Structural Characteristics of Phospholipid Multilamellar Liposomes

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Abstract □ The relative proportion of lipid on the external surface of spherical multilamellar vesicles and the aqueous volume trapped within them, can be computed as a function of a liposome's outer radius, interlamellar aqueous spacing, and the number of bilayers. When known experimental data is put into these calculations, the results lead to the conclusion that spontaneously formed liposomes are on the average composed of up to 10 lamellae, whose total thickness is ~0.1 μm, and traps an aqueous sphere whose average radius is ~0.5 μm. Most of the aqueous medium entrapped within the multi-bilayers is contained in the internal core of the liposome. When assuming spherical liposomes and using percent exposure data, this calculation overestimates the experimentally detected trapped volumes.

Keyphrases □ Liposomes—phospholipid, multilamellar, structural characteristics, determination of external lipid layer and aqueous volume □ Vesicles—multilamellar phospholipid liposomes, structural characteristics, determination of external lipid layers and aqueous volume □ Delivery systems—phospholipid liposomes, structural characteristics, determination of external lipid layers and aqueous volume

A wide variety of phospholipids spontaneously form multilamellar vesicles (liposomes) on hydration which are constructed of stacked concentric bimolecular lamellae interspersed with an aqueous medium (1, 2). These aggregates, often referred to as "onion-like" structures, are relatively impermeable to entrapped ions (3) and other solutes, which has generated interest in their use as an experimental tool for drug delivery (4). Despite extensive studies, the structural characteristics of these liposomes are not well defined. The properties of the liposomes depend on the composition and concentration of constituent phospholipids and the ionic strength of the aqueous medium (5, 6), as well as the method of phospholipid suspension and the time of hydration (5, 7).

A common feature of all dispersions made by shaking phospholipids in aqueous medium is the size heterogeneity of the resultant multilamellar liposomes. In egg phosphatidyl choline dispersions, electron microscopy reveals the existence of liposomes with diameters <0.2 μm as well as some with diameters >3 μm (5). Although average radius and average number of bilayers have limited significance as physical characteristics, attempts have been made to measure these parameters. Electron microscopic data are helpful for such studies; however, for these purposes the usefulness of electron microscopy is limited both by the need for vesicle fixation and by difficulties in the analysis of heterogeneous dispersions. Alternatively, two experimentally available parameters are the average trapped aqueous volume (V_T) and percent of lipid exposed on the external surface (E). Both these parameters have been examined for various liposome preparations, and the results have been interpreted in terms of the average liposome radius (R), number of bilayers (n), and/or the thickness of the intervening aqueous layers (d). For this kind of analysis, a relationship between the observed parameters and the intrinsic features of the liposomes must either be assumed or calculated on the basis of certain assumptions.

Assuming that the outer radius of the multi-bilayers is large when compared with the bilayer thickness, and that successive lamellae, therefore, have very similar surface areas, Schwartz and McConnell (7) calculated the average number of lamellae from the percent exposure of an ESR spin label to an externally added reducing agent. They concluded that this number varies between 6 and 10, depending on the exact method of liposome preparation. For large multilamellar vesicles (MLV) with an average radius of ~0.7 μm (5), successive lamellae of <50 Å thickness, and interlammelar spacing of <100 Å, the assumptions made by Schwartz and McConnell seem to be justified by their experimental results. However, in a recent review, Szoka and Papahadjopoulos (8) commented that accounting for the decreasing surface areas of successive lamellae and for the interlamellar spacing "would tend to considerably increase the calculated number of lamellae."

Formulas for calculating the number and surface areas of liposomes in any suspension from the trapped aqueous volume and the partial specific volume of the constituent lipids have recently been published by Pidgeon and Hunt (9). They have used these formulas to simulate electron microscopic results for number of lamellae and average radius in various lipid dispersions. In these calculations, Pidgeon and Hunt used combinations of liposomes with different interlamellar distances. This assumes *a priori* that significantly different interlamellar distances can occur within a liposome preparation. In fact, lipid dispersions made from a wide variety of lipids and lipid mixtures, both artificial and natural, show distinct X-ray diffraction bands. The presence of these bands strongly

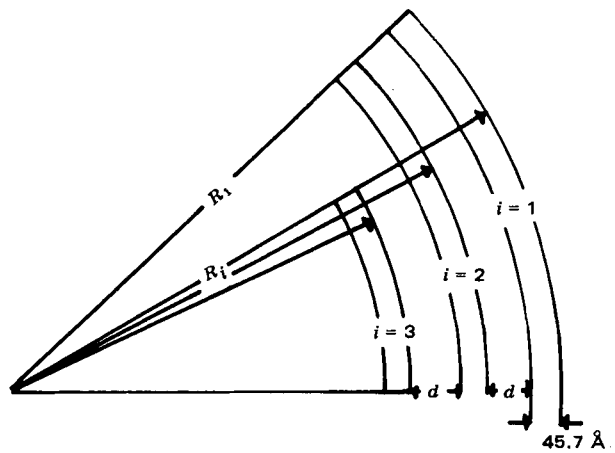

Figure 1—*Multilamellar structure of bilayers. Key: (R_1) outermost radius of the liposome; (i) serial number of the bilayer; (d) constant thickness of intervening aqueous shells.*

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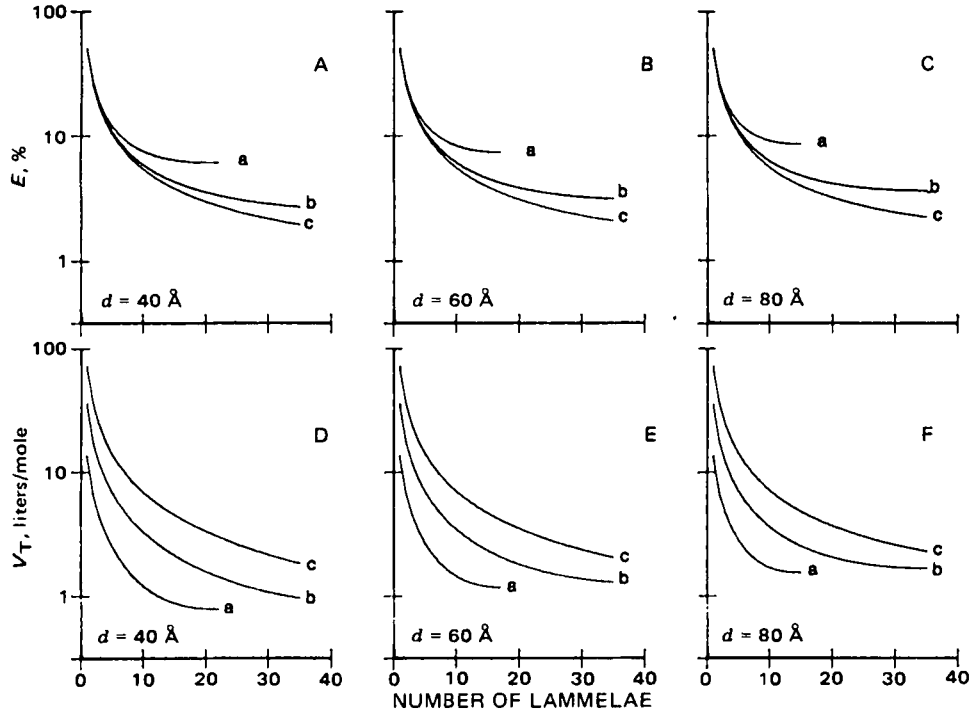

Figure 2—*Calculated dependence of percent exposure (E, Eq. 2) (A–C) and the total aqueous trapped volume (V_T, Eq. 3) (D–F) as a function of the number of lamellae, for various fixed interlamellar spacings (d). Key: (a) R_1 = 0.2 μm, (b) R_1 = 0.5 μm, (c) R_1 = 1.0 μm).*

argues that the majority of vesicles have a fixed repeat distance in a given preparation of liposomes (10).

In our view, there are several problems with the Pidgeon and Hunt model. Because they chose the innermost lamellae to begin their calculations, they were forced to assume that all of the vesicles had a fixed minimum radius common to all liposomes. In all cases they chose the radius determined for homogeneous sonicated vesicles, the smallest and most accurately known radius for any lipid vesicle (10), but one with no apparent constitutive relationship to multilamellar dispersions. The Pidgeon and Hunt formulas also are highly sensitive to deviations from the assumption of vesicle sphericity. This is because of the third-power relationship of entrapped volume (or encapsulation efficiency) to vesicle radius.

Percent exposure calculated from surface area measurements ($\propto r^2$) should be less sensitive to deviations from sphericity than trapped-volume measurements ($\propto r^3$).

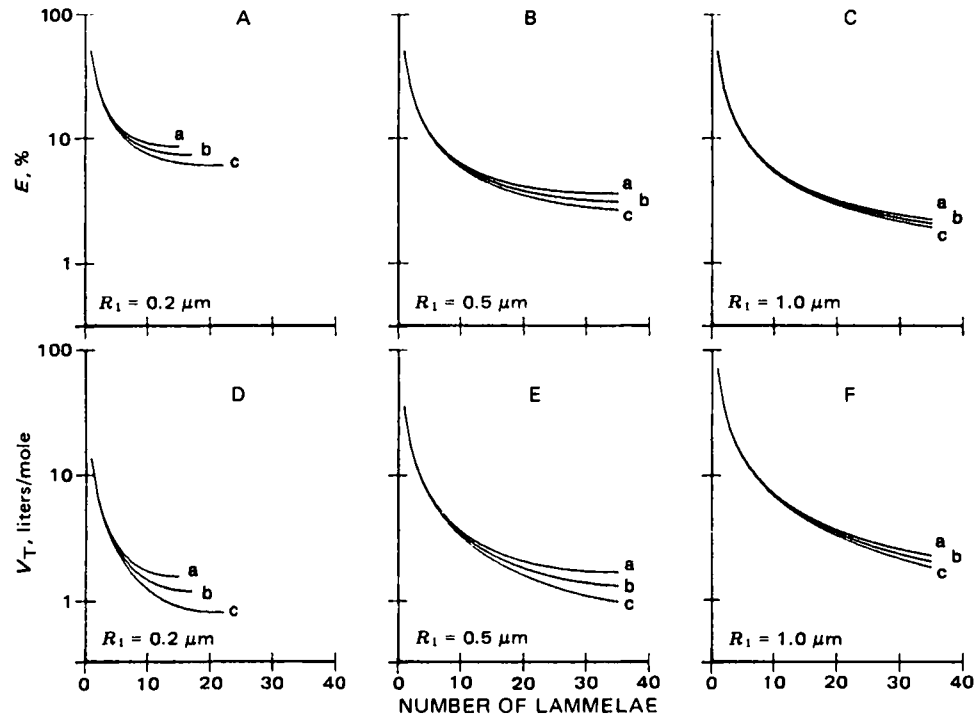

Figure 3—*Data in Fig. 2 plotted with various fixed* R_1 *radii. Key: (a)* d = 40 Å, *(b)* d = 60 Å, *(c)* d = 80 Å. *Calculations were terminated when* $R_n \leq 100$ Å *or* $n \geq 35$ *lamellae.*

Calculations based on percent exposure therefore, can be regarded as a more reliable basis for evaluating the number of lamellae in the multilamellar liposomes because this evaluation depends on the calculated radius. This is the approach which Schwartz and McConnell used. To avoid the criticism by Szoka and Papahadjopoulos, the differences in surface areas of successive lamellae and the existence of interlamellar spacing have been taken into consideration in the following treatment.

THEORETICAL

We adopted the assumption of vesicle sphericity as a first-order approximation. We also assumed that the thickness of the hydrated phospholipid bilayer is constant at 45.7 Å, and that the surface area per phospholipid molecule is constant at 72.5 Å^2 (11). In addition, we assumed that the bilayers are separated by intervening aqueous shells of constant thickness, d.

For a multilamellar structure of n bilayers, the radius of the outer surface of any given bilayer is:

$$R_i = R_1 - [(i - 1)(45.7 + d)] \qquad \text{(Eq. 1)}$$

where R_1 is the outermost radius of the liposome and i is the serial number of that bilayer, counted inward from the outermost bilayer (Fig. 1). The surface areas of both faces of the ith bilayer are closely approximated by $4\pi R_i^2$. The number of molecules for this bilayer is then twice this value divided by the surface area per molecule. The percent exposure is taken as equal to the external surface area of the outer bilayer ($4\pi R_1^2$) divided by the total surface area of all the bilayers and multiplied by 100:

$$E = \frac{100R_1^2}{\sum_{i=1}^{n} 2[R_1 - (i - 1)(45.7 + d)]^2}\% \qquad \text{(Eq. 2)}$$

Aqueous medium is trapped within the innermost bilayer shell (bilayer n) forming the aqueous core and between the concentric bilayers. The aqueous core has a radius of $R_n = R_1 - [45.7n + d(n - 1)]$ and a volume of $V_n = 4/3(\pi R_n^3)$. The volume of each of the inter-bilayer compartments can be calculated from the difference between the volumes of the two spheres defined by the inner radius of any given bilayer (i) and the outer radius of the next bilayer ($i + 1$). The moles of phospholipid per multibilayer are calculated as the sum of the surface areas of all bilayers divided by the assumed area per molecule and Avogadro's number. If the sum of all the trapped volume elements is divided by the moles of phospholipid, the following expression is obtained for the trapped volume as a function of R, d, and n:

$$V_T = 0.007274 \frac{\{R_1 - [45.7n + d(n - 1)]\}^3 + \sum_{i=1}^{n-1} \{[R_1 + d - i(45.7 + d)]^3 - [R_1 - i(45.7 + d)]^3\}}{\sum_{i=1}^{n} [R_1 - (i - 1)(45.7 + d)]^2} \frac{\text{L}}{\text{mol}} \qquad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

The dependence of the trapped volume (V_T) and the percent exposure (E) on the number of lamellae (n) is presented in Figs. 2 and 3. These curves are computed for various outermost radii (R_1) and interlamellar spacing (d) using Eqs. 2 and 3. These calculations suggest that both the trapped volume and percent exposure depend on R, d, and n. As previously proposed by Szoka and Papahadjopoulos (8), both the computed trapped volume and percent exposure decrease sharply as the number of lamellae increase. However, the dependence of the calculated trapped volume on the various parameters is quite different from the dependence of the calculated percent exposure.

Consider liposomes with ≤10 bilayers and radii >0.2 μm. The calculated percent exposure primarily depends on the number of lamellae and is insensitive to both the liposome outer radius (Fig. 2A) and the interlamellar distance (Fig. 3A). Furthermore, in liposomes with >10 bilayers, but radii of 0.5–1.0 μm and interlamellar distances of 40–80 Å, the major determinant of the calculated percent exposure is the number of lamellae and not the outer radius (R_1) (Fig. 2A–C) or interlamellar distance (Fig. 3A–C). On the other hand, the calculated trapped volume is very sensitive to R and d (Figs. 2D–F and 3D–F). For liposomes of ≤10 bilayers, the calculated trapped volume primarily depends on the number of lamellae and the outer radius of the liposome (Fig. 2D–F). In liposomes with more bilayers, the trapped volume becomes less sensitive to the number of lamellae (Figs. 2D–F and 3D–F), and the major factor is the outer radius of the liposome (Fig. 2D–F). The interlamellar spacing (d) in vesicles with an outer radius of 0.5 μm is significant only in multi-bilayers with >20 lamellae (Fig. 3E).

Average Number of Bilayers in Multilamellar Liposomes—The number of lamellae (not the radius nor interlamellar distance) is the major factor in determining the percent exposure. The radius, and to a lesser extent the interlamellar distance, markedly affect the trapped volume in spherical multi-bilayers. Thus, for multilamellar liposomes any approximation of the average number of lamellae would best be based on the fraction of exposed lipid and not on the encapsulated volume.

Schwartz and McConnell found in their ESR spin label experiments that liposomes made under specific conditions from dimyristoyl phosphatidyl choline, dipalmitoyl phosphatidyl choline, and binary mixtures of dipalmitoyl phosphatidyl choline and cholesterol "all have the same portion of external lipids" (7). The variation of the percent exposure in these uncharged liposomes was within the range of 5–9%, depending on the conditions of liposome preparation. This is similar to the value of 8.5% obtained previously for the percent of lipid exposed to UO_2^{2+} in liposomes composed of egg phosphatidyl choline (6). For liposomes of an average radius which is >0.4 μm (5, 6) and in this range of exposure (5–9%), the fraction of exposed lipid primarily reflects the average number of lamellae (Figs. 2 and 3). Therefore, we conclude that these liposomes are constructed of 5–10 bilayers on the average.

It should be pointed out that liposomes made of various phospholipids differ in their gel–liquid crystalline phase transition temperature. Thus, at room temperature egg phosphatidyl choline is in a liquid crystalline state, whereas dipalmitoyl phosphatidyl choline is in a gel state. The close similarity of the percent exposure, therefore, suggests that the average number of lamellae in the liposomes can only be slightly affected by the physical state of the phospholipid.

For negatively charged liposomes composed of 10% dicetyl phosphate and 90% dipalmitoyl phosphatidyl choline, 11% of the lipid is exposed to an externally added reagent (7). This may indicate that in the charged liposomes the average number of lamellae is smaller than in the uncharged liposome average.

The number of lamellae found by Schwartz and McConnell closely agrees with our calculations. Contrary to the suggestion of Szoka and Papahadjopoulos (8), accounting for the decreasing surface areas of successive bilayers and for the presence of interlamellar space does not increase the number of lamellae by more than one over the simple approximation of Schwartz and McConnell, which represents the minimal number of lamellae for any given percent exposure, since it models a liposome in which the radius of the inner aqueous core is very close to the external radius. Our computations support the suggestion of Schwartz and McConnell that for realistic values of the membrane thickness and aqueous interlamellar spacing, the multilamellar liposome can be described as having a large aqueous cavity entrapped within compact multilamella.

Trapped Volume and Shape of Multilamellar Liposomes—Consider egg phosphatidyl choline multilamellar liposomes with six bilayers [$E \simeq 8.5\%$ (6)] with an average radius in the range of 0.4–1.0 μm. The calculated trapped volume range of 4–12 L/mol is higher than that observed previously [1.5 L/mol (6)]. Moreover liposomes made of charged lipids, which are probably composed of fewer bilayers (7), have an average trapped volume that varies from 3 to 8 L/mol (5, 6). The upper limit of this range is obtained only in low ionic strength suspensions, where the liposomes are postulated to have fewer lamellae (12). In all other cases, the experimental values for the entrapped volumes are 1.5–4 times lower than predicted by our calculations.

Two factors may contribute to this large discrepancy. One possibility is a lower internal concentration of the trapped volume indicator within the liposome relative to the indicator concentration in the bulk medium. This could be due to the permanent exclusion of small solute molecules,

such as sucrose or other volume indicators, from a layer of water of hydration of the phospholipid. For dimyristoyl phosphatidyl choline liposomes, this "nonsolvent water" has been shown to be 11.5 mol of water/mol of phosphatidyl choline, or approximately one-third of the enclosed liposomal water (13). The second factor is the difference between the true liposomal structure and the geometric approximations made in our model. For example, any change in the shape of the model to a nonspherical geometry will decrease the trapped volume relative to the percent exposure (r^2 *versus* r^3). This does not imply that multi-bilayers are nonspherical, but deviations from sphericity can account for this discrepancy.

In conclusion, our calculations clearly show that the estimation by Schwartz and McConnell for the number of lamellae in multilamellar vesicles is essentially correct. This, in fact, implies that the wall of the average liposome spontaneously formed by hydration of phospholipids, consists of 5–10 bilayers and has a total thickness $< \sim 0.2$ μm. Thus, "fully swollen" (spherical) liposomes of a diameter of 1.4 μm, on the average, contain an inner aqueous cavity >1.0 μm. When viewed obliquely, a nonspherical multi-bilayer could result in the observed space-filling "onion-like" structures seen in some electron micrographs and give a somewhat misleading impression of the large central cavity which we calculate to be present in multilamellar liposomal structures. Finally, we conclude that therapeutic strategies using encapsulation of aqueous components into simple multilamellar lipid dispersions must take into account the fact that the vast majority of encapsulated material will reside in the central cavity of these liposomes.

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Epimerization of Benzylpenicilloic Acid in Alkaline Media

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Abstract □ 5*R*,6*R*-Benzylpenicilloic acid was found to epimerize slowly in alkaline media to 5*S*,6*R*-benzylpenicilloic acid until equilibrium was established. Epimerization proceeded *via* the imine tautomer of penamaldic acid rather than the enamine form and was found to favor the 5*S*,6*R*-epimer at equilibrium. The conversion process was monitored using both reverse-phase high-performance liquid chromatography and NMR spectroscopy.

Keyphrases □ Benzylpenicilloic acid—epimerization, alkaline media, imine tautomer of penamaldic acid as intermediate □ Epimerization—benzylpenicilloic acid in alkaline media, imine tautomer of penamaldic acid as intermediate □ Penamaldic acid—imine tautomer, intermediate in the epimerization of benzylpenicilloic acid

Penicilloic acid has been reported to be the principal hydrolysis and excretion product of penicillin (1, 2). It has also been cited as a minor antigenic determinant, although it is structurally incapable of reacting directly with proteins to form the highly reactive penicilloyl conjugate (3). This compound is believed to exert its antigenic activity by reacting with disulfide linkages of proteins (4). Despite the important role which penicilloic acid may play as an antigenic determinant and its reported existence as a mixture of isomers in alkaline media (5, 6), virtually no attempt has been made either to separate the isomers or to investigate the isomerization process.

In this paper, a combination of high-performance liquid chromatography (HPLC), NMR, polarography, and UV spectroscopy was used to separate the 5*R*,6*R*- and 5*S*,6*R*-epimers of benzylpenicilloic acid, monitor the conversion process, and examine the epimerization mechanism.

EXPERIMENTAL

Chemicals and Reagents—Penicillin G potassium[1] was obtained commercially and used without further treatment. Acetonitrile[2] was HPLC grade, while all other chemicals were either USP or reagent grade. Double-distilled water was used to prepare buffer solutions.

Preparation of Disodium Penicilloate—The disodium salts of 5*R*,6*R*- and 5*S*,6*R*-benzylpenicilloic acids were prepared by procedures similar to those used in the preparation of penicic and epipenicic acids (7).

Disodium 5R,6R-Benzylpenicilloate—An 8.64-g sample of penicillin G potassium was dissolved in 15 mL of double-distilled water and allowed to cool in an ice-salt bath. After the solution temperature reached ~0°C, 4.64 mL of cold 10 M NaOH was added in one increment. Fifteen minutes later, the pH was adjusted to 8.7 [the equivalence point of the disodium salt of benzylpenicilloic acid (7)] using 1 M HCl, and the reaction mixture was immediately lyophilized. ^{13}C-NMR: δ 26.4 and 26.7 (C-2$^{\alpha}$ and C-2$^{\beta}$), 42.5 (C-9), 58.6 (C-2), 59.9 (C-6), 66.1 (C-5), 75.4 (C-3), 127.4 (C-4′), 129.0 (C-3′), 129.4 (C-2′), 135.0 (C-1′), and 173.9, 175.4, and 175.9 (C-3$^{\alpha}$, C-6$^{\alpha}$, and C-8). ^{1}H-NMR (D_2O): δ 1.07 (s, CH_3), 1.34 (s, CH_3), 3.26 (s, H-3), 3.53

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Waters Associates, Milford, Mass.

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