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# A SEQUENTIAL DIALYSIS METHOD FOR MEASURING PERMEABILITY COEFFICIENTS OF PHOSPHOLIPID VESICLES

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SUMMARY

A sequential dialysis device was constructed to measure the permeability coefficient of phospholipid vesicles. Its advantages are automation and good accuracy. During the dialysis the external solution is changed repeatedly at constant intervals.

A general mathematical treatment of this sequential dialysis is given. Preliminary results of measurements of the D-glucose permeability coefficient of single-compartment bimolecular vesicles made of soy-bean lecithin are presented. At 25 °C a value of $6.8 \cdot 10^{-12}$ cm·s$^{-1}$ $\pm$ 25 % was found and is compared with results obtained with lipid bilayer membranes.

## INTRODUCTION

Phospholipid bilayer structures are widely used as model systems for biological membranes and their properties are of considerable interest. One of these is the permeability, which can be determined by tracer techniques. Usually the efflux of radioactively marked molecules across the membrane is measured and a time constant, $K$, is obtained. The latter is correlated to the permeability coefficient, $P$, by the formula

$$P = K \frac{V}{A} \frac{V_0}{V + V_0} \tag{1}$$

containing the surface of the penetrated membrane, $A$, and the outer and inner volumes $V_0$ and $V$, respectively. In many cases the outer volume is large compared with the inner volume and Eqn 1 reduces to

$$P = K \frac{V}{A} \tag{2}$$

Since the permeability of lipid bilayers to hydrophilic substances is known to be very low, one has to minimize the ratio $V/A$ in order to obtain reasonable time constants.

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Permeability constants of planar black lipid membranes were measured by Petkau and Chelack[1], Lippe[2] and Wood *et al.*[3], but even the best chamber constructions give a comparatively high value of $V/A$ (*cf.* Table I). Pagano and Thompson[4], and more recently Jung[5], used spherical bilayer membranes of some millimetres diameter and obtained a more satisfactory value for $V/A$.

TABLE I

SOME GEOMETRICAL DATA AT PERMEABILITY MEASUREMENTS

The ratio of the enclosed volume, $V$, to the penetrated area, $A$, multiplied by the time constant (determined by measurement) gives the permeability coefficient.

| *Membrane* | $V$ | $A$ | $V/A$ | *Permeant* | *Ref.* |
|---|---|---|---|---|---|
| Lipid bilayer planar | 3000 $mm^3$ | 6 $mm^2$ | 500 mm | $Na^+$ | 1 |
| | — | 7 $mm^2$ | — | Thiourea | 2 |
| | 500 $mm^3$ | 1.5 $mm^2$ | 330 mm | Urea, sorbitol, glucose | 3 |
| Lipid bilayer spherical | 4–100 $mm^3$ | 10–100 $mm^2$ | 0.4–1 mm | $Na^+$, $Cl^-$ | 4 |
| | 30–120 $mm^3$ | 47–120 $mm^2$ | 0.64–1 mm | D-Glucose, various sugars | 5 |
| Human erythrocyte | 87 $\mu m^3$ | 155 $\mu m^2$ | 0.56 $\mu m$ | | 14 |
| Lipid vesicle | 2140 $nm^3$ | 1260 $nm^2$ | 1.7 nm | $K^+$ | 10, 11 |
| | — | — | — | $Na^+$, $K^+$, $Rb^+$ | 12, 13 |

The smallest values of this ratio are found in suspensions of lipid vesicles, produced by ultrasonication of aqueous lipid dispersions. The physical properties of such systems were investigated by Huang[6], Schwan *et al.*[7], Seufert[8] and Miyamoto and Stoeckenius[9]. Permeability measurements were carried out by Johnson and Bangham[10,11] and Papahadjopoulos[12,13]. A schematic list of the above permeability measurements together with the corresponding $V/A$ values is given in Table I.

Because of their small size the vesicles cannot be washed by centrifugation techniques commonly used in the treatment of biological systems. Therefore, the tracer molecules not initially trapped by the vesicles and the ensuing tracer efflux must be removed by a different means, for instance by dialysis. In this paper a new dialysis method is described to determine the permeability time constant of phospholipid vesicles.

## METHOD OF MEASUREMENT

A cell was developed which allows the vesicle suspension to be repeatedly dialysed against comparatively small volumes of tracer-free solution; these are sampled and counted separately. In this way the tracer concentration in the bathing solution is maintained at low levels and the net flux therefore kept as high as possible.

Further, the dialysis procedure results in a large number of small independent samples and the dialysed radioactivity is completely available for measurement. Finally, it was possible to automatize the measuring procedure to save time and increase the reproducibility of each step.

After presetting the number and duration of the dialysis steps on the counter of a fraction collector, the sampling and the refilling of the dialysis cell are controlled

by two magnetic valves. The volume of the dialysate is limited by a pair of suitably mounted electrodes. The temperature of the dialysis cell and the stock solution is thermostatically controlled.

A suitable dialysis cell should have a large and reproducible time constant. This can be achieved by a low ratio of volume to membrane surface, by a well-defined geometry, and by sufficient stirring. These requirements are fulfilled by a dialysis tube tightened in a perspex cage, closed by a teflon disk and filled by a teflon piston (*cf.* Fig. 1). The small space between tube and piston containes the vesicle suspension.

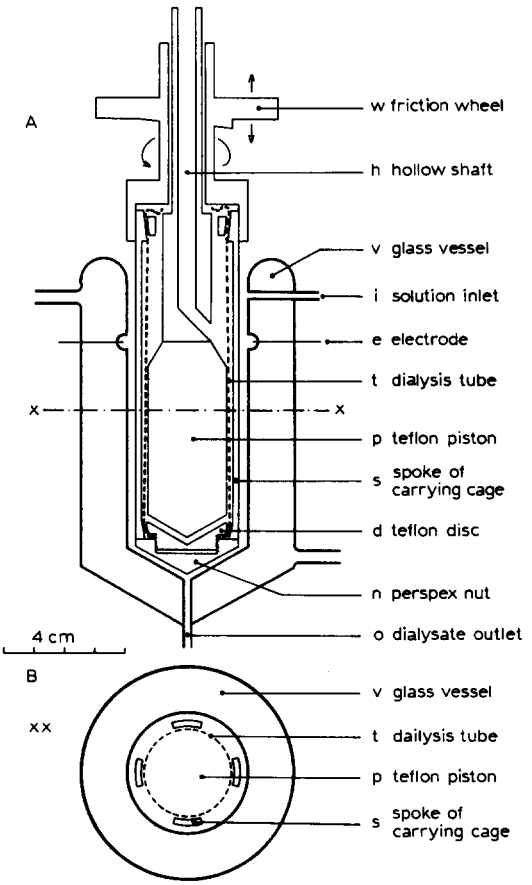


Fig. 1. Dialysis cell: A, longitudinal section; B, cross section. The dialysis tube, t, is suspended in the carrying cage, c. It is fixed at the upper end by a conical ring and at the lower end by teflon disc, d. This disc is pressed in its seat by nut, n, and thus seals and tightens the dialysis tube as well. The upper and lower ends of the carrying cage are connected by four spokes, s. The lower part of the dialysis tube is almost completely occupied by teflon piston, p, fitted to the hollow shaft, h. At its upper end this shaft is fixed to a support (not shown). The vesicle suspension is poured into the small space between piston and dialysis tube through the hollow shaft. The carrying cage with dialysis tube, fitted to friction wheel, w, rotates around the piston. An additional up and down motion generated separately provides the mixing of the vesicle suspension ($v_2$). Details of the bearing and driving device are not shown. The outer solution ($v_1$), stirred by the spokes of the rotating carrying cage, is contained by the temperature controlled double-walled glass vessel, v, with outer solution inlet, i and outlet, o. The filling hight of the outer compartment is limited by short-circuiting the electrodes, e, through the outer solution. Unless otherwise stated, all parts of the apparatus are made of colourless perspex.

Cage and dialysis tube rotate together around the teflon piston and stir essentially the outer compartment, whereas the stirring of the inner compartment is achieved by an additional up and down motion of the cage and the tube against the piston. This device was shown to have a reproducible time constant at constant temperature and rotation rates >30 per min. Using an inner volume of 3 ml the obtained value of $V/A = 0.63$ mm is fairly satisfying. This is shown by a comparison with Table I.

The assembled dialysis cell is filled with the vesicle suspension (through the hollow shaft of the teflon piston) and simultaneously immersed in the external solution within the glass vessel (*cf.* Fig. 1). At the same time the counter is started. In a preparative phase the radioactivity of the suspending medium inside the dialysis tube is washed out by a number of short dialysis steps. (Normally thirty steps of 2 min duration each.) In the subsequent measurement phase the efflux of tracer molecules from the vesicles is observed by dialysis steps of longer duration (*e.g.* 60 min).

## MATHEMATICAL TREATMENT

The described arrangement of external solution, dialysis tube, and vesicles can be treated as a concentric three compartment system. In the same sequence, the three volumes are labeled $v_1$, $v_2$, $v_3$ and the corresponding amounts of radioactivity $y_1$, $y_2$, $y_3$. The (not normalized) permeabilities of the dialysis tube and the vesicles are named $p_1$ and $p_3$. These give the permeability coefficients after division by the penetrated surface. With these notations one obtains for $y_1$ the differential equation:

$$0 = \frac{d^2y_1}{dt^2} + \frac{p_1v_3(v_1 + v_2) + p_3v_1(v_2 + v_3)}{v_1v_2v_3}\frac{dy_1}{dt} + \frac{p_1p_3(v_1 + v_2 + v_3)}{v_1v_2v_3}y_1 - \frac{p_1p_3}{v_2v_3}(y_1 + y_2 + y_3) \tag{3}$$

Since the total inner volume of the vesicles, $v_3$, is very small compared with $v_2$ and $v_1$ the approximation $v_2 \pm v_3 \approx v_2$ may be used. Together with the following substitutions,

$$v_0 = v_1 + v_2 + v_3 \qquad y_0 = y_1 + y_2 + y_3 \tag{4}$$

$$K = \frac{p_1v_0}{v_1v_2} \qquad L = \frac{p_3}{v_3} \tag{5}$$

the differential equation becomes:

$$0 = \frac{d^2y_1}{dt^2} + (K + L)\frac{dy_1}{dt} + KLy_1 - KL\frac{v_1}{v_0}y_0 \tag{6}$$

Equivalent equations can be derived for the quantities $y_2$ and $y_3$.

With the initial conditions at the time $t = 0$

$$y_1(0) = 0, \qquad y_2(0) = \alpha y_0, \qquad y_3(0) = (1 - \alpha)y_0 \tag{7}$$

$$\frac{dy_1}{dt}(0) = \alpha K\frac{v_1}{v_0}y_0, \qquad \frac{dy_2}{dt}(0) = \left(L(1 - \alpha) - \alpha K\frac{v_1}{v_0}\right)y_0$$

$$\frac{dy_3}{dt}(0) = -y_0 L(1-\alpha) \tag{8}$$

Integration of these differential equations gives:

$$y_1 = y_0 \frac{v_1}{v_0}\left[1 - \frac{(1-\alpha)K}{K-L}e^{-Lt} + \frac{L-\alpha K}{K-L}e^{-Kt}\right] = y_0 w(t) \tag{9}$$

$$y_2 = y_0 \frac{v_2}{v_0}\left[1 - (1-\alpha)\frac{K - \frac{v_0}{v_2}L}{K-L}e^{-Lt} - \frac{v_1}{v_2}\frac{L-\alpha K}{K-L}e^{-Kt}\right] \tag{10}$$

$$y_3 = y_0 \frac{v_3}{v_0}\left[1 + \left(\frac{v_0}{v_3}(1-\alpha) - \frac{K - \frac{v_0}{v_2}L}{K-L}\right)e^{-Lt} - \frac{v_1}{v_2}\frac{L}{K-L}e^{-Kt}\right] \tag{11}$$

The quantity $\alpha$, introduced by the initial conditions (Eqns 7) denotes the fraction of total radioactivity present in the volume $v_2$ surrounding the vesicles at the time $t = 0$. The substitutions $K$ and $L$ (Eqn 5) represent the time constants of the dialysis tube and the vesicles respectively. The function $w(t)$ in Eqn 9 is an abbreviation for the term $y_1(t)/y_0$.

In the measuring procedure described above the dialysis is stopped after a period $\tau$, the radioactivity in the volume $v_1$ is removed from the system and the next dialysis step is started at $t = 0$ with changed initial conditions. At the end of the first step one obtaines:

$$y_{1,1}(\tau) = y_{1,1} = w y_{0,1} \tag{12}$$

with $w = w(\tau)$ and $y_{0,1} = y_0$. Here the second index denotes the step number. It should be noted that $w = w(\tau)$ does not depend on $y_{0,1}$ $(i = 1,2,\ldots,n)$.

The second step yields:

$$\begin{aligned} y_{1,2}(\tau) = y_{1,2} &= w y_{0,2} \\ &= w(y_{0,1} - y_{1,1}) \\ &= w y_{0,1}(1-w) \end{aligned} \tag{13}$$

With these relations the following equation holds for the end of the $(n+1)^{st}$ step:

$$\begin{aligned} y_{1,n+1}(\tau) = y_{1,n+1} &= w y_{0,n+1} \\ &= w y_{0,n}(1-w) \\ &= w y_{0,1}(1-w)^n \end{aligned} \tag{14}$$

A logarithmic plot of $y_{1,i}$ $(i = 1,2,\ldots,n)$ gives a straight line with a slope of $\log(1-w)$. Thus a value for $w$ can be obtained.

The expression $w = w(\tau)$ defined by Eqn 9 is a transcendental equation for the desired quantity $L$, and can be rearranged to:

$$L = \frac{1}{\tau}\ln\frac{K-L}{K-\alpha K}\left(1 - \frac{v_0}{v_1}w + \frac{L-\alpha K}{K-L}e^{-K\tau}\right) = f(L) \tag{15}$$

Eqn 15 containes two unknowns, the dialysis time constant $K$, which can be determined by measurements without vesicles, and the quantity $\alpha$, which has to be calculated.

In the described sequential dialysis the beginning of a new step does not change the radioactivity $y_2$:

$$y_{2,n+1}(0) = y_{2,n}(\tau) \tag{16}$$

The definition of $\alpha$, Eqn 7, combined with Eqns 14 and 16 yields:

$$\alpha = \frac{y_{2,n+1}(0)}{y_{0,n+1}} = \frac{1}{1-w}\frac{y_{2,n}(\tau)}{y_{0,n}}$$

Introducing Eqn 10 one obtains:

$$\alpha = \frac{1}{1-w}\frac{v_2}{v_0}\left[1-(1-\alpha)\frac{K-\dfrac{v_0}{v_2}}{K-L}e^{-L\tau} - \frac{v_1}{v_2}\frac{L-\alpha K}{K-L}e^{-K\tau}\right] \tag{17}$$

This expression does not depend on $n$ and can be rewritten as an explicit equation for $\alpha$:

$$\alpha = \frac{v_2(K-L)-(v_2K-v_0L)e^{-L\tau} - v_1Le^{-K\tau}}{(1-w)v_0(K-L)-(v_2K-v_0L)e^{-L\tau}-v_1Ke^{-K\tau}} \tag{18}$$

This result can be inserted into Eqn 15 and the transcendental equation can then be solved by an iterative method according to:

$$L_{i+1} = f(L_i) \qquad (i = 0,1,2,\ldots)$$

with an estimated value for $L_0$, which may be zero in all cases where $L < K$.

In the treatment given above $w = w(\tau)$ defined by Eqn 7 is taken as a constant. Such however is the case only if $\alpha$ does not depend on $n$ and *vice versa*, as can be seen by Eqn 18. In actual fact the system, started with an arbitrary value of $y_{2,1}(0)$ will need a few steps until $\alpha$ attains its equilibrium value. Within these steps $w$ will not be constant, *i.e.* the first $y_{1,i}$ will not form a straight line (*e.g.* see Fig. 2, the first point of Curve B).

As the total inner volume of the vesicles, $v_3$, is very small compared with $v_2$, the permeability coefficient, $P$, can be calculated from the time constant, $L$, according to Eqn 2:

$$P = \frac{v_3}{a_3}L$$

Here $a_3$ is the total penetrated membrane surface of the vesicles.

The total volume, $v_3$, as well as the total surface, $a_3$, are the sums of volumes and surfaces of the individual vesicles. In the case of a uniform vesicle size, the expression $v_3/a_3$ can be calculated from the data of one vesicle. Otherwise the surface, $a_3$, and the volume, $v_3$, have to be determined separately, *e.g.* as it was done by Johnson and Bangham[10,11].

The method described above differs in one essential point from that presented by Johnson and Bangham[10] and also from the computer analysis of a five compart-

ment system given by Klein *et al.*[15]: Sequential dialysis permits the initial conditions of the three compartment system (*i.e.* the parameter $\alpha$) to be determined exactly.

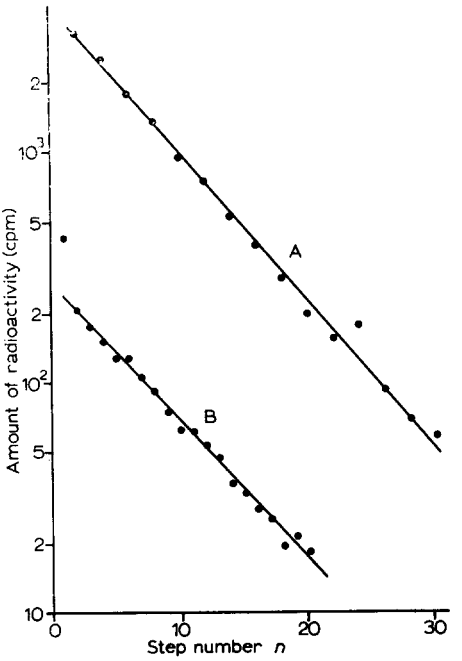


Fig. 2. Radioactivity of dialysate samples *versus* dialysis step number $n$. A, radioactivity decrease of the dialysates during the preparative period: the tracer is washed out from the suspending medium around the vesicles. The duration of each step was 2 min. The radioactivity of every second sample was counted. The slope of the curve gives the time constant $K$ of the dialysis tube. B, radioactivity decrease of the dialysates during the measuring period. The counts $y_{1,n}$ form a straight line the slope of which determines the quantity $w$ (*cf.* text). The duration of the dialysis steps was 60 min each.

Thus it becomes possible to experiment with systems where vesicle time constants ($L$) are of the same order of magnitude as the dialysis time constant ($K$). Calculations showed that the method remains practicable up to $L$ values of $0.75K$, if sufficiently short time steps $\tau$ are used.

A more detailed comparison with the computer analysis of Klein *et al.*[15] is excluded by the brevity of their presentation.

## MEASUREMENTS

First measurements using the described method were carried out with D-[U-$^{14}$C]-glucose on soy-bean lecithin vesicles. The lecithin was chromatographically pure. All other chemicals were of reagent grade, the water was destilled twice in quartz.

The lecithin was dissolved in carbon tetrachloride (50 mg/ml) and refrigerated below —20°C until used. The necessary quantities containing 150 mg lecithin were pipetted into 50 ml round bottomed flasks and dried in a rotatory evaporator under nitrogen. To each sample 3 ml of a 1 mM Tris buffer (pH 7.0) containing 27.7 mM

D-glucose, 10 mM KCl, 1 mM cysteine, 0.5 mM sodium ethylene diamine tetraacetate, 0.01 % sodium azide and 20 $\mu$Ci/ml D-[U-$^{14}$C]glucose were added.

The flask *plus* content was then sonicated under $N_2$ in a bath sonicator (Bandelin Sonorex GR 60, 40 kHz) for at least 3 h below 20 °C. To remove greater particles, the clear suspension was centrifuged (3000 × *g*) and equilibrated over night at 4 °C. The suspensions prepared in this manner contained about 50 mg/ml lecithin which was almost completely in the form of vesicles of various diameters bounded by one lipid bilayer (Huang[6], Miyamoto and Stoeckenius[9], Johnson and Bangham[11]). They were fractionated on a column of large pore agarose gel (Sepharose 2B, 2.5 cm × 20 cm) according to Huang. The separation was controlled photometrically at 300 nm. A fraction taken from the principal peak was selected for the permeability measurement. It was concentrated with dry Sephadex G-50 fine, approx. 3 ml of the concentrate were placed in the dialysis cell and the dialysis started.

Thirty dialysis steps of 2 min duration each proved to be sufficient to separate tracer molecules from the suspending medium $v_2$. This procedure was followed by 30 measurement steps of 60 min duration each. The dialysates were sampled individually and from each one 5 ml were pipetted into scintillation vials. These samples were dried in an oven at 80 °C, redissolved in 0.2 ml destilled water, mixed with 10 ml scintillation fluid (POPOP *plus* PPO in toluene–methanol (1:1, v/v)) and counted for 10 min in a liquid scintillation counter (Packard Tri-Carb).

A logarithmic plot of the counting rates *versus n* (Fig. 2) yielded a straight line. From its slope the quantity $1 - w$ was determined and the time constant $L$ was calculated iteratively on a desk computer according to the Eqns 15 and 18.

Three measurements at 25 °C gave a mean value of

$$L_{25} = 3.67 \cdot 10^{-5} s^{-1} \pm 5\%$$

The corresponding value of $K$ was

$$K_{25} = 1.55 \cdot 10^{-3} s^{-1} \pm 1\%$$

To evaluate the permeability coefficient, $P$, from the time constant, $L$, the geometric data of the vesicles are needed. Since a determination of the vesicles size parameters was not yet possible, the necessary data were taken from the literature. Huang[6] demonstrated that the particles separated in the described manner are spherical vesicles bounded by single bilayer membranes with a relatively uniform diameter. He gives a mean value for the outer diameter of 250 ± 25 Å. In contrast to an earlier paper[10], Johnson and Bangham recently[11] confirmed these findings and give an outer diameter of 247 ± 17 Å. Miyamoto and Stoeckenius[9] found a somewhat higher value of 300 ± 50 Å. By combining these values one can estimate an outer diameter of 266 ± 35 Å.

Because of the relatively uniform size of the vesicles, the ratio $v_3/a_3$ may be calculated from the above value. If a thickness of 45 Å for the bilayer vesicle wall (Miyamoto and Stoeckenius[9]) is taken into account, the volume trapped by a single vesicle is given by an inner radius of $r_i = 88$ Å and the vesicles effective membrane surface by a mean radius of $r_m = 110$ Å. Hence $v_3/a_3$ becomes $1{,}86 \cdot 10^{-8}$ cm ± 20 % and one finally obtains the permeability coefficient $P = Lv_3/a_3$:

$$P_{25} = 6{,}8 \cdot 10^{-12} cm \cdot s^{-1} \pm 25\%$$

## DISCUSSION

The result may be compared with the upper limit of the D-glucose permeability coefficient of $10^{-10}$ cm·s$^{-1}$ given by Wood *et al.*[3] and the value of $2.35 \cdot 10^{-10}$ cm·s$^{-1}$ ± 55 % reported by Jung[5]. Both authors worked with black lipid membranes (planar[3] and spherical[5]) made from a total lipid extract of human erythrocytes. Their values are about 1.5 orders of magnitude higher as the result reported here.

The major reason for this difference may be seen in the additional organic components in the membrane-forming solutions of these authors *e.g.* chloroform, methanol and α-tocopherol, which were still to some degree present in the black lipid membranes. Another reason may be that membranes with different lipid compositions are being compared. Finally the degree of curvature of the vesicles is quite high and the resulting assymmetry in their bilayer structure may influence their permeability properties. If for instance the penetration across the inner surface of the vesicles is assumed to be the rate-limiting step for the diffusion of the permeant, one gets a $v_3/a_3$ ratio of $2.93 \cdot 10^{-7}$ cm and therefore a somewhat higher permeability coefficient of about $1 \cdot 10^{-11}$ cm·s$^{-1}$.

The permeability coefficient of phospholipid vesicles obtained by the sequential dialysis method also indicates a very low and unspecific permeability of protein-free phospholipid bilayer membranes to neutral lipophobic molecules.

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