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POTASSIUM PERMEABILITY OF SINGLE COMPARTMENT LIPOSOMES WITH AND WITHOUT VALINOMYCIN

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SUMMARY

Methods which permit permeability coefficients of 4% phosphatidic acid–phosphatidyl choline liposomes to be measured over the region $1 \cdot 10^{-9}$–$1 \cdot 10^{-13}$ cm·sec^{-1} have been developed and are here described. The K^+ permeability of liposomes in the presence of very small quantities of valinomycin has been studied, and the results indicate a carrier hypothesis.

INTRODUCTION

The diffusion of univalent cations through the spontaneously formed liquid crystals (liposomes) of phosphatidyl choline and phosphatidic acid has been described and measured by previous workers, in particular by BANGHAM *et al.*[1] and by PAPAHADJOPOULOS AND WATKINS[2]. However, their data suffered from four serious disadvantages. Firstly, the results could not be expressed as true permeabilities or fluxes, since the surface area of the dispersions was not measured. Secondly, the reproducibility of the measured variables was very poor; for example PAPAHADJOPOULOS AND WATKINS[2] quote K^+ self-diffusion flux values for phosphatidyl choline liposomes varying from 0.4 to 4.0 mequiv per mole of lipid per hour, depending on the method of preparation of the dispersion. Higher K^+ flux values might be expected from sonicated lipid dispersions with larger surface areas, but the opposite results were obtained. This indicates the third disadvantage, namely the susceptibility of large liposomes to mechanical rupture. And finally, the fourth disadvantage arises from the many concentric components of the large liposomes; if the permeability of the lipid is high, the isotope in the outermost compartment is reinforced by diffusion from the inner compartments, and the exchange kinetics become too complex for analysis.

To overcome these difficulties a method has been developed which gives the true permeability or flux from a one-compartment system, giving more repeatable results and allowing the measurement of permeability coefficients over four orders of magnitude.

MATERIALS AND METHODS

Reagents

Phosphatidyl choline was extracted from egg yolks and purified by using alumina and silicic acid chromatography[1]. Phosphatidic acid was prepared from the phospha-

tidyl choline by enzymic hydrolysis with an extract of Savoy cabbage, followed by chromatography on silicic acid[3]. Valinomycin was a gift from Dr. J. MacDonald, Prairie Research Laboratory, Saskatoon, Saskatchewan, Canada. All other reagents were A.R.; distilled water was redistilled from $KMnO_4$ in borosilicate glass apparatus.

The Forbes bar

A 12 cm × 13.5 cm × 43.5 cm steel bar, well insulated by polystyrene except for the top surface, was thermostatically controlled at either end by insertion into the sides of water baths. Bored into the top surface of the bar was a double row of 15 holes (1.9 cm × 10 cm) to hold test tubes and a central row of smaller holes for temperature measurement. Liquid paraffin in the holes helped thermal contact and prevented rusting. When the two water baths were set at 64 and 16° respectively, the apparatus gave a linear temperature gradient between 22 and 52°.

Preparation of liposomes

A mixture containing a 4% molecular proportion of phosphatidic acid to phosphatidyl choline in chloroform was used as the stock lipid solution. For experiments with liposomes of low permeability, less than $1 \cdot 10^{-11}$ cm·sec^{-1}, the simple exchange method was used. 100 μmoles of lipid were taken to dryness under reduced pressure and shaken with 1 ml of 0.155 M KCl solution containing about 40 μC $^{42}K^+$. The milky suspension was then sonicated for between 1 and 1.5 h below 30° in a flat-bottomed 150-ml flask under N_2, using a Kerry's ultrasonic cleaning bath type KB 80/1 at 80 kcycles/sec; it was then left to equilibrate for at least 16 h at room temperature. The now faintly opalescent blue-tinged suspension was passed down a 1.5 cm × 30 cm column containing 3 g Sephadex G-50 in 0.16 M KCl, and twelve 1-ml portions of the ionically filtered dispersion were measured into 8/32 Visking dialysis tubing, which was knotted to form a bag enclosing a small bubble to assist stirring. Meanwhile 10-ml portions of isotope-free 0.16 M KCl had been allowed to equilibrate in the Forbes bar at 12 incremental temperatures between 27 and 52°. Then the bags were each dropped into a tube at a particular temperature and left for 20–30 min in two sequential 10-ml portions, to ensure thermal equilibrium, before finally being allowed to leak isotope for 1–3 h in a third tube, during which period they were shaken at least once every 15 min. At the end of the leak, the bags were removed and the liposomes released into a further set of tubes containing 10 ml of isotope-free 0.16 M KCl. The samples were counted in a 10-ml M6 tube with Panax counting equipment.

For the experiments using high permeability liposomes with, for example, valinomycin, the cumulative exchange method was used. Stock solution containing 80 μmoles lipid was mixed with the appropriate volume of a 182 nM valinomycin solution in chloroform, evaporated to dryness and dispersed in 1 ml of 0.155 M KCl with 40 μC $^{42}K^+$. After the usual sonication and equilibration, the liposomes were passed over a Sephadex column equilibrated with 0.16 M KCl, and the ionically filtered liposomes pipetted into 4 Visking tubing bags. The time was noted as each bag was dropped into 20 ml 0.16 M KCl in a 100-ml round bottomed flask and shaken continually at one of the four required temperatures. Six 1-ml samples were withdrawn at appropriate intervals, made up to 10 ml with cold 0.16 M KCl and the isotope counted. Afterwards, the Visking bag was cut open in the 100 ml flask, and 10 ml of the dispersion counted.

When the permeability of the liposomes was as high as $5 \cdot 10^{-11}$ cm·sec^{-1} at

room temperature it was necessary to note the time interval from when lipid came off the dialysis column until the bags were dropped into the flasks, usually about 6 min.

The amount of lipid in the dialysis bags was found by phosphate analysis of the dispersions, firstly by the method of FISKE AND SUBBAROW[4], then by a method developed by MCCLARE (C. W. F. MCCLARE, personal communication), which proved to be more sensitive and gave more reproducible results.

0.2-ml samples were evaporated to complete dryness. The tubes were loosely stoppered and the samples digested for 20 min with 0.5 ml of 70% $HClO_4$ at 220° in a fume chamber. Meanwhile 35 ml of $HClO_4$ were diluted to 400 ml. 5 ± 0.5 g ammonium molybdate and 1 ± 0.1 g ascorbic acid both in aqueous solution were added to the dilute $HClO_4$ in the order stated. The mixture was made up to 500 ml. 9.5 ml of this solution were added to the cold digested samples which were then heated at $49 \pm 0.1°$ for 1 h, cooled in cold water and read within 10 min on a SP-500 spectrometer at 825 mμ. Temperature control is important. Standards were 0.2 ml of a solution containing 21.8 μg P per ml as KH_2PO_4 dissolved in water with 10% formaldehyde. A blank was also prepared. The reproducibility should depend only upon volumetric accuracy. The reagents were discarded after use.

The surface area of the liposomes was measured by the method described originally by BANGHAM *et al.*[5], except that the sensitivity of the method was increased by carrying out the UO_2^{2+} titrations on 10-ml aliquots of dispersion in small (5.5-cm diameter) freshly waxed petri dishes. Titrations were followed using UO_2^{2+} concentrations producing changes in the surface potential of a monolayer of up to 70–80 mV at 22°, and the surface areas were calculated from the gradient of the graph of $\sinh \Delta\psi_G/50.86$ against s.

$$\sinh \frac{\Delta\psi_G}{50.86} = \frac{134 \times 6.023}{a\sqrt{0.16}} s$$

$\Delta\psi_G$ was the change in surface potential, a the surface area of the dispersion in cm^2 and s the number of $1 \cdot 10^{-7}$-mole aliquots of UO_2^{2+} added. $\sqrt{0.16}$ is the square root of the concentration of the KCl electrolyte. The relation was derived from the Gouy equation linking surface potential to surface charge density.

Calculation of flux and permeability

Experiments showed that prolonged sonication of the 4% phosphatidic acid – phosphatidyl choline liposomes at the energy level of the cleaning bath eventually gave double-walled liposomes with a relatively large central compartment; the isotope diffusion was therefore interpreted as representing a K^+ exchange between single compartment liposomes of equal volume and the bulk solution. Such a system is very much easier to describe than the diffusion from the earlier multilayered liposomes.

In the experimental system, however, the counts escaping from the liposomes also have to pass through a dialysis bag into the external solution and in so doing may be retarded. A general equation describing the system is deduced below, from which special relations for particular conditions can readily by obtained. Fig. 1 is a diagram of the system at a time t from the start of the diffusion experiment.

The liposomes permeability p and the volume v_c contain $N-n$ counts.

The dialysis bag permeability p_1 and the volume v_1 contains $n-n_2$ counts.

The external solution volume v_2 contains n_2 counts. As v_c is extremely small, the

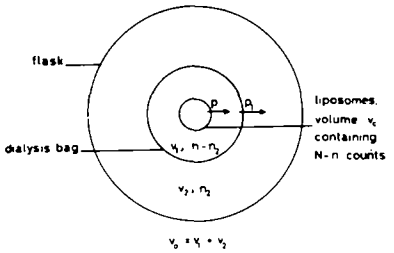

Fig. 1.

total volume v_0 is given by the relation: $v_0 = v_1 + v_2$. Originally all N counts in the system were inside the liposomes. The isotope diffusion permeability coefficient of the liposomes is P, measured in cm·sec^{-1}

$$P = \frac{p}{\text{liposome surface area}}$$

The ion flux in moles·cm^2·sec^{-1} across the membrane in either direction is obtained by multiplying P by the ion concentration in moles·cm^{-3}. The concentration difference Δc at time t across the liposome is

$$-\left(\frac{N-n}{v_c} - \frac{n-n_2}{v_1}\right)$$

Since $dn/dt = -p\Delta c$

$$\frac{dn}{dt} = p\left(\frac{N-n}{v_c} - \frac{n-n_2}{v_1}\right) \tag{1}$$

Since $v_c \ll v_1$,

$$v_1 + v_c \approx v_1$$

Using this approximation and rearranging

$$\frac{dn}{dt} = \frac{p}{v_c}\left(\frac{v_c n_2 + v_1 N}{v_1}\right) - \frac{pn}{v_c} \tag{2}$$

At the dialysis bag surface at time t, the concentration difference is

$$-\left(\frac{n-n_2}{v_1} - \frac{n_2}{v_2}\right)$$

and

$$\frac{dn_2}{dt} = p_1\left(\frac{n-n_2}{v_1} - \frac{n_2}{v_2}\right) \tag{3}$$

Differentiating Eqn. 3 with respect to t,

$$\frac{d^2n_2}{dt^2} = p_1\left(\frac{1}{v_1}\frac{dn}{dt} - \frac{1}{v_1}\frac{dn_2}{dt} - \frac{1}{v_2}\frac{dn_2}{dt}\right) \tag{4}$$

By substituting for dn/dt from Eqn. 2 and n from Eqn. 3, rearranging and using the approximation $v_c v_2 \ll v_0 v_1$,

$$\frac{d^2n_2}{dt^2} + \left(\frac{p_1v_0v_c + pv_1v_2}{v_1v_2v_c}\right)\frac{dn_2}{dt} + \frac{v_0p_1pn_2}{v_1v_2v_c} - \frac{pp_1N}{v_1v_c} = 0 \tag{5}$$

This integrates to

$$Ae^{-pt/v_c} + Be^{-p_1v_0t/v_1v_2} + \frac{v_2}{v_0}N = n_2 \tag{6}$$

(Providing $p_1v_0v_c \neq pv_1v_2$). Differentiating Eqn. 6 with respect to t

$$-\frac{p}{v_c}Ae^{-pt/v_c} - \frac{p_1v_0}{v_1v_2}Be^{-p_1v_0t/v_1v_2} = \frac{dn_2}{dt} \tag{7}$$

When $t \to 0$, the exponentials tend to 1 and $n_2 \to 0$. However, there may be some free counts in the dialysis bag, produced by the liposomes after leaving the Sephadex column but before the start of the run. Let these be αN when $t = 0$.
Then $dn_2/dt = \alpha Np_1/v_1$. From Eqns. 6 and 7 we obtain

$$A + B + \frac{v_2}{v_0}N = 0 \tag{8}$$

$$-\frac{p}{v_c}A - \frac{p_1v_0}{v_1v_2}B = \frac{\alpha Np_1}{v_1} \tag{9}$$

From Eqns. 8 and 9

$$A = \frac{v_2v_cp_1(1-\alpha)N}{pv_1v_2 - p_1v_0v_c}$$

$$B = \frac{(\alpha p_1v_cv_0 - pv_1v_2)v_2N}{(pv_1v_2 - p_1v_0v_c)v_0}$$

And the equation becomes

$$\frac{v_2}{v_0} - \frac{n_2}{N} = \frac{p_1v_2v_c(1-\alpha)}{p_1v_0v_c - pv_1v_2}e^{-pt/v_c} - \frac{v_2}{v_0}\cdot\frac{(pv_1v_2 - p_1v_0v_c\alpha)}{(p_1v_0v_c - pv_1v_2)}e^{-p_1v_0t/v_1v_2} \tag{10}$$

The expression is much simplified if the following substitutions are made:

$$L = p/v_c \qquad K = p_1v_0/v_1v_2$$

Then we have

$$\frac{v_2}{v_0} - \frac{n_2}{N} = \frac{v_2}{v_0(K-L)}[(1-\alpha)Ke^{-Lt} - (L-\alpha K)e^{-Kt}] \tag{10a}$$

Special cases

(1) α is negligibly small. This occurs if $P < 5 \cdot 10^{-11}$ at room temperature, as is the case in the experiments using a very small quantity of valinomycin. Then Eqn. 10a becomes

$$\frac{v_2}{v_0} - \frac{n_2}{N} = \frac{v_2}{v_0(K-L)}(Ke^{-Lt} - Le^{-Kt}) \tag{11}$$

(2) The permeability of the dialysis bag is very much greater than that of the liposomes: $p_1 \gg p$, therefore $K \gg L$. And Eqn. 10a becomes

$$\frac{v_2}{v_0} - \frac{n_2}{N} = \frac{v_2}{v_0}(1-\alpha)e^{-Lt} \tag{12a}$$

Usually α is also small and so we obtain

$$\frac{v_2}{v_0} - \frac{n_2}{N} = \frac{v_2}{v_0} e^{-Lt} \tag{12}$$

or

$$-Lt = \ln\left(\frac{v_2}{v_0} - \frac{n_2}{N}\right) - \ln\frac{v_2}{v_0} \tag{12b}$$

(3) The permeability coefficient of the liposomes is less than $1 \cdot 10^{-11}$ cm·sec^{-1}. This is the condition in the simple exchange experiments. As $n_2 v_0 / N v_2 \ll 1$, the logarithm in Eqn. 12b can be expanded. Then we have

$$L = \frac{v_0 n_2}{v_2 N t} \tag{13}$$

or substituting for L

$$\frac{p}{v_c} = \frac{v_0 n_2}{v_2 N t} \tag{13a}$$

The volume inside the liposomes was found from the relation

$$v_c = \frac{N \times 0.01}{\text{standard}} \tag{14}$$

where the standard was the count rate of 0.01 cm^3 of the initial isotope solution used to disperse the liposomes.

Substituting for v_c in Eqn. 13a,

$$p = \frac{v_0 \times n_2 \times 0.01}{v_2 \times t \times \text{standard}} \tag{15}$$

The permeability of the dialysis bags can also be obtained from Eqn. 10a by letting $L \to \infty$

Eqn. 10a becomes

$$\frac{v_2}{v_0} - \frac{n_2}{N} = \frac{v_2}{v_0} e^{-Kt} \tag{16}$$

or

$$\frac{-p_1 v_0}{v_1 v_2} t = \ln\left(1 - \frac{v_0 n_2}{v_2 N}\right) \tag{16a}$$

Isotope was put inside the bag, and the external solution was sampled at known times t.

It was found, for example, that the permeability of the bags varied between different samples of Visking tubing, and was higher at high temperatures or in the presence of small amounts of the organic solvents, chloroform, butanol or ether.

Experimental test of the mathematical model

The validity of the model was tested experimentally by studying the isotope exchange rate in the presence of 300 mM *n*-butanol which was added to the lipid before dialysis over Sephadex. Under these conditions the dialysis bag permeability was high and p_1 could be neglected, so Eqn. 12b was used. The number of counts in a given sample was multiplied by the volume of the solution from which it was extracted, and the counts removed in the previous samples were added to it. This gave n_2. N was

found by multiplying the 10 ml dispersion count at the end by 1.5 and adding the counts removed by the previous 6 samples. v_2/v_0 was the ratio of the average volumes during the period of the leak. It varied from 0.952 (20/21) to 0.946 (17/18). Ideally it should be constant, but the variation is less than 1% and can be neglected. Fig. 2 shows a plot of ln $[(v_2/v_0) - (n_2/N)]$ against t for two lipid samples; one much more highly sonicated than the other. In both cases the regression line through the first five or six points passed through (0, ln 0.952) within the limit of experimental error; but later points showed a marked deviation from this line, presumably due to the presence of some multicompartment liposomes and/or variation in liposome radius. However, in the highly sonicated dispersion this did not occur until 69% of the isotope had exchanged. In all other experiments, only values of n_2/N up to 0.6 were considered, in dispersions which had a value of v_c per μmole less than 0.0007 cm^3.

The gradients of the two regression lines gave values for p/v_c of 0.713 $\pm$ 0.016 and 1.467 $\pm$ 0.022 from which p could be calculated as v_c was known. The values of v_c per μmole were 0.001126 and 0.0006 cm^3, and using the area values from Fig. 4, the permeability coefficients for the less and more sonicated dispersions were found to be 2.54 $\pm$ 0.06 $\cdot$ 10^{-10} and 2.17 $\pm$ 0.03 $\cdot$ 10^{-10} cm $\cdot$ sec^{-1}.

In the valinomycin experiments, values of log $[(v_2/v_0) - (n_2/N)]$ were plotted against t. The plot was curved convex to the Y axis in the opposite direction to Fig. 2. This was due to the retarding action of the dialysis bags. An approximate value of p/v_c could be obtained from the gradient of the chord joining the intercept on the Y axis and the last experimental point. This was used to find α.

$$\alpha = 1 - e^{-pt_0/v_c}$$

t_0 was the time between the lipid coming off the column and the start of the experiment.

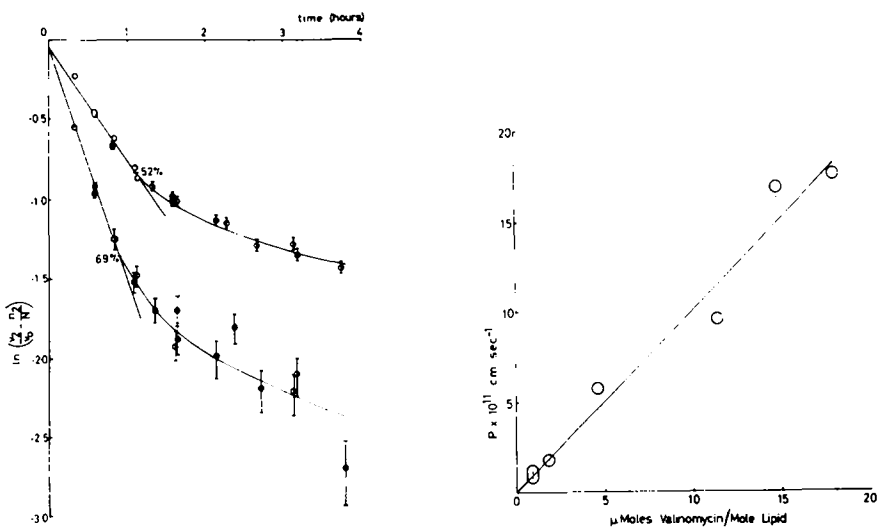

Fig. 2. Isotope exchange profile. $^{42}K^+$ exchange from 4% phosphatidic acid–phosphatidyl choline liposomes in 0.16 M KCl in the presence of 300 mM n-butanol. The upper curve is a less sonicated system.

Fig. 3. The permeability coefficient for 4% phosphatidic acid–phosphatidyl choline liposomes with valinomycin at 22°.

Values of t and $[(v_2/v_0) - (n_2/N)]$ were fitted by computer to Eqns. 10a or 11 to give the best values of L and K.

The values of K and L found by the computer were quite consistent with the approximate values of p and p_1 found independently.

p for the Forbes bar experiments was found by direct substitution in Eqn. 14.

RESULTS

A plot of the values of P against the initial valinomycin concentration per μmole of lipid is shown in Fig. 3. The regression line through the eight points goes through zero within the limits of experimental error. The experiments were done with three different lipid preparations, and the results are reasonably consistent. One point was obtained where P was extremely high; this was believed to be due to the accidental oxidation of the lipid during sonication. It is not shown in the plot.

Using one preparation of lipid, four values for the permeability coefficient at 37° without valinomycin gave a mean of $3.3 \cdot 10^{-13}$ cm·sec^{-1}, and varied between 2.7 and 4.0. Other values of the liposome permeability at 37° for a different lipid preparation gave a mean of $7.3 \cdot 10^{-13}$ cm·sec^{-1}, varying between 5.2 and 8.5.

Permeability coefficients between $1 \cdot 10^{-13}$ and $1 \cdot 10^{-11}$ cm·sec^{-1} could be measured using the simple exchange method and Eqn. 14; whereas permeability coefficients between $1 \cdot 10^{-11}$ and at least $1 \cdot 10^{-9}$ cm·sec^{-1} could be found from the cumulative exchange method and Eqns. 10a or 11.

DISCUSSION

Fig. 5 shows an electronmicrograph of the highly sonicated 4% phosphatidic acid–phosphatidyl choline liposomes used in the experiment with 300 mM butanol. They were negatively stained with isotonic ammonium molybdate and photographed at 40 kV with a Siemens Elmiskop 1. From the photograph, the thickness of the liposome boundary layer is 107 Å. As the isotope exchange profile indicated that the system behaved as a collection of single compartments, the boundary which consisted of two bilayers must contain only a small quantity of isotope. The dark line on the photograph indicates the head group region between the unstained hydrocarbon cores.

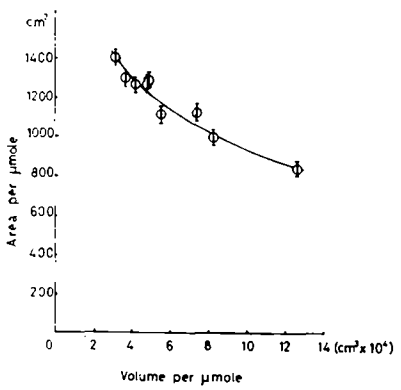

Fig. 4. Relation between surface area and internal volume for 4% phosphatidic acid–phosphatidyl choline liposomes.

Fig. 4 shows that the limiting liposome volume of 0.0003 cm^3 per μmole had a surface area of 1415 cm^2. If the liposomes are assumed to be spherical and r is the radius of the internal compartments,

$$\frac{r^3}{3(r + 107)^2} = \frac{0.0003}{1415}$$

from which $r = 169$ Å. In view of the general uncertainty in measurement, no allowance has been made for the invisible head groups on the inside or outside of the boundary.

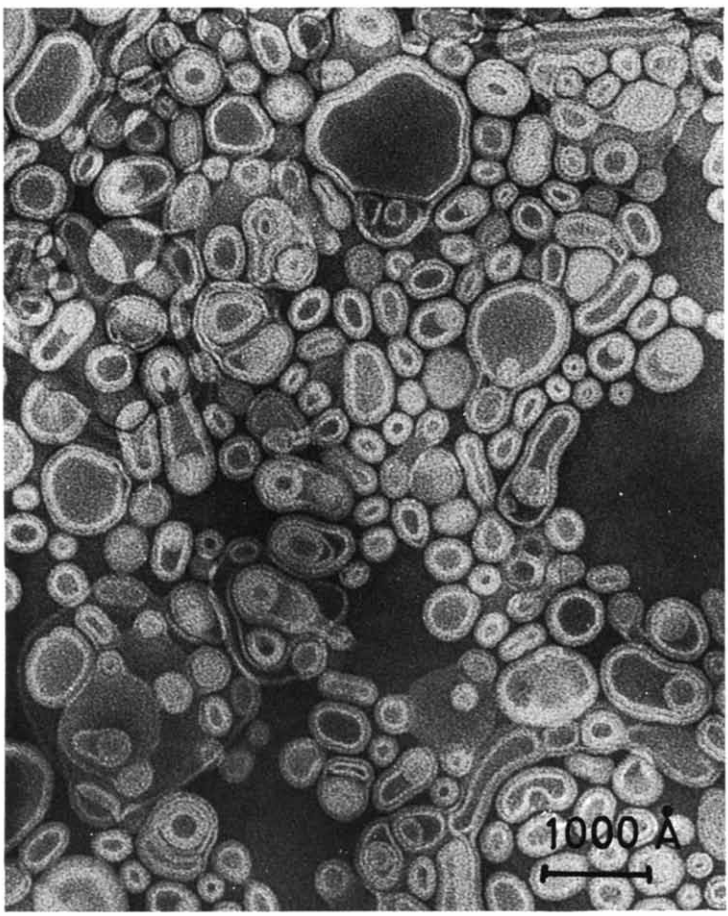

Fig. 5. Electronmicrograph of highly sonicated 4% phosphatidic acid–phosphatidyl choline liposomes, negatively stained with ammonium molybdate in the presence of 300 mM *n*-butanol.

The proportion of lipid molecules on the outside of the outer bilayer for these small liposomes is a function of r and the boundary thickness. The centre of each bilayer was taken as being 107/6 Å from the inside or outside of the boundary. Then the proportion required is

$$\frac{\text{area of outer bilayer}}{2(\text{area of outer and inner bilayer})}$$

which is 0.328. Since the number of molecules in 1 μmole is $6.023 \cdot 10^{17}$ and since 0.328 of them face outwards, the surface area per molecule must be

$$\frac{1415 \times 10^{16}}{6.023 \times 0.328 \times 10^{17}} \text{ Å}^2 = 71.6 \text{ Å}^2$$

SMALL[6] quotes a value of 71.7 Å^2 for a fully hydrated lecithin molecule which he obtained from X-ray data. Owing to the approximations made, the excellent agreement between these two values must be partly due to chance.

The mean number of molecules of lipid in one double-walled liposome should be 35 612. In the experiments when the molar concentration of valinomycin to lipid was $1:1 \cdot 10^6$, only 1 in 28 of the liposomes could contain a valinomycin molecule at a given time. However, the results clearly showed that all the liposomes became permeable, so presumably the valinomycin molecules diffused rapidly from one liposome to another. TOSTESON *et al.*[7] give the partition coefficient for valinomycin between decane and 0.1 M KCl as 3400. Using this value with the ratio of lipid to aqueous phase in the present system, it can be calculated that about 3 % of the valinomycin molecules was in the aqueous phase. Nevertheless this very low concentration of valinomycin produced an 100-fold increase in K^+ permeability, which shows the remarkable efficiency of the carrier molecule. Little change in Na^+ permeability was noted.

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