## BBA Report

BBA 71080

# Single bilayer liposomes

S.M. JOHNSON, A.D. BANGHAM, M.W. HILL and E.D. KORN*

*Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge (Great Britain)*

(Received March 26th, 1971)

SUMMARY

Further investigation has revealed that the liposomes formed by highly sonicated 4% phosphatidic acid/96% phosphatidyl choline in 0.16 M KCl are single bilayer spherical shells, with a Stokes' radius of 120 ± 4 Å.

Johnson and Bangham[1] recently described the preparation of highly sonicated liposomes consisting of 96% phosphatidyl choline and 4% phosphatidic acid which showed permeability properties characteristic of vesicles bounded by a single skin, but which by electron microscopy appeared to have a large space surrounded by two closely applied concentric bimolecular membranes. It was argued from the permeability data and from the measured values of surface area, obtained by the method described by Bangham *et al.*[2], that the space between the two apparent skins was negligible compared to the central compartment and that in effect the permeability was being measured across two membranes. We now believe that the liposomes are indeed bounded by a single bilayer. In support of our reappraisal we have measured the diffusion coefficient of the liposomes in 0.16 M KCl and from it calculated the equivalent sphere radius to be 120 ± 4 Å, a value too low for any vesicle with a double bilayer.

Phosphatidyl choline was extracted from hens eggs, and phosphatidic acid prepared from it by enzymic hydrolysis, the phospholipids were stored in chloroform under nitrogen at -10°. Water was twice distilled in borosilicate glass apparatus, the second time from $KMnO_4$. Other chemicals used were reagent grade.

Liposomes were prepared by evaporating the chloroform under vacuum from up to 120 μmoles of mixed phospholipids in a 100-ml round-bottomed flask. 0.6 ml of 0.16 M NaCl or other salt, *e.g.* ammonium acetate was added to the dry phospholipid and the flask filled with nitrogen. The viscous suspension was transferred to a 1.3-cm-diameter flat-bottomed tube, and sonicated under an atmosphere of nitrogen for 0.5 to 1.5 h in

*National Institutes of Health, Bethesda, Md. 20014, U.S.A.

a Kerry's ultrasonic cleaning bath type KB 80/1 at 80 kcycles·sec$^{-1}$ until the suspension became clear and bluish. It was left overnight in the unopened vials before use.

*Electron microscopy*

Sonicated liposomes negatively stained with ammonium molybdate typically seem to have two limiting membranes (single arrows, Fig. 1, and ref. 1). But when the same preparation of sonicated, stained liposomes is mounted on the grid in the presence of as little as 0.02% albumin (1 mg of albumin:4 mg of phospholipid) only one limiting membrane is seen (Fig. 2). We believe both images can be derived from a sonicated liposome whose structure is that of a single spherical bilayer membrane as illustrated in Fig. 3.

When negatively stained in the absence of protein the spherical bilayers

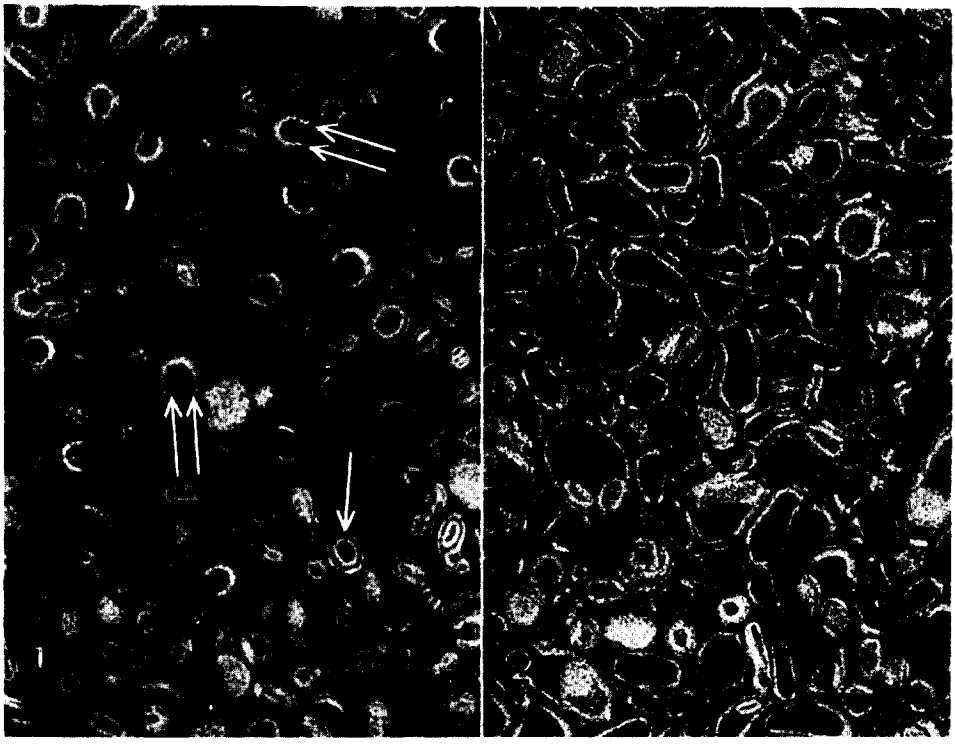

Fig. 1 (left). Negatively stained sonicated liposomes. Sonicated liposomes were diluted to a concentration of 1 μmole of phospholipid per ml of 0.5% ammonium molybdate, gently sprayed onto a carbon–colloidon grid, and allowed to dry in air. Micrographs were taken on a Siemens Elmiskop 1A at an instrumental magnification of 40 000. The micrograph is interpreted in the text. The bar is 0.1 μm × 140 000.

Fig. 2 (right). Sonicated liposomes negatively stained in the presence of protein. The same preparation of sonicated liposomes used in Fig. 1 was diluted to 1 μmole of phospholipid per ml of 0.5% ammonium molybdate which contained 0.02% albumin. Other procedures were the same as in Fig. 1. The bar is 0.1 μm × 140 000.

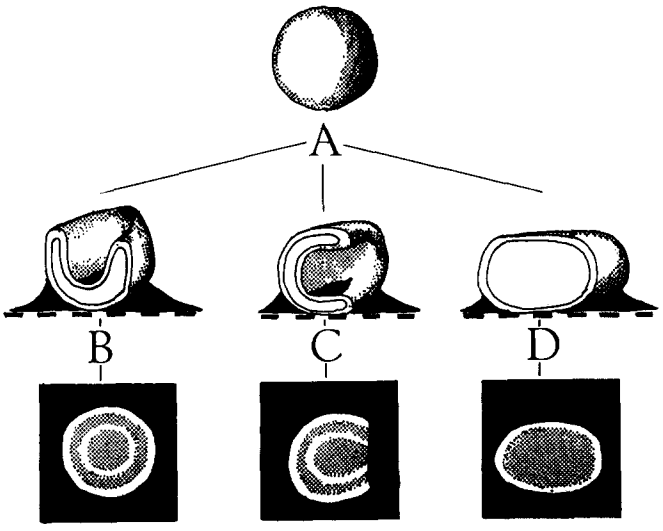


Fig. 3. Schematic representation of events which might occur during negative staining of spherical, single bilayer sonicated liposomes to give rise to the micrographs shown in Figs. 1 and 2. The top row illustrates a spherical liposome in suspension. The second row illustrates configurations the liposomes might assume on the electron microscopic grid in the absence of albumin (B and C) and in the presence of albumin (D). In each case the liposome has been bisected to reveal the configuration of the single bilayer membrane. The bottom row illustrates the electron microscopic image to be expected when an electron beam passes through the liposomes from top to bottom. It is assumed that electron dense ammonium molybdate surrounds the liposome and fills its aqueous interior.

(Fig. 3A) may collapse, much as footballs from which the air has been removed, to form cup-like structures (Fig. 3B). These could give an electron microscopic image (Fig. 3C, Fig. 1) essentially indistinguishable from that of a true double membrane liposome. In some cases the collapsed sphere would lie on its side and give rise to the images identified by the double arrows in Fig. 1. When negatively stained in the presence of albumin the spherical bilayers must flatten (Fig. 3D) to produce the microscopic image illustrated in Fig. 3E and seen in Fig. 2.

We assume that ammonium molybdate fills the aqueous interior of the liposome and surrounds the flattened or collapsed liposome. The electron translucent lines are taken to be the central hydrocarbon regions of the phospholipid bilayers.

*Surface area measurements*

Uranyl ions adsorb specifically onto a 4% phosphatidic acid/96% phosphatidyl choline monolayer even in the presence of 0.16 M univalent salt. The surface charge of the monolayer is altered and hence the surface potential $\psi_G$ (refs. 1, 2).

The relation between surface potential and surface charge density is given by the Gouy equation

$$\psi_G = \frac{2kT}{\epsilon} \sinh^{-1}\left\{\frac{\sigma}{c_i^{1/2}}\left(\frac{500\pi}{DRT}\right)^{1/2}\right\} \quad (1)$$

$c_i$ is the uni/univalent ionic concentration in moles $\cdot$ $l^{-1}$, $k$ is Boltzmann's constant, $T$ the absolute temperature, $\epsilon$ the electronic charge, $R$ the gas constant and $D$ the dielectric constant of water. $\sigma$ is the surface charge density. As before uranyl nitrate was used to change the surface potential of a monolayer of phospholipid above an unknown area (about 2 $\mu$moles) of highly sonicated phospholipid in 10 ml 0.16 M KCl at pH 5.8 (ref. 2). The surface potential and charge were taken as zero initially, and the change in $\psi_G$ noted as the concentration of uranyl ion is increased. At 20° the Gouy equation gives for $\Delta\psi_G$ in mV:

$$\sinh \frac{\Delta\psi_G}{50.54} = \frac{\sigma \times 2.8312 \times 10^{-5}}{\sqrt{0.16}} \qquad (2)$$

Now the amount of uranyl free in solution and on the phospholipid monolayer is negligible in comparison to the amount on the very large surface area of the liposomes in this system. Let the surface area of the liposomes be $a$ cm$^2$, and the number of moles of uranyl nitrate added be $u$. $z$ is the number of charges added to the monolayer per uranyl group.

$$\sigma = \frac{z \epsilon u \times 6.024 \times 10^{23}}{a} \qquad (3)$$

If $z = 2$ and $\epsilon = 4.803 \cdot 10^{-10}$ e.s.u. then $\sigma = \dfrac{u \times 5.787 \times 10^{14}}{a}$

Substituting for $\sigma$ in (2),

$$\sinh \frac{\Delta\psi_G}{50.54} = \frac{u \times 1.638 \times 10^{10}}{a\sqrt{0.16}}$$

$a$ can be calculated from the gradient of the graph of sinh $\Delta\psi_G/50.54$ against $u$. Values of $a$ calculated in refs. 2–4 are too low by a factor of 2 because the areas were worked out for $z = 1$. However, in none of these papers is the conclusion materially affected.

The measured limiting surface area per $\mu$mole quoted in ref. 1 now becomes 2830 cm$^2$, for an internal volume of $3.08 \cdot 10^{-4}$ cm$^3$. The thickness, $\delta$, of a single bilayer of phosphatidyl choline was measured by Bangham and Horne[5] from an electronmicrograph of a myelin preparation negatively stained with sodium phosphotungstate. They obtained a value of 44 Å, consistent with the bilayer thickness in Figs. 1 and 2.

Assuming a spherical particle the internal radius $r_c$ can be calculated from the volume area ratio as in ref. 1.

$$\frac{r_c^3}{3(r_c + \delta)} = \frac{\text{Volume per } \mu\text{mole}}{\text{Area per } \mu\text{mole}}$$

if $\delta = 44$, $r_c = 79$, and the external radius $r = r_c + \delta = 123$ Å.
The fraction of phospholipid molecules facing outwards

$$= \frac{r^2}{r^2 + r_c^2}$$

$$= 0.708$$

Hence the surface area per molecules is $\frac{2830 \times 10^{16}}{6.024 \times 10^{17} \times 0.708}$

$$= 66 \text{ Å}^2$$

Values of $r_c$, $r$ and $A$ were calculated for a ± 5% error in the thickness, volume and area measurements. The most extreme combination of all three uncertainties gave limits of

$$73 < r_c < 86$$
$$115 < r < 132$$
$$64 < A < 69$$

The area per molecule should be compared to that reported by Small[6], who from X-ray diffraction data obtained a value of 71.7 Å for unsonicated phosphatidyl choline in water. The difference between these two values could be due to a real physical difference between the two systems, or to an error inherent in the uranyl method of measuring surface area. One such source of error can be described. The surface area per egg phosphatidyl choline molecule in a collapsed monolayer is given by Shah and Schulman[7] as 62 Å$^2$, and by Watkins[8] as 56 Å$^2$, which is appreciably smaller than that for the same molecule in a liposome. Hence the surface charge densities at a given uranyl ion concentration probably differ. The liposome internal volume quoted above is that for trapped $K^+$, but the sucrose volume is similar. Using Small's[6] value for the thickness of a fully hydrated phosphatidyl choline bilayer, 45.6 Å, together with his surface area per molecule, the values of $r_c$ and $r$ can be calculated as 76 and 121.6, respectively.

The diffusion of the sonicated 4% phosphatidic acid/96% phosphatidyl choline liposomes in 0.16 M KCl at 20° was studied in a Beckman Model E ultracentrifuge using a synthetic boundary cell. The diffusion coefficient $D$ was measured as a function of liposome concentration and $D_{20,\,KCl}$ found by extrapolation (see Fig. 4).

$$D_{20,w} = \frac{\eta_{KCl}}{\eta_{H_2O}} \cdot D_{20,KCl} = 1.76\ (\pm 0.05)\ \cdot 10^{-7}$$

The Stokes' radius $r_s$ was found from the Stokes' Einstein diffusion equation

$$r_s = \frac{kT}{6\pi\eta D}$$

$\eta$ is the viscosity of the solvent.

$$r_s = 120 \pm 4 \text{ Å}$$

where ± indicates the 95% confidence limits.

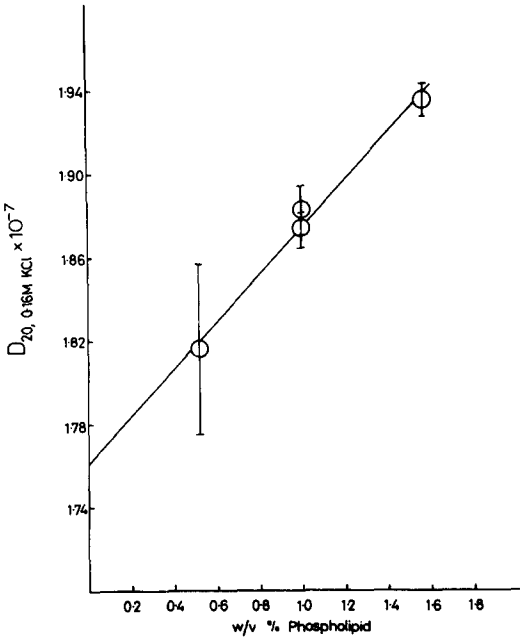


Fig. 4. The diffusion coefficient in 0.16 M KCl at 20° for 4% phosphatidic acid/96% phosphatidyl choline liposomes, plotted as a function of liposome concentration. The error bars show the standard deviation of each $D$ value. $D$ in $cm^2 \cdot sec^{-1}$.

This result is comparable to the Stokes' radius values given by Huang[9] for phosphatidyl choline liposomes, which is 114 ± 5 Å.

The unusual increase of the diffusion constant with liposome concentration shown in Fig. 4 does not occur in 0.16 M NaCl and is believed to be due to the water structure breaking property of the $K^+$ (see Johnson and Buttress[10]).

The agreement of the Stokes' radius $116 < r_s < 124$ with the value of $r$ calculated from the volume/area ratio, $115 < r < 132$ indicates that the particles are probably spherical. If they were ellipsoidal $r$ would become smaller for a given particle area whereas $r_s$ would become larger, giving a ratio of $r_s/r > 1$. However, the possibility of some asymmetry cannot be eliminated on these figures.

We conclude from these results that the 4% phosphatidic acid/96% phosphatidyl choline liposomes in 0.16 M KCl are single bilayer shells, external radius 120 ± 4 Å, internal radius 73–86 Å.

REFERENCES

1 S.M. Johnson and A.D. Bangham, *Biochim. Biophys. Acta*, 193 (1969) 82.
2 A.D. Bangham, J. De Gier and G.D. Greville, *Chem. Phys. Lipids*, 1 (1967) 225.
3 A.D. Bangham, *Progr. Biophys. Mol. Biol.*, 18 (1968) 31.
4 S.M. Johnson and A.D. Bangham, *Biochim. Biophys. Acta*, 193 (1969) 92.
5 A.D. Bangham and R.W. Horne, *J. Mol. Biol.*, 8 (1964) 660.

6 D.M. Small, *J. Lipid Res.*, 8 (1967) 551.
7 D.O. Shah and J.H. Schulman, *J. Lipid Res.*, 6 (1965) 341.
8 J.C. Watkins, *Biochim. Biophys. Acta*, 152 (1968) 293.
9 C. Huang, *Biochemistry*, 8 (1969) 344.
10 S.M. Johnson and N. Buttress, in preparation.

*Biochim. Biophys. Acta*, 233 (1971) 820–826