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DIELECTRIC STUDIES ON HOMOGENEOUS PHOSPHATIDYLCHOLINE VESICLES

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SUMMARY

Dielectric measurements carried out on phospholipid vesicles about 280 Å in diameter suspended in dilute aqueous salt solution show a dispersion centered at about 40 MHz which is analogous to the "β-dispersion" observed in suspensions of biological cells and organelles. Measurements over a wide frequency range reveal no "α-dispersion", provided the vesicles have zero net charge. Analysis of the electrical characteristics of the suspension based on the Pauly–Schwan equations gives values of 3.3 to 4.2 μF/cm^2 for the capacitance of the phospholipid shell. The homogeneous suspensions of phospholipid vesicles were prepared from purified egg phosphatidylcholine according to the procedure of HUANG[3].

INTRODUCTION

Aqueous dispersions of phospholipids have been used in recent years as model membrane systems[1]. However, most of these preparations consisted of heterogeneous populations of multilamellar structures[2]. HUANG[3] has described the formation and physical properties of a dispersion of single-walled, homogeneous phosphatidylcholine vesicles in dilute aqueous salt solution that is suitable for investigation by the general physico-chemical techniques utilized in the study of macromolecules and aqueous suspensions of small cells, subcellular organelles and viruses. Recently the preparation and dielectric investigation of single-walled vesicles of fairly uniform size formed from the mixed soybean phospholipids have been described[4–6].

Dielectric measurements on suspensions of biological tissues have provided valuable information about the electrical impedances and structures of cellular membranes and also the conductances of the intercellular fluids and cytoplasms[7,8]. It was considered that similar studies on the homogeneous phospholipid system[3] would yield information concerning the electrical properties of the phospholipid shell and the interior aqueous compartment. These data could then be compared with the literature data for cellular suspensions and planar bilayers, since this homogeneous dispersion can be regarded as a model for an aqueous suspension of cell membrane ghosts, albeit the size of the phospholipid vesicles is approximately one order of magnitude smaller than the pleuropneumonia-like organisms[9].

In a previous dielectric study of vesicle membranes, membrane dielectric constants in the range 10–30 have been reported[6]. However, these vesicles carried a fixed net charge and a pronounced dielectric dispersion due to counter ion relaxation was observed. This made it difficult to determine the static dielectric capacitance value needed to evaluate the shell capacitance and dielectric constant. The system reported here has zero fixed net charge and thus affords a more precise determination of these parameters.

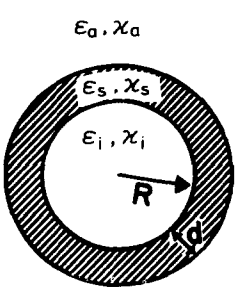
THEORY

The application of an electric field to a suspension of particles surrounded by a phospholipid shell will result in the charging of the shells, which act like electrical condensers. Since the accumulation of the charges on the lipid shell is a kinetic process, a frequence dependence of the moment of the induced dipoles will occur, and a Maxwell–Wagner relaxation of the impedance of the suspension will be observed. The general theory of the electrical impedance of a suspension of spherical particles surrounded by a shell has been formulated by PAULY AND SCHWAN[10]. In the brief outline of this theory given below the following symbols will be employed: ε, relative dielectric constant; κ, conductivity; ω, angular frequence of the applied field; T, relaxation time, which is inversely proportional to the characteristic angular frequence; R_i, inner radius of the vesicle; d = thickness of vesicle wall; $R_0 = R_i + d$, outer radius of the vesicle; P, volume fraction of vesicles in the suspension; v, ratio of aqueous interior compartment of the vesicle to the total vesicle volume.

The dielectric constant of the three-phase system represented by the aqueous suspension of uncharged phospholipid vesicles (see Fig. 1) can be described by Eq. 1. Explicit expressions have been formulated for each of the parameters ε_{10}, ε_{20}, ε_{200}, T_1 and T_2 in terms of the properties of the vesicle suspension. These expressions derive from the general solution of the Maxwell equations for this system, with no assumptions made about the relative magnitudes of R_0 and d[10,18].

$$\varepsilon = \frac{\varepsilon_{10} - \varepsilon_{20}}{1 + (\omega T_1)^2} + \frac{\varepsilon_{20} + (\omega T_2)^2 \varepsilon_{2\infty}}{1 + (\omega T_2)^2} \qquad (1)$$

The parameters used in this equation are defined in Fig. 2.



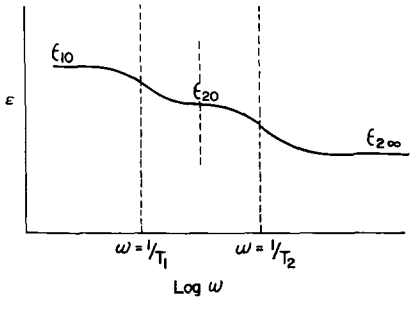


Fig. 1. Diagrammatic representation of a spherical phospholipid vesicle.

Fig. 2. Frequency dependence of the dielectric constant of a suspension of spherical particles surrounded by nonconducting shells, according to PAULY–SCHWAN theory.

EXPERIMENTAL

The electrical measurements over the frequency range 0.5–150 MHz were made with a Boonton Radio Corporation RX-meter, type 250-A. This instrument is designed to measure the equivalent parallel resistance and capacitance of the sample. The first cell to be used, a pin cell, contained approximately 1 ml of sample and the impedance of the suspension was measured between two cylindrical platinum electrodes. A smaller cell of 0.1-ml sample capacity with coaxial platinum electrodes was also employed to evaluate possible artifacts in the measured sample impedance arising from cell design factors. Both the pin cell[12] and the coaxial cell[9] have been described elsewhere.

The theory of the operation of the RX-meter and the calculation of the sample impedance from raw data have been described in detail in a previous publication[12]. The accuracy of the RX-meter declines markedly above 100 MHz and impedance measurements at higher frequencies were unreliable with this instrument.

The dielectric measurements at higher frequencies (280–500 MHz) were accomplished with the aid of a parallel wire transmission line. The theory of the operation of this apparatus and the calculation of sample impedance using the standing wave pattern technique have also been described previously[12]. The sample cell employed with the transmission line had a 20 ml sample capacity.

Phospholipid vesicles were prepared as follows[3]. Chromatographically pure egg yolk phosphatidylcholine, was lyophilized from benzene, then sonicated at 4° under nitrogen for 2.5 h in 10^{-3}–10^{-2} M aqueous KCl solution. The undispersed lecithin and titanium fragments from the sonicator probe were removed by ultracentrifugation at 105000 × g for 60 min at 4°. The clear suspension resulting from the ultracentrifugation step was then subjected to molecular sieve chromatography on a Sepharose 4B column (2.5 cm × 100 cm) at 4°. The absorption of column effluent at 300 nm was continuously monitored with a recording spectrophotometer and the effluent was collected in a refrigerated fraction collector. The elution pattern of the phosphatidylcholine suspension was similar in all respects to that reported by HUANG[3] and was comprised of two distinct fractions. The first fraction was eluted with the void volume of the column, while the second fraction distributed with the internal volume and was eluted as a broad symmetrical peak. The homogeneous phospholipid vesicle samples used for the dielectric studies were obtained from center cuts of the second fraction. These suspensions were concentrated by ultrafiltration using a diaflow apparatus (Amicon Co.) and stored at 4° under nitrogen. Before dielectric measure-

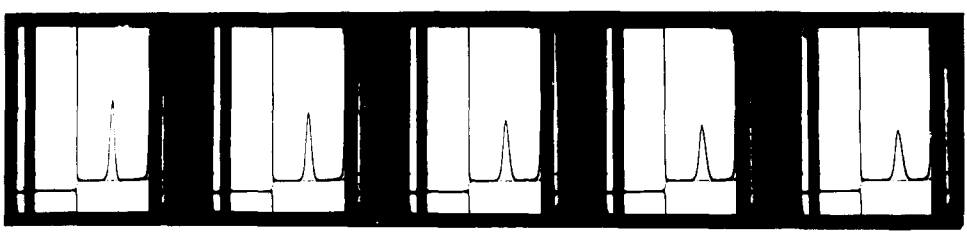


Fig. 3. Ultracentrifugal Schlieren pattern of a sedimentation velocity experiment on the phosphatidylcholine vesicle suspension. Initial concentration 1.0 g phospholipid per 100 ml. Rotor speed 42040 rev./min. Time between frames, 8 min. Phase plate angle, 70°.

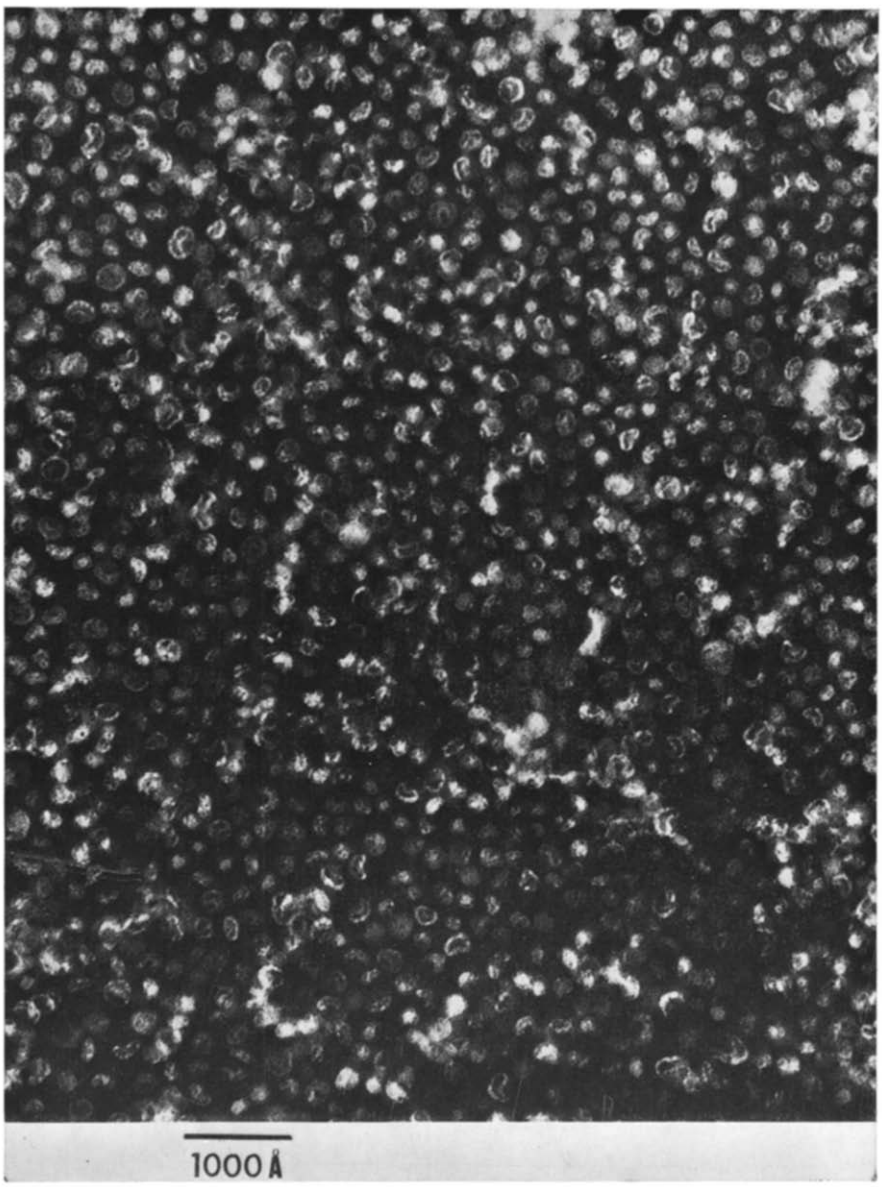


Fig. 4. Electron micrograph of phospholipid vesicles negatively stained with potassium phosphotungstate.

ments were taken, the samples were recentrifuged at 105 000 × *g* for 30 min at 4°, and then filtered through a 0.1-μm Sartorius membrane. Phospholipid concentrations were determined by assay of the inorganic phosphate content[14]. A small sample of the concentrated vesicle preparation was diluted with isotonic aqueous KCl solution and sedimentation velocity measurements were carried out at 20 ± 0.05° in a Spinco Model E ultracentrifuge, equipped with a RTIC temperature control unit, and a

Schlieren optical system with phase plate. A double sector capillary type synthetic boundary cell with a 12-mm optical path was used. Kodak metallographic plates were used to photograph Schlieren patterns which were then read on a two dimensional microcomparator. The homogeneity of the vesicle preparation was determined from the sedimentation velocity data using the Fujita analysis[15].

RESULTS AND DISCUSSION

A typical ultracentrifugal Schlieren pattern of a sedimentation velocity experiment on the phospholipid vesicle solution is shown in Fig. 3. A Fujita analysis of these patterns gave a value of less than $0.5 \cdot 10^{-14}$ sec for the heterogeneity parameter characterizing the size distribution of the vesicles. Although these vesicles were formed in 1 mM KCl, the sedimentation results are in accord with those obtained by HUANG[3] for phospholipid dispersions in 10^{-1} M NaCl. An electron micrograph of this homogeneous preparation negatively stained with potassium phosphotungstate is presented in Fig. 4. At this magnification, it is possible to observe the uniformity of vesicle size in the suspension. In addition, each vesicle can be seen to be surrounded by a single phospholipid lamella. Quantitative information regarding the size of the vesicles, however, requires higher magnification.

The frequency dependence of the dielectric constant of the phospholipid vesicle dispersion is illustrated in Fig. 5a. These data were obtained with the pin cell. Results obtained with the coaxial cell were essentially identical. A small but measurable dielectric dispersion occurs over the frequency range shown. No measurable increase in dielectric constant occurs with this system in the frequency region 100 kHz to 1 MHz. This was confirmed using a Siemens Radio Frequency bridge. The existence of this "β-dispersion" in the dielectric properties of the homogeneous phospholipid dispersion supports the view that the vesicles are indeed closed structures bounded by a non-conducting shell[7].

If approximately 5 % by weight of stearic acid is added to the purified phosphatidylcholine prior to sonication, however, and the procedure for the vesicle preparation followed, an additional dielectric dispersion below 1 MHz is then observed (Fig. 5b). Sedimentation velocity experiments on this charged vesicle preparation indicated that the sample was homogeneous. Incorporation of stearic acid into the homogeneous vesicles was confirmed by thin layer chromatographic analysis of the aqueous suspension, using Brinkman silica gel G plates and the solvent system: chloroform–methanol–water (65:25:4, by vol.). This low frequency dispersion can, therefore, be attributed to relaxation of the counterion atmosphere around the negatively charged vesicles. It is analogous to the "α-dispersion" seen with biological material,[11] and similar to the large "α-dispersion" described by SCHWAN *et al.*[6] for charged vesicles formed from mixed soybean phospholipids.

The absence of the "α-dispersion" in the homogeneous vesicle system prepared from purified phosphatidylcholine permits quantitative measurements to be made on the "β-dispersion". Fig. 6 shows the dielectric increment of the "β-dispersion" for these uncharged phospholipid vesicles to be approximately proportional to the volume fraction of the vesicles in the suspension as predicted by the Pauly-Schwan theory[10]. In order to determine the unknown parameters defined in Fig. 1, it is expedient to to assume that the phospholipid shell has an extremely low conductivity, similar to

the value for wet hydrocarbons. Then there are essentially only three unknown electrical parameters. These are the dielectric constants of the phospholipid shell (ε_s) and the dielectric constant (ε_i) and the conductivity (κ_i) of the interior aqueous compartment. For each set of dielectric constant *versus* frequency measurements, the dielectric constant (ε_a) and conductivity (κ_a) of the suspending medium are recorded and the weight concentration of the phospholipid in the suspension is determined by inorganic phosphate assay.

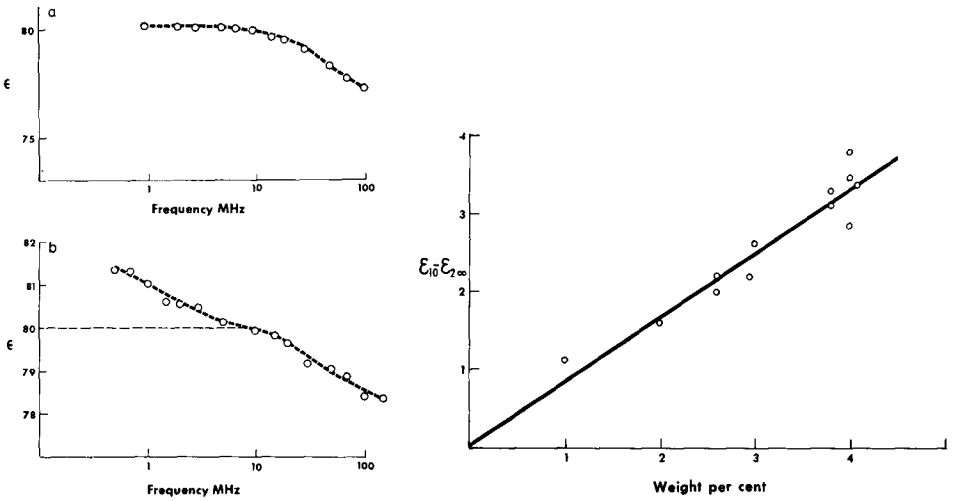


Fig. 5. Frequency dependence of the dielectric constant for phospholipid vesicle suspensions in 10^{-3} M aqueous KCl at 17°. a. 0.040 g phospholipid per ml. b. 0.0246 g phospholipid + 0.0014 g stearic acid per ml.

Fig. 6. Dielectric increment of the "β-dispersion" as a function of the concentration of the phospholipid vesicles in the suspension. Temperature 20 ± 1°. Correlation coefficient, 0.975.

The two parameters P and v, employed in the general theory developed by PAULY AND SCHWAN,[10] are functions of the vesicle diameter R_0 and shell thickness d. Thus P, the volume fraction of vesicles in suspension, is related to the weight concentration of phospholipid Q, by the following equation:

$$P = \frac{\bar{v}R_0^3Q}{R_0^3 - R_i^3} \tag{2}$$

Here $R_i = R_0 - d$ and $\bar{v}$ is the partial specific volume of the phospholipid. v, the ratio of the volume of the interior aqueous compartment to the total volume of the vesicle (see Fig. 1), is given by the expression:

$$v = (R_i/R_0)^3 \tag{3}$$

Values of v and the factor $\bar{v}R_0^3/(R_0^3 - R_i^3)$ calcultated for values of R_0 and d determined by several different experimental methods are given in Table I.

The phosphatidylcholine vesicles can only be approximated by the simple three layer dielectric model of Fig. 1. The electron density of the phospholipid bilayer,

in fact, varies from a maximum in the polar head group region to a minimum at the terminal methyl groups[16]. However, in order to apply the analysis of PAULY AND SCHWAN[10] it is necessary to consider the phospholipid shell as a dielectric element of definite thickness (d) and uniform electrical properties (ε_s, κ_s). In Table I the thickness (d) is taken as the peak to peak distance in the electron density profile for hen egg phosphatidylcholine determined by recent X-ray diffraction studies[16,21].

TABLE I

SIZE PARAMETERS FOR PHOSPHOLIPID VESICLES

$\bar{v}$ = 0.9885 ml/g (ref. 3).

$R°$ (Å)	d (Å)	$\frac{\bar{v}R_0^3}{R_0^3-R_i^3}$ (ml/g)	v
150*	35+	1.80	0.451
150	40++	1.63	0.394
140**	35	1.71	0.422
140	40	1.55	0.369
125***	35	1.58	0.373
125	40	1.44	0.314

* Electron microscopy, negative staining[3].
** Molecular sieve chromatography[3].
*** Electron microscopy, freeze etching[3].
\+ High resolution X-ray diffraction[16].
++ X-ray diffraction[21].

The analysis to determine the unknown electrical properties of the vesicles proceeds as follows. The size parameters (Table I) and the experimental values for ε_a, κ_a and the vesicle concentration are programmed as input parameters into a Fortran subroutine for the complete PAULY–SCHWAN[10] equations describing the impedance of a suspension of spherical particles surrounded by a shell. A value of $10^{-14}\ \Omega^{-1}\cdot cm^{-1}$ assumed for κ_s, as indicated previously*, and realistic estimates of the parameters ε_s, ε_i and κ_i are inserted. The experimental data for the frequence dependence of the dielectric constant of the suspension are programmed into a non-linear least squares analysis designed to fit the theoretical dielectric dispersion (eqn. 1) to the experimental data. The computer program uses a NEWTON–RAPHSON[17] iterative procedure to minimize the sum of the squares of the residues, namely, $\Sigma[\varepsilon(\text{calculated})-\varepsilon(\text{experimental})]^2$ by systematically changing the values of the three unknown electrical parameters ε_s, ε_i and κ_i until the best estimates are obtained. The program output provides the frequency profile of the dielectric constant for the best fit of the experimental data according to the PAULY–SCHWAN theory, together with a listing of the corresponding computer estimates of the unknown electrical parameters describing the phospholipid shell and interior aqueous compartment of the phospholipid vesicle.

* The choice of this value can be shown to have virtually no effect on the computed values of ε_s, ε_i, and κ_i.

An example of the theoretical fit to the experimental data is represented by the solid line in Fig. 7. It should be noted that the computed frequency profile for the dielectric constant generates the double dielectric dispersion predicted by the PAULY–SCHWAN theory as shown in Fig. 2. Moreover, the computer fit to the data appears quite satisfactory. (This computed line should not be compared with the dotted lined drawn to smooth the data in Fig. 5a and b.) The computed values of ε_s, ε_i and κ_i for the experimental data shown in Fig. 7 are listed in Table II. The P values of 0.073 and 0.059 in Table II correspond to the upper and lower values of $\bar{v}R_0^3/(R_0^3-R_1^3)$ listed in Table I; the P value of 0.066 corresponds to the mean value of this ratio, 1.62 ml·g^{-1}. The computed frequency profile of the dielectric constant in Fig. 7 corresponds to the P-value of 0.066 with a statistical sum of the squares of the residues (minSSR) of $3.24 \cdot 10^{-1}$.

Inspection of Table II reveals that the choice of v does not influence the sum of the squares of the residues (minSSR) in the computer fits. The computer fit for a given value of P is, in fact, common to the class of solutions of the PAULY–SCHWAN

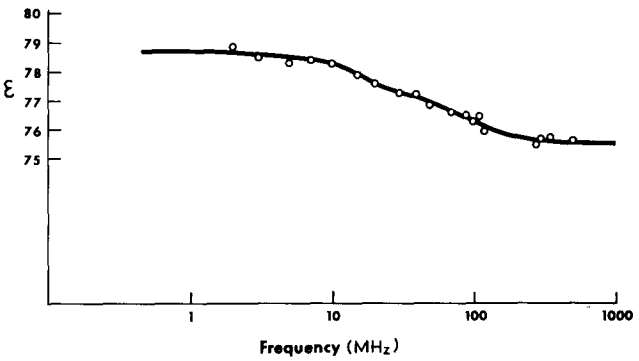


Fig. 7. Frequency dependence of the dielectric constant for a phospholipid suspension in $7.5 \cdot 10^{-3}$ M aqueous KCl at 21.3°, 0.0408 g phospholipid per ml. The solid line indicates the computer fit for $P = 0.066$ in Table II.

TABLE II

COMPUTER ESTIMATES OF ELECTRICAL PARAMETERS OF VESICLES

Vesicle concentration, 0.0408 g/ml. ε_a, 79.7; $\kappa_a = 9.7 \cdot 10^{-4}\,\Omega^{-1}\cdot cm^{-1}$; $\kappa_s = 10^{-14}\,\Omega^{-1}\cdot cm^{-1}$; temp., 21.3° (data from Fig. 7).

P	v	ε_i	κ_i ($\cdot 10^3$) ($\Omega^{-1}\cdot cm^{-1}$)	ε_s	*MinSSR*
0.073	0.451	104 ± 9.4	9.5 ± 0.8	14.0 ± 0.1	$3.35 \cdot 10^{-1}$
0.073	0.422	102 ± 9.4	9.5 ± 0.8	15.2 ± 0.1	$3.35 \cdot 10^{-1}$
0.073	0.373	97.6 ± 9.3	9.3 ± 0.8	17.4 ± 0.1	$3.35 \cdot 10^{-1}$
0.066	0.451	74.3 ± 6.4	6.7 ± 0.5	13.7 ± 0.1	$3.24 \cdot 10^{-1}$
0.066	0.422	72.5 ± 6.4	6.7 ± 0.5	14.8 ± 0.1	$3.24 \cdot 10^{-1}$
0.066	0.373	68.5 ± 6.4	6.6 ± 0.5	17.0 ± 0.2	$3.24 \cdot 10^{-1}$
0.059	0.451	49.1 ± 4.5	4.7 ± 0.3	13.3 ± 0.1	$3.36 \cdot 10^{-1}$
0.059	0.422	47.5 ± 4.5	4.7 ± 0.3	14.4 ± 0.2	$3.36 \cdot 10^{-1}$
0.059	0.373	44.0 ± 4.5	4.6 ± 0.3	16.5 ± 0.2	$3.36 \cdot 10^{-1}$

equations for different values of ν. It is, therefore, possible to generate sets of ε_s values for different choices of d, each corresponding to the computer fit shown in Fig. 7.

ε_s can also be calculated directly from the values of P, ν, ε_a and the dielectric constant of the suspension at low frequency, ε_{10}, using the following equation developed by OLDENBURG[18]:

$$\varepsilon_s = \frac{(2 + P)(1 - \nu)[\varepsilon_{10}(2 + P) - 2(1 - P)\varepsilon_a]}{9P(1 + 2\nu)} \quad (4)$$

Examples of values obtained in this manner are listed in Table III for the values $P = 0.066$, $\varepsilon_{10} = 78.78$ and $\varepsilon_a = 79.70$. It is apparent that they are identical to the computer fit values within the limits of experimental error. It should be noted that ε_s is significantly larger than the value of about 2 reported for planar bilayer membranes generated from hen egg phosphatidylcholine in decane[19]. The higher value of ε_s may be due to the absence in the vesicle bilayer of free hydrocarbon solvent of low dielectric known to be present in the planar bilayer[1]. The high value may also reflect a difference in molecular packing in the two systems caused by the enormously different radii of curvature of the two types of bilayers. The large value of ε_s may possibly reflect the association of water with the phospholipid shell of the vesicles[20]. Measurements of the preferential hydration of similar phospholipid vesicles recently reported by HUANG AND CHARLTON[20] indicate that about 0.51 g of water per g phospholipid vesicles is preferentially bound to the vesicles in dilute monovalent salt solutions.

In a description of the dielectric properties of membrane systems, it is conventional to refer to C_M, the capacitance per unit of membrane. An approximate expression for C_M in $\mu F/cm^2$ is[22]:

$$C_M = \frac{\varepsilon_s}{9\pi 10^{11}} \cdot \frac{R_0 R_i}{R_0 - R_i} \frac{1}{(R_0 + R_i)^2} \quad (5)$$

Values of C_M listed in Table III are calculated with Eqn. 5. It can be seen that the shell capacitance lies between 3.3 and 4.2 $\mu F/cm^2$ and to a first approximation is not a function of d. The range of C_M values reflects the uncertainty in R_0. These values are significantly higher than the values reported for planar lipid bilayer mem-

TABLE III

ESTIMATES OF SHELL PROPERTIES OF VESICLES

Calculated from Eqn. 4. P, 0.066; ε_{10}, 78.78; ε_a, 79.70.

R_0 (Å)	d (Å)	ν	ε_s	C_M ($\mu F/cm^2$)
150		0.451	13.9	3.4
140	35	0.422	15.1	3.7
125		0.373	17.3	4.2
150		0.512	11.6	3.4
140	30	0.485	12.6	3.7
125		0.439	14.4	4.2
150		0.579	9.4	3.3
140	25	0.554	10.2	3.6
125		0.512	11.6	4.1

branes containing neutral hydrocarbon solvent[1], yet lie within the range observed for biological membranes (0.5 to 5 μF/cm^2)[8].

The computed values of ε_i and κ_i unlike the values for ε_s are quite sensitive to the choice of P (see Table II). On the other hand, for a given value of P there is little dependence on ν. The computed values of ε_i for the mean value of $P = 0.066$ lie close to the value for ε_a, 79.7. Within the permissible range of R_0, however, the computed values of ε_i vary considerably, and consequently it is not possible to decide whether ε_i is significantly different from the solvent value, ε_a. It is interesting to note that the computed value of κ_i is consistently higher than the solvent value, κ_a.

Whether or not a higher ion concentration exists within the interior aqueous compartment has not been determined. A significantly higher internal salt concentration would be reflected in a lower value for ε_i than the bulk value, ε_a. This possibility cannot be precluded due to the uncertainty in R_0, which results in the range of ε_i values reported in Table II. On the basis of the available data, therefore, it is not possible to elucidate the structure of the water in the micro compartment within the phospholipid vesicles. It appears improbable, however, that a significant proportion of this trapped aqueous volume is of the "bound" type.

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