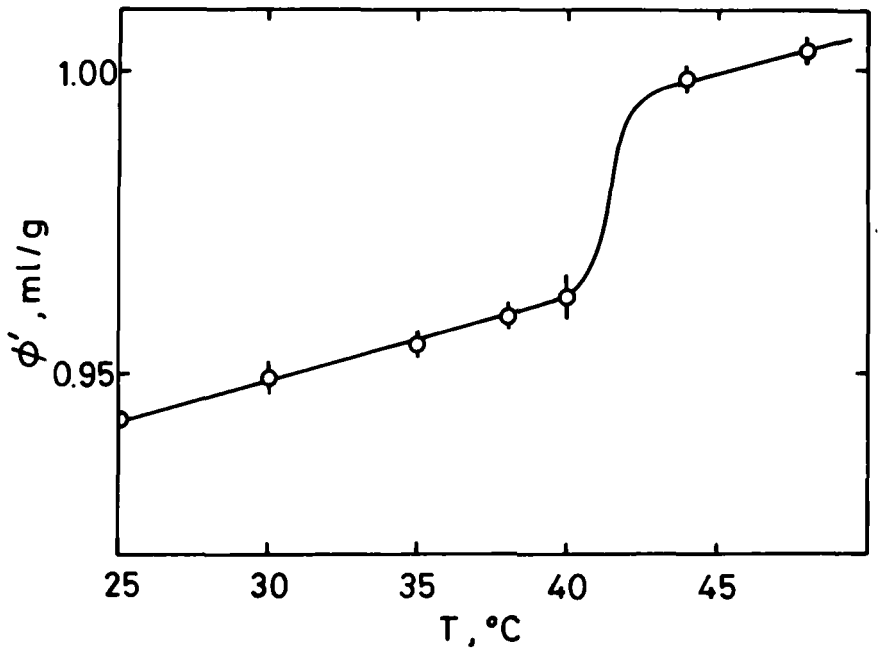

FIGURE 3 Temperature dependence of effective partial specific volume of DPPC dispersed in a buffered solution (50 mM KCl, 5 mM tris-Cl, pH = 8). O; present data, ——; Nagel *et al.*

In the calculation of $\overline{M}_w$ for DPPC liposomes, $\phi' = 0.9560$ ml/g obtained at 35°C was used. While for egg PC liposomes, $\phi' = 0.9831$ at 20°C reported by Haung *et al.*[17,18] was used.

Figure 4 shows a relationship between $\overline{M}_w$ and K_{av} for the liposomes in comparison with globular proteins[19] and dextran.[20] The data of egg PC liposomes prepared by the two different methods fall on single straight line, suggesting that these liposomes have essentially same structure, presumable single walled liposome, over wide molecular weight range. Although the values of $\overline{M}_w$ obtained for DPPC liposomes involve larger experimental error than those for egg PC due to the aggregation of the liposomes during the sedimentation equilibrium experiment, K_{av} vs. $\overline{M}_w$ plots satisfy the same linear relationship as egg PC liposomes. It is obvious, however, that the data for the PC liposomes are not in agreement with the relations reported not only for dextran, but for globular proteins. Judging from the present results, we have to point out that the estimation of the molecular weights of the liposomes using the chromatographic characteristics of globular proteins on Sepharose gels leads to the erroneous results. Finally we are just concerned with the

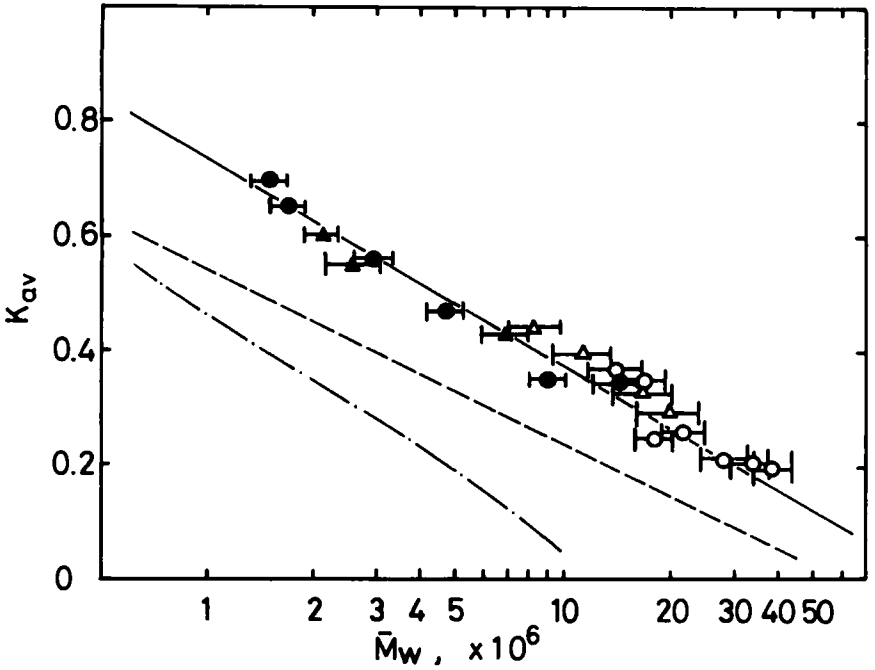

FIGURE 4 A relationship between K_{av} on Sepharose 2B gel and molecular weight of PC liposomes. ●; egg PC "sonicated" liposomes, ○; egg PC "ethanol", ▲; DPPC "sonicated", △; DPPC "ethanol". ——————; the corresponding relationship for globular protein, ——— ; for dextran.

structure of the liposomes with which we have worked. In a succeeding work of this series, the phase transition behavior of the DPPC liposomes prepared by the present methods have been examined by using an amphiphilic fluorescence probe (N,N'-distearyloxacarbocyanine iodide) incorporated into the liposomes. Bangham type multilamellar DPPC liposomes have exhibited an abrupt change in the fluorescence intensity associated with the main transition at 41.5°C and a smaller but significant change accompanied by the pretransition around 30°C. On the other hand, all the liposomes prepared by the present methods have shown only a single characteristic change around each main transition temperature, provided that they were submitted immediately to the phase transition experiments when they came out from the second Sepharose 2B column. If they had been stored below 35°C for longer than 3 hrs. prior to the phase transition experiments, the fluorescence intensity change associated with the pretransition became to be observed and its magnitude increased with the incubation time, reflecting the aggregate formation of the liposomes. These facts suggest that the liposomes prepared by the present methods are essentially in the single lamellar structure rather than the multilamellar. A more detailed discussion will appear in Part II of this series.

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