Calculating Number and Surface Area of Liposomes in Any Suspension

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Abstract □ Simple formulas for calculating the total surface area and number of liposomes in any liposome population are presented. The necessary parameters for calculating these values are the encapsulation ratio (or efficiency) of the population, the partial specific volume of the lipids used to prepare the liposomes, and an estimate of the frequency distribution of the population. When tested on theoretical liposome populations, excellent results are obtained regardless of the heterogeneity of the population.

Keyphrases □ Liposomes—simple formulas for calculating number and surface area of liposomes, heterogeneous populations □ Drug delivery systems, potential—liposomes, simple formulas for calculating number and surface area □ Models, mathematical—simple formulas for calculating number and surface area of liposomes

Liposome is the generic term for any lipid bilayer vesicle that traps a volume of water, regardless of its size, composition, and number of lamellae (Fig. 1). In part, because of their ability to entrap and transport drugs *in vivo*, liposomes have been studied extensively.

The disposition properties of intravenously injected liposomes are complicated and depend in part on size (1), dose (2–4), charge (1, 4), number of particles, and total surface area. Pharmacological experiments based solely on the number of particles and total surface area have not been pursued because these parameters are not easily estimated for a given vesicle population. Although dose and size taken together are related to the number of particles and surface area, the tendency of liposome populations to be heterogeneous allows for differences in number and surface area for any given total lipid dose. Even for homogeneously sized vesicles, the relationships among dose, size, number, and surface area are ambiguous.

Methods for estimating the number of particles and surface area for solid spheres are available (5, 6), but liposomes are not solid spheres. A method is presented here for calculating the number and total surface area for any liposome population given the liposome encapsulation efficiency, an estimate of the liposome frequency *versus* radius distribution, and the total effective volume of lipid used to prepare the vesicles.

THEORETICAL

Any given liposome population, whether artificially generated or real, may be classified into one of three categories: a homogeneous population, a fixed-lamella, variable-radius population, or a heterogeneous population. The liposomes in a homogeneous population are the same in every parameter, *i.e.*, radius, surface area, and number of layers if multilamellar. An example of this type of population is unilamellar liposomes that have been sized carefully (6). A fixed-lamella, variable-radius population is similar to a homogeneous population in that the lipid bilayer and water layer thicknesses are the same in all liposomes, but the number of repeating water–lipid layers and, consequently, the liposome radii vary between liposomes. In contrast, a heterogeneous liposome population is the most diverse; every liposome parameter can differ within and between vesicles.

The properties of each population can be rigorously described

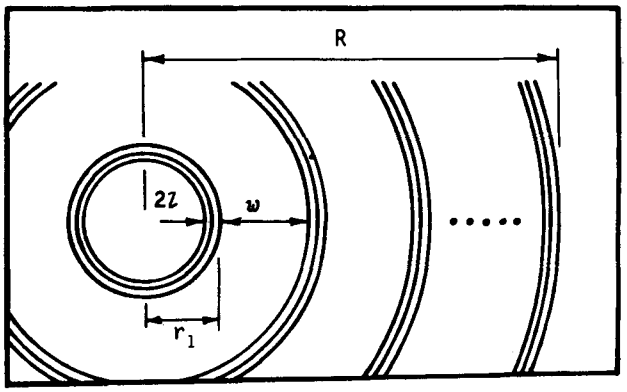

Figure 1—*Schematic drawing of liposome structure. Each multilamellar liposome consists of* n *lamellae, each of which is composed of a lipid bilayer of thickness 2l. Each lamella alternates with water layers of average thickness* w*; the core lamella forms a unilamellar liposome of radius* r_1 *(Eq. 19).*

mathematically. The volume of water encapsulated in a unilamellar (single-layered) liposome, VW_1, is:

$$VW_1 = \frac{4}{3}\pi R^3 - VL_1 \qquad \text{(Eq. 1)}$$

where VL_1 is the volume of lipid in the single-layered liposome and R is the effective outside radius. If a second layer of water and lipid is added, then:

$$VW_2 = \frac{4}{3}\pi R^3 - VL_2 \qquad \text{(Eq. 2)}$$

where VW_2 is the volume of water encapsulated in a bilamellar liposome and VL_2 is the volume of lipid required for its formation. Equation 2 can be generalized for a n-lamellar liposome (Fig. 1) as:

$$VW_n = \frac{4}{3}\pi R^3 - VL_n \qquad \text{(Eq. 3)}$$

In a homogeneous population, the values of VW_n and VL_n are identical between vesicles. Therefore, multiplying Eq. 3 by the number of liposomes in the population, N, gives the total volume of trapped water TVW:

$$TVW = NVW_n = N\frac{4}{3}\pi R^3 - NVL_n = N\frac{4}{3}\pi R^3 - TVL \qquad \text{(Eq. 4)}$$

where TVL is the total effective volume of lipid used to prepare the liposome population. TVL, in turn, is calculated from:

$$TVL = \bar{v}M(\overline{MW}) \qquad \text{(Eq. 5)}$$

where $\bar{v}$ is the partial specific volume, M is the number of moles, and $\overline{MW}$ is the average molecular weight of the hydrated lipids. Dividing Eq. 4 by Eq. 5 and defining the encapsulation ratio (or efficiency), E, as the ratio of the entrapped water volume to the total volume of lipid yield:

$$E = \frac{\frac{4}{3}\pi NR^3}{TVL} - 1 \qquad \text{(Eq. 6)}$$

Solving Eq. 6 for N gives:

$$N = \frac{(E+1)TVL}{\frac{4}{3}\pi R^3} \qquad \text{(Eq. 7)}$$

Equation 7 is derived for a homogeneous population of liposomes where

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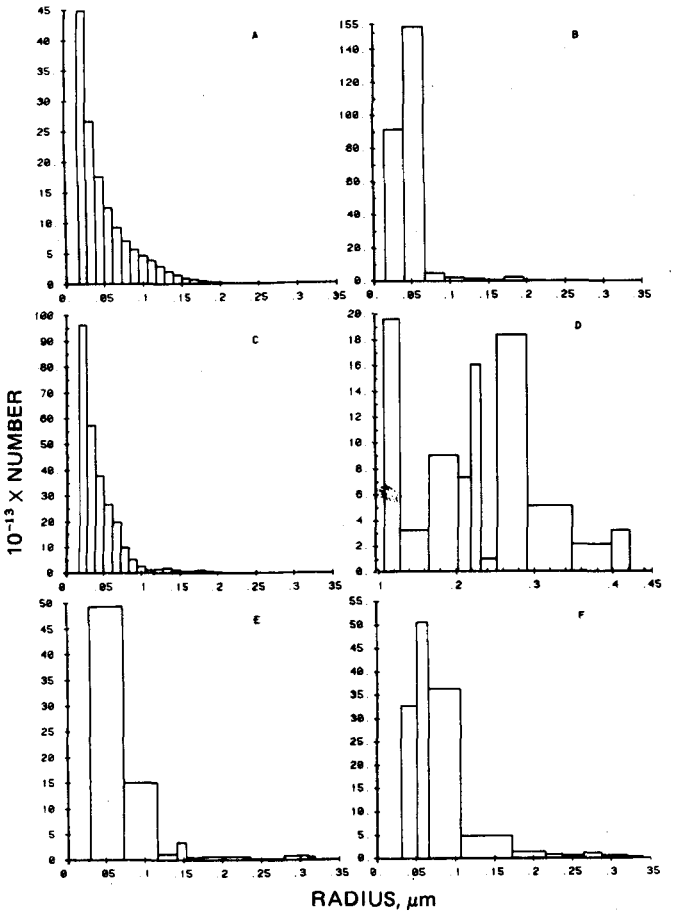

Figure 2—*Frequency* versus *radius for six hypothetical liposome populations used to illustrate the validity of Eqs. 15 and 18. In each frame, the number of liposomes in each subpopulation of fixed radius is indicated. Each liposome population is composed of 1 mmole of lipid, and each bar represents a homogeneous subpopulation. The variable liposomal parameters used to construct each population were described in the text. Key: A,* w = 7.4 *nm and* r_1 = 22.5 *nm, with* n *ranging from one to 18 in steps of one; B,* w = 92.5 *nm and* r_1 = 15 *nm, with* n *ranging from two to 18 in steps of two; C,* w = 7.4 *nm and* r_1 = 22.5 *nm, with* n *ranging from one to 18 in steps of one; D,* w *ranges from 7.4 to 333 nm and* r_1 *ranges from 15 to 100 nm, with* n *ranging from eight to 15 (values shown on the ordinate are reduced by a factor of 10); E,* w *ranges from 7.4 to 333 nm and* r_1 *ranges from 15 to 80 nm, with* n *ranging from two to 18; and F,* w *ranges from 7.4 to 259 nm and* r_1 *ranges from 15 to 80 nm with* n *ranging from two to 14.*

every liposome parameter is identical between liposomes. If many homogeneous subpopulations are combined to give a heterogeneous population or a fixed-lamella, variable-radius population, Eq. 7 will hold for each ith subpopulation and can be rewritten as:

$$N_i = \frac{(E_i + 1)TVL_i}{\frac{4}{3}\pi R_i^3} \qquad \text{(Eq. 8)}$$

Rearranging Eq. 8 and summing the subpopulations up to the kth population give:

$$\frac{4}{3}\pi \sum_{i=1}^{k} N_i R_i^3 = \sum_{i=1}^{k} (E_i + 1)TVL_i = \sum_{i=1}^{k} E_i TVL_i + \sum_{i=1}^{k} TVL_i \qquad \text{(Eq. 9)}$$

Recognizing that $\Sigma_{i=1}^{k} TVL_i$ is the total volume of lipid in the entire population, one can write Eq. 9 as:

$$\frac{\frac{4}{3}\pi \sum_{i=1}^{k} N_i R_i^3}{TVL} = \frac{\sum_{i=1}^{k} E_i TVL_i}{TVL} + 1 \qquad \text{(Eq. 10)}$$

Since TVL_i/TVL is the mole fraction, x_i, of lipid in the ith population,

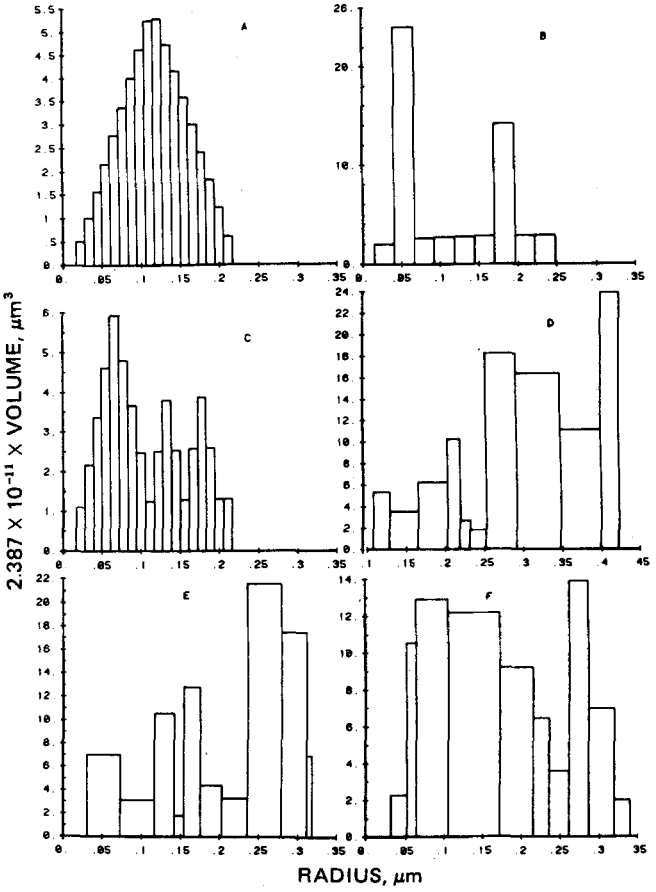

Figure 3—*Volume* versus *radius for the same liposome populations shown in Fig. 2 (*$10^{-10}/[(4/3)\pi] = 2.387 \times 10^{-11}$*). Each bar gives the total volume in cubic micrometers (*10^{11} μm^3 = 0.1 ml*) of a homogeneous subpopulation of fixed radius.*

Eq. 10 can be rewritten as:

$$\frac{\frac{4}{3}\pi \sum_{i=1}^{k} N_i R_i^3}{TVL} = \sum_{i=1}^{k} x_i E_i + 1 \qquad \text{(Eq. 11)}$$

The encapsulation efficiency of the entire population, E_p, is equal to $\Sigma_{i=1}^{k} x_i E_i$; therefore, Eq. 11 reduces to:

$$\sum_{i=1}^{k} N_i R_i^3 = \frac{(E_p + 1)TVL}{\frac{4}{3}\pi} \qquad \text{(Eq. 12)}$$

Dividing both sides of Eq. 12 by $\Sigma_{i=1}^{k} N_i = N_p$ and rearranging give:

$$N_p = \frac{(E_p + 1)TVL}{\left(\frac{4}{3}\pi \sum_{i=1}^{k} N_i R_i^3\right) \Big/ \sum_{i=1}^{k} N_i} \qquad \text{(Eq. 13)}$$

where the term $(4/3)\pi\Sigma_{i=1}^{k} N_i R_i^3$ is the total volume of all liposomes in the entire population; therefore, $[(4/3)\pi\Sigma_{i=1}^{k} N_i R_i^3]/\Sigma_{i=1}^{k} N_i$ is the average volume per liposome. This average volume can be calculated directly from a frequency *versus* radius distribution of the population obtained from electron micrographs (7). It follows that the liposome with the average volume will have a radius $r_{\bar{v}}$ as is given in:

$$\frac{\frac{4}{3}\pi \sum_{i=1}^{k} N_i R_i^3}{\sum_{i=1}^{k} N_i} = \frac{4}{3}\pi r_{\bar{v}}^3 \qquad \text{(Eq. 14)}$$

Substitution of Eq. 14 into Eq. 13 gives:

$$N_p = \frac{(E_p + 1)TVL}{\frac{4}{3}\pi r_{\bar{v}}^3} \qquad \text{(Eq. 15)}$$

Equation 15 allows the total number of liposomes to be calculated for any

Table I—Typical Example [a] of a Complete Set of Properties for Homogeneous Liposome Subpopulations

n	R, μm	$10^5\ vl_n$, μm³	$10^5\ VL_n$, μm³	$10^5\ vw_n$, μm³	$10^5\ VW_n$, μm³	E	$10^{-14}\ N$	$10^{-13}\ TSA$, μm²	$10^{-12}\ TVW$, μm³
1	0.0225	1.99	1.99	2.78	2.78	1.4	404	25.7	1.12
2	0.0336	4.69	6.68	6.43	9.21	1.38	120	17.0	1.11
3	0.0447	8.54	15.2	13.0	22.2	1.46	52.7	13.2	1.17
4	0.0558	13.5	28.8	21.8	44.0	1.53	27.9	10.9	1.23
5	0.0669	19.7	48.4	33.0	77.0	1.59	16.6	9.32	1.27
6	0.0780	26.9	75.4	46.4	123	1.64	10.6	8.13	1.31
7	0.0891	35.4	111	62.1	185	1.67	7.24	7.22	1.34
8	0.1002	45.0	156	80.1	265	1.70	5.15	6.50	1.37
9	0.1113	55.7	211	100	366	1.73	3.79	5.91	1.39
10	0.1224	67.6	279	123	489	1.75	2.87	5.41	1.41
11	0.1335	80.6	360	148	637	1.77	2.23	5.00	1.42
12	0.1446	94.7	454	175	812	1.79	1.76	4.64	1.43
13	0.1557	110	564	204	1020	1.80	1.42	4.33	1.44
14	0.1668	126	691	236	1250	1.81	1.16	4.06	1.45
15	0.1779	144	835	270	1520	1.82	0.961	3.82	1.46
16	0.1890	163	998	307	1830	1.83	0.804	3.61	1.47
17	0.2001	182	1180	345	2180	1.84	0.680	3.42	1.48
18	0.2112	204	1380	386	2560	1.85	0.579	3.25	1.48

[a] Each row gives the properties of a homogeneous, n-lamellar liposome population composed of 1 mmole of lipid (8.0226×10^{11} μm³). For this example, w, the thickness of each water layer ($n > 1$) is fixed at 7.4 nm; the outside radius of the innermost liposome is fixed at 22.5 nm. The column headings are defined in the text.

type of liposome population whenever valid measures of the parameters on either side of Eq. 14 can be obtained.

A similar expression can be obtained for calculating the total surface area of the population. Rearranging Eqs. 12 and 15 to:

$$\sum_{i=1}^{k} 4\pi N_i R_i^3 = 3(E_p + 1)TVL = 4\pi N_p r_{\bar{v}}^3 \qquad \text{(Eq. 16)}$$

and dividing by the total surface area of the population, TSA_p, gives:

$$\eta = \frac{r_{\bar{v}}^3}{r_{\bar{s}}^2} = \frac{3(E_p + 1)TVL}{TSA_p} = \frac{\sum_{i=1}^{k} 4\pi N_i R_i^3}{\sum_{i=1}^{k} 4\pi N_i R_i^2} \qquad \text{(Eq. 17)}$$

where:

$$TSA_p = \sum_{i=1}^{k} 4\pi N_i R_i^2 = 4\pi N_p r_{\bar{s}}^2$$

and $r_{\bar{s}}$ is the radius of the liposome with the average surface area and $\eta = r_{\bar{v}} = r_{\bar{s}}$ for a homogeneous population. Solving for TSA_p and canceling terms give:

$$TSA_p = \frac{3(E_p + 1)TVL}{\left(\sum_{i=1}^{k} N_i R_i^3\right) \Big/ \sum_{i=1}^{k} N_i R_i} = \frac{3(E_p + 1)TVL}{\eta} \qquad \text{(Eq. 18)}$$

Thus, to calculate N_p and TSA_p for any liposome population, only four parameters (E_p, TVL, $r_{\bar{s}}$, and $r_{\bar{v}}$) are needed.

RESULTS

Data Generation—Data to test Eqs. 15 and 18 were generated from lipid molecular parameters reported by Mason and Huang (8) for phosphatidylcholine liposomes. Briefly, data generation involved specifying the outside radius of the first lamella and the length of the lipid molecule and then calculating the volume of lipid and the volume of water in the liposome. The remaining liposome parameters were calculated, then another layer of water and lipid was added, and the same calculations were performed. Data tables thus were generated with 18 rows, each row corresponding to a homogeneous population of liposomes composed of 1 mmole of phospholipid. All liposome input and output parameters, along with the method of calculation, are outlined here.

The outside radius, R, in micrometers of any liposome (Fig. 1) is given by:

$$R = r_1 + (n - 1)(2l + w) \qquad \text{(Eq. 19)}$$

where r_1 is the outside radius of the first lamella (*i.e.*, the outside radius of the innermost liposome in a multilamellar liposome), n is the number of lamella or lipid layers, l is the average length of a lipid molecule (it is also one-half of the lipid bilayer thickness), and w is the water layer thickness (only when $n \geq 2$).

The volume, in cubic micrometers, of lipid in the nth lamella or lipid layer, vl_n, is:

$$vl_n = \frac{4}{3}\pi[R^3 - (R - 2l)^3] \qquad \text{(Eq. 20)}$$

The volume, in cubic micrometers, of lipid in any n-lamellar liposome, VL_n, is:

$$VL_n = \sum_{i=1}^{n} vl_i \qquad \text{(Eq. 21)}$$

The volume of water in the nth aqueous compartment or layer, vw_n, is:

$$vw_n = \frac{4}{3}\pi\{[r_1 + (n - 1)w + 2l(n - 2)]^3 - [r_1 + (n - 2)w + (n - 2)2l]^3\} \qquad \text{(Eq. 22)}$$

The volume of water in any n-lamellar liposome, VW_n, is:

Table II—Example [a] of Subpopulation Parameter Values Used to Construct Heterogeneous Liposome Populations

$10^4\ w$, μm	$10^4\ r_1$, μm	n	R, μm	$10^{-14}\ N_i$	$10^{-13}\ TSA_i$, μm²	$10^{-12}\ TVW_i$, μm³	X_i	$X_i\ 10^{-14}\ N_i$	$X_i\ 10^{-13}\ TSA_i$, μm²	$X_i\ 10^{-11}\ TVW_i$, μm³
74	300	2	0.041	73.9	15.7	1.35	0.044	3.25	0.698	0.600
74	800	5	0.224	0.854	5.40	3.24	0.067	0.057	0.360	2.16
92.5	225	10	0.139	2.27	5.51	1.75	0.200	0.454	1.10	3.50
92.5	1400	6	0.205	0.974	5.12	2.69	0.111	0.108	0.569	2.99
111	150	4	0.059	28.3	12.5	1.68	0.178	5.03	2.22	2.99
111	2200	3	0.250	1.06	8.27	6.08	0.022	0.024	0.184	1.35
148	150	4	0.071	20.5	12.8	2.21	0.178	3.64	2.28	3.93
148	1000	14	0.341	0.231	3.37	3.02	0.022	0.005	0.075	0.670
185	300	12	0.274	0.505	4.77	3.56	0.133	0.067	0.636	4.75
259	1800	5	0.298	0.594	6.64	5.81	0.044	0.026	0.295	2.58
Population value							1.0	12.7	8.417	25.52

[a] These data are for population F and correspond to Figs. 2F and 3F. This heterogeneous population is composed of 10 homogeneous subpopulations (each corresponding to one of the 10 rows and one bar in Figs. 1 and 2), where their relative proportion in the total population (X_i) is randomly selected. The total lipid concentration in each final heterogeneous population is 1 mM.

Table III—Comparison of Actual Values of the Total Number and Total Surface Area with Values Calculated Using Eqs. 15 and 18, Respectively, for the Six Liposome Populations Shown in Figs. 2 and 3

Population	$r_{\bar{v}}$, μm	η, μm	E_p	Actual Value of N_p	Calculated Value of N_p	Actual Value of TSA_p, μm²	Calculated Value of TSA_p, μm²
A	0.0714	0.098	1.71	1.41×10^{15}	1.42×10^{15}	6.64×10^{13}	6.56×10^{13}
B	0.0604	0.079	1.96	2.58×10^{15}	2.57×10^{15}	9.04×10^{13}	9.00×10^{13}
C	0.0579	0.080	1.66	2.62×10^{15}	2.62×10^{15}	7.99×10^{13}	8.00×10^{13}
D	0.2272	0.260	4.19	8.48×10^{13}	8.48×10^{13}	4.79×10^{13}	4.80×10^{13}
E	0.1072	0.156	3.64	7.21×10^{14}	7.21×10^{14}	7.14×10^{13}	7.16×10^{13}
F	0.0857	0.119	3.18	1.27×10^{15}	1.27×10^{15}	8.42×10^{13}	8.40×10^{13}

$$VW_n = \sum_{i=1}^{n} vw_i \qquad \text{(Eq. 23)}$$

The total volume of lipid used is given by Eq. 5 and by:

$$TVL = (6.023 \times 10^{23}) Ml\alpha \qquad \text{(Eq. 24)}$$

where M is the number of moles of lipid used and α is the area per lipid molecule.

Approximately 3500 homogeneous liposome populations were generated. A typical set of homogeneous liposome populations with the number of lamella ranging from one to 18 is given in Table I.

Fixed-lamella, variable-radius populations were generated by combining an entire data table such as Table I. Each row of the table was generated using 1 mmole of lipid. So that the final fixed-lamella, variable-radius population also would contain only 1 mmole of lipid, each row in the table was multiplied by X_i, the fraction of the total lipid contained in the ith homogeneous subpopulation, where $\Sigma X_i = 1$ for each fixed-lamella, variable-radius population. Three examples of such populations are included in Figs. 2 and 3.

Since w and r_1 in Eq. 19 are constant in any one data table, combining rows from different data tables allows generation of heterogeneous populations composed of i homogeneous populations. Again, X_i is the fraction of the total lipid contained in the ith homogeneous population (values of X_i were selected at random). Table II is an example of one heterogeneous population in Figs. 2 and 3.

Testing of Eqs. 15 and 19—Six heterogeneous populations were generated (Figs. 2 and 3). Each population was treated as if it were a sample of a larger population. For each population, the total volume of lipid, TVL, was the same and was calculated from Eq. 5, where $\bar{v} = 1$ ml/g $= 10^{12}$ μm³/g, $M = 10^{-3}$ (or 1 mmole), and $\overline{MW} = 802.26$ g/mole; thus, $TVL = 8.0226 \times 10^{11}$ μm³/mmole. The encapsulation ratio, E_p, for each population was calculated as the ratio of TVW to TVL, $r_{\bar{v}}$ was calculated from Eq. 14, and η was calculated from Eq. 17. Actual and calculated values of N_p and TSA_p for the six liposome populations in Figs. 2 and 3 are compared in Table III.

DISCUSSION

Whenever liposomes are used as drug delivery systems, either *in vivo* or *in vitro*, the number and surface area of the liposomes should be known. These parameters can be calculated using Eqs. 15 and 18. Number and surface area values from Eqs. 15 and 18 are theoretically exact as was demonstrated in Table III, regardless of the population heterogeneity. However, experimental estimates of the parameters E_p, TVL, $r_{\bar{v}}$, and $r_{\bar{s}}$ will have associated error, which will be reflected in the final values of number and surface area. Thus, how the estimates of the four dependent variables in Eqs. 15 and 18 are obtained should be discussed. It is assumed that estimates of number and surface area within 90% of the actual values can be obtained and are adequate for most purposes.

The encapsulation ratio is defined as the ratio of the total volume of trapped water to the total volume of lipid. Typically, however, the encapsulation ratio is measured relative to some solute; *e.g.*, X% of an aqueous glucose solution is encapsulated by Y moles of lipid. When it is known that the solute does not interact with the lipid bilayer, the general assumption is that the encapsulated concentration of solute is the same as in the original solution; therefore, the encapsulated volume of aqueous phase can be calculated directly. For inert solutes, this assumption is generally reasonable. The exceptions are small unilamellar liposomes (8) and poorly hydrated multilamellar liposomes in which the water of hydration, which may exclude the solute somewhat, is a significant fraction of the total encapsulated aqueous phase. This should not be the case as long as $E_p \geq 1.0$.

The value of the partial specific volume of the hydrated lipids (Eq. 5) will never be significantly less than or greater than unity. For example, the value for phosphatidylcholine is 0.98 (8). A reasonable assumption is that $\bar{v} = 1$ (9, 10), even though the area and volume per molecule in mixed lipid liposomes can vary substantially. Thus, the total volume of lipid can be approximated reasonably by the total mass of lipid used, regardless of the molecular weights of the component lipids.

An estimate of the frequency *versus* size distribution is essential for these calculations. This estimate can be obtained by electron microscopy using either negative stain or freeze–fracture techniques. Olson *et al.* (7) showed that both procedures give essentially the same frequency *versus* size distributions over a wide range of liposome diameters. (They also discussed the limitations of both techniques.) Estimates of both $r_{\bar{v}}$ and η are obtained directly from the electron photomicrographs of the liposome sample. An estimate of $r_{\bar{v}}$ also can be obtained using laser light-scattering techniques (11). The value of $r_{\bar{v}}$ is calculated using Eq. 14, and the value of η is calculated from:

$$\eta = \frac{\sum_{i=1}^{k} N_i R_i^3}{\sum_{i=1}^{k} N_i R_i^2} \qquad \text{(Eq. 25)}$$

where k is the sample size.

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