Water Permeability of Thin Lipid Membranes

ALBERT CASS and ALAN FINKELSTEIN

From The Rockefeller University, New York, and the Departments of Neurology and Physiology, Albert Einstein College of Medicine, New York

ABSTRACT The osmotic permeability coefficient, P_f, and the tagged water permeability coefficient, P_d, were determined for thin (<100 A) lipid membranes formed from ox brain lipids plus DL-α-tocopherol; their value of approximately 1×10^{-3} cm/sec is within the range reported for plasma membranes. It was established that $P_f = P_d$. Other reports that $P_f > P_d$ can be attributed to the presence of unstirred layers in the experimental determination of P_d. Thus, there is no evidence for the existence of aqueous pores in these thin phospholipid membranes. The adsorption onto the membrane of a protein that lowers its electrical resistance by a factor of 10^3 was found not to affect its water permeability; however, glucose and sucrose were found to interact with the membrane to modify P_f. Possible mechanisms of water transport across these films are discussed, together with the implications of data obtained on these structures for plasma membranes.

I. INTRODUCTION

The rate of water movement across plasma membranes has long been of special interest to cellular physiologists concerned with their selective permeability characteristics. Two kinds of measurements of this rate have been made on various types of cells, yielding the osmotic and tagged water permeability coefficients. In the osmotic type of experiment, the solution surrounding the cell is made anisotonic and the water permeability is determined from the rate of change of cell volume (Lucké et al., 1931; Sidel and Solomon, 1957). In the second type of experiment, isotopic water is introduced outside (or inside) the cell, and its rate of appearance on the other side is measured (Paganelli and Solomon, 1957; Nevis, 1958). Both methods, and particularly a comparison of the two results for the same cell, have yielded information which has been used to deduce the physical mechanism by which water crosses plasma membranes and to draw certain conclusions about membrane ultrastructure; these points will be commented upon later in the paper.

In recent years a technique has been developed for forming from phospholipids (plus additives) thin (<100 A) films which separate two aqueous phases. Because of the similarities in structure between these films and

plasma membranes (Mueller et al., 1962; Huang et al., 1964),[1] it appears that investigation of the water permeability of these synthetic structures would be useful in understanding and interpreting the data obtained from cells; in particular, the relative role of lipid and protein in water transport can be assessed, since these films can be made either with or without protein adsorbed to them.

It is the purpose of this paper to present data on the tagged water and osmotic permeability of thin lipid membranes with and without adsorbed protein, and to discuss the results in terms of possible mechanisms involved in water transport. We shall also compare our data with those obtained by other

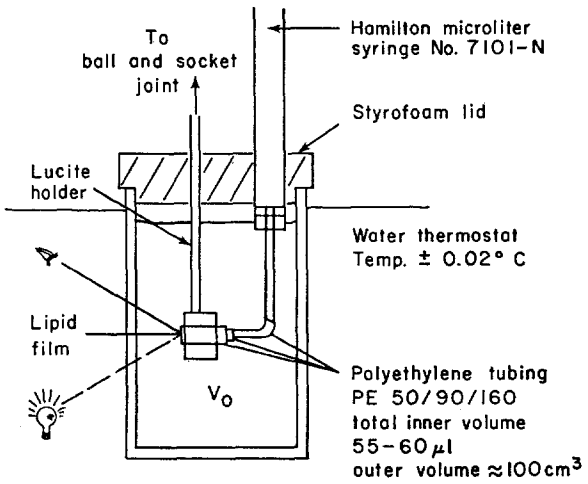

FIGURE 1. Cell used for measuring osmotic flux across thin lipid membranes.

workers on similar membranes (Hanai et al., 1965; Hanai and Haydon, 1966; Huang and Thompson, 1966) and demonstrate that the discrepancy between certain of their results and ours can be attributed to the existence of unstirred layers in their tagged water experiments. In addition, the effect of sugars on water transport in these films will be presented and discussed.

II. METHODS

A. *General*

All films studied were generated by the brush technique of Mueller et al. (1963) in a solution of 100 mM NaCl, 0.2 mM $MgCl_2$, 5 mM histidine, pH = 7 at 35-37°C. The phospholipids used were twice split lipids extracted from fresh ox brain white matter (Mueller et al., 1963); residual protein was less than 0.5%. The membrane solutions

[1] As a consequence of these studies plus a priori energy considerations, it has been generally assumed in the literature that these membranes are bilayer structures. Without questioning this conclusion, we have chosen throughout this paper to use the more conservative expression "thin lipid membranes (or films)" in referring to these structures.

consisted of 3-4.4% lipids in 2:1 (v/v) chloroform-methanol, plus 150-200 mg of DL-α-tocopherol and 10-60 mg of additional cholesterol per ml of membrane-forming solution. Such a solution, kept at $-30°C$ when not in use, could be used for several weeks, in most cases, to generate films with reproducible water permeabilities.

B. *Osmotic Flow*

Measurements of the net flow of water with concentration differences of NaCl, urea, sucrose, and glucose were made by a technique similar in principle to that used by Hanai and Haydon (1966) and by Huang and Thompson (1966).

The cell we used was simple, convenient to use, and very rapid in coming to temperature equilibrium (Fig. 1). It consisted of joined lengths of polyethylene tubing, terminated at one end by a Hamilton microliter syringe, No. 7101. The lipid film, when formed on the other end,[2] closed off the volume of the tube from the external solution.[3] Movement of the syringe changed this inner volume, and thus adjusted the degree of curvature of the film. The tubing and syringe were placed in a 100 ml Lucite box, immersed in a thermostat regulating to $\pm 0.02°C$. The tubing was mounted in a holder attached to a ball and socket joint. Thus, the film could be positioned for optimal viewing of the light reflected from it. The pattern of the light from the fully black film was dependent on the degree of curvature of the film; when the film was bulged by syringe displacements smaller than 0.005 μl, easily detectable changes in the pattern of reflected light occurred. With identical solutions inside and out and the system in thermal equilibrium, the syringe reading, which is the volume of the tube less an additive constant, remained the same for hours within the limits ± 0.01 μl. In practice, 10 min controls in symmetrical solutions were sufficient.

At the end of the control period, a small volume of concentrated solution was added to the Lucite box, and mixed thoroughly. After a small temperature transient, the film began to bulge inward, as water left the inner cell. By movement of the syringe to keep the pattern of light unchanged, and thus the area constant, the volume as a function of time was obtained. It was usually possible to make two to three changes of concentration on one film, as in Fig. 3.

Samples of the outer volume were analyzed gravimetrically, by chloride titration, and by freezing point depression. Water activities obtained from these data were checked by computation from the known volumes and concentrations employed. Osmotic coefficients for NaCl were obtained from Harned and Owen (1958); for urea and sucrose from Scatchard et al. (1938).

C. *THO Diffusion*

Two different arrangements for measurement of THO exchange with no net volume flow across the film were used. The second technique was developed from the first in our effort to solve stirring problems encountered. The general design of both methods is shown in Fig. 2.

[2] The internal cross-sectional area of tubing used was either 0.54 mm^2 or 1.02 mm^2.

[3] For each experiment a new piece of tubing was used; thus, elaborate cleaning rituals were unnecessary. Chemically clean surfaces were generated by cutting the new sterile tubing with a clean razor blade.

The first type of design used a 5 ml polyethylene cup, which could be sealed gas-tight, as the chamber containing the high concentration of isotope. The cup was placed in a 60 × 20 mm Petri dish, whose volume was partly occluded with paraffin to give a volume of ~6 ml. The film was formed across a circular hole about 1 mm in diameter in the thinned sidewall of the cup.

The experimental procedure was to form the film on the unsealed cup, allow it to become fully black, and then pipette 0.1 ml (100 μc) of tritiated water into the cup through an opening in the cover. The cup was then sealed by placing a lid over the opening to prevent communication between the inside and outside through the vapor phase.

Regular, intermittent stirring was accomplished by magnetic fleas inside and out; the fleas were in motion approximately 15 sec of each minute. 3 to 5 min after the addition of the isotope, a 0.05 ml sample was removed from the outside at a time defined as 0 min and placed in 15 ml of Bray's phosphor (Bray, 1960). Additional 0.05 ml samples were taken at 5 min intervals for counting (with replacement of

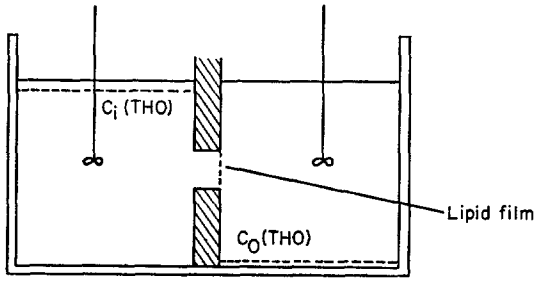

FIGURE 2. Design of THO diffusion experiment. c_i and c_o represent the concentrations of THO in the inside and outside compartments, respectively ($c_i \gg c_o$). Note the aqueous channel behind the membrane (see Discussion).

fluid to prevent bulging of the film) for as long as the film survived; this was often 60-70 min. At the end of the experiment, a 0.05 ml sample was taken from the more radioactive compartment (which generally had a specific activity of approximately 5×10^6 counts/min/ml), and all samples were then counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co.).

In this early design, two different thicknesses of sidewall, 0.2 and 0.9 mm, were used in order to test the degree of stirring behind the film.

In the second design both inner and outer compartments were milled from a solid block of Lucite. Stainless steel plates 0.125 mm thick were used to divide the two compartments of 5 ml each. The films were formed on a 0.62 mm^2 circular hole in the plate. In this design, constant stirring by bubbling on the outside with nitrogen and by a magnetic flea on the inside was routinely used. Other aspects of the protocol were similar to those of the early design.

In all experiments, controls were run to insure that THO exchanged between the compartments only by crossing the film.

III. THEORY

A. *Osmotic Flow of Water*

When the membrane is completely impermeable to the solute (which is the case of interest here), then the volume flow, J_v, of water is given by

$$J_V \equiv \frac{dV}{dt} = -\frac{L_P A}{\bar{V}} \Delta\mu_w \tag{1}$$

where L_P is the so-called hydrodynamic coefficient of the membrane, A is its area, and μ_w is the chemical potential of water given by

$$\frac{\mu_w}{\bar{V}} = \phi \frac{RT}{\bar{V}} \ln X_w \approx -\phi RT c_s \tag{2}$$

where,

X_w = mole fraction of water
c_s = concentration of solute
ϕ = osmotic coefficient
R = gas constant
T = absolute temperature
$\bar{V}$ = partial molar volume of water

Combining (1) and (2) we obtain

$$J_V = -L_P ART [\phi_2 c_{s_2} - \phi_1 c_{s_2}] \tag{3}$$

where the subscripts 1 and 2 refer to sides 1 and 2 of the membrane. The flux, Φ_w, of water (in moles per unit time), is

$$\Phi_w \equiv \frac{J_V}{\bar{V}} \tag{4}$$

From (3) and (4)

$$\Phi_w = -\frac{L_p ART}{\bar{V}} [\phi_2 c_{s_2} - \phi_1 c_{s_1}] = -P_f A[\phi_2 c_{s_2} - \phi_1 c_{s_1}] \tag{5}$$

where P_f is the so-called filtration (or osmotic) permeability coefficient of the membrane and one of the two permeability coefficients we wish to determine. For the solutes and their range of concentrations used in the present experiments, $\phi_2 \approx \phi_1 \equiv \phi$.

Defining:

$$c_{s_2} - c_{s_1} \equiv \Delta c_s$$

equation (5) is then rewritten as:

$$\begin{aligned} \Phi_w &= -P_f A \Delta c_s \times \phi \text{ (nonelectrolytes)} \\ \Phi_w &= -P_f A \Delta c_s \times 2 \times \phi \text{ (NaCl)} \end{aligned} \tag{5 a}$$

From the slope of V vs. t, Φ_w is readily obtained from (4) and P_f is then calculated from equation (5 *a*).

B. *Tagged Water Flow*

The flux, Φ_w^*, of tritiated water across a membrane is given by Fick's law

$$\Phi_w^* = -D_w A \frac{dc_w^*}{dx} \tag{6}$$

where c_w^* is the concentration of THO at any point x in the membrane and

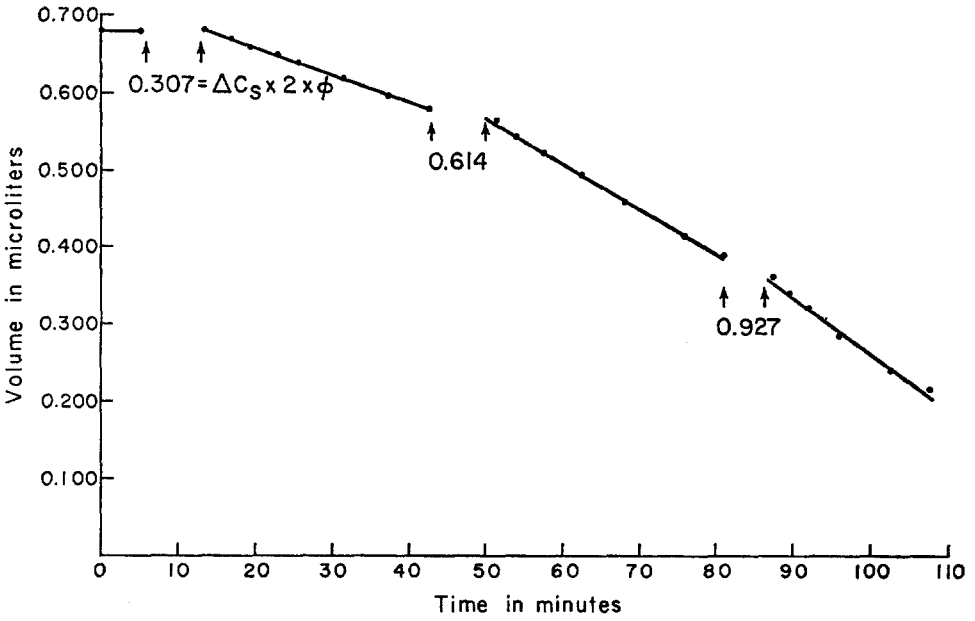

FIGURE 3. Volume flow of water across a single membrane for three concentration differences of NaCl. The pairs of arrows mark the time during which the salt concentration was changed. Area of film = 1.02 mm²; temperature = 36 ± 0.02°C.

D_w is the apparent diffusion coefficient of water (assumed to be the same for THO) in the membrane. Integrating (6) we have[4]

$$\Phi_w^* = -\frac{D_w A}{\Delta x}(c_{w_2}^* - c_{w_1}^*) = -P_d A(c_{w_2}^* - c_{w_1}^*) \tag{7}$$

where P_d is the *diffusion* permeability coefficient of water for the membrane, and the other quantity we wish to determine. From the slope of $c_{w_1}^*$ vs. time and the volume of side 1, we obtain Φ_w^*, and P_d is then calculated from (7). (Because of the small fluxes involved, $[c_{w_2}^* - c_{w_1}^*] \approx c_{w_2}^*$ throughout the course of the experiment.)

[4] The effect of any partitioning of water between the aqueous and membrane phases at the boundaries is already included in the coefficient D_w, so that it is permissible to integrate from sides 1 and 2 without concern for the details of the boundary processes.

IV. RESULTS

A. *Osmotic Flow*

1. NaCl AS SOLUTE The results of a typical experiment *on a single membrane* with NaCl as the solute are plotted in Fig. 3. Two aspects of this figure should be noted: first, $\frac{dV}{dt}$ (the rate of water movement) is constant for a given con-

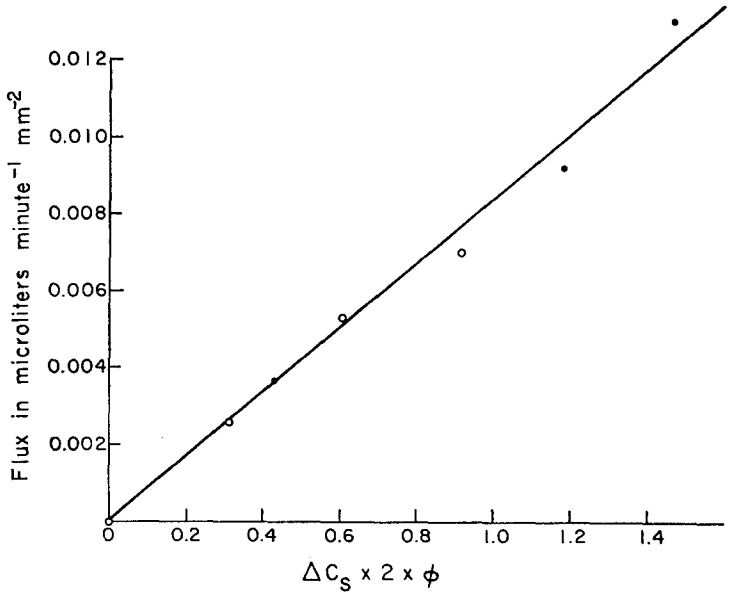

FIGURE 4. Flux, per unit area, of water (across films made from the same lipid solution used in the experiment of Fig. 3) as a function of concentration difference of NaCl. Each filled circle refers to a different membrane; the three open circles are from the experiment depicted in Fig. 3. The area of the films was 1.02 mm^2 for all points except the two at the largest Δc_s's, which was 0.54 mm^2. Temperature = 36 ± 0.02°C. From the coordinates of any point on the line, we obtain, using equation (5 *a*), $P_f = 0.78 \times 10^{-3}$ cm/sec.

centration difference (Δc_s) of NaCl, thus enabling an unambiguous P_f to be calculated;[5] second, the value of P_f so determined is invariant to Δc_s over a range of from 0.16 M to 0.5 M. (The values of P_f for this experiment are 0.77, 0.79, and 0.69 $\times$ 10^{-3} cm/sec for Δc_s's of molarity 0.16, 0.32, and 0.50, re-

[5] This is true provided the membrane is impermeable to NaCl and electroosmotic effects can be neglected. From the high electrical resistance of these films and independent tracer studies, it is clear that NaCl permeability is negligible compared with that of water, and thus the first condition is satisfied. In separate experiments with gradients of NaCl across the membrane, negligible membrane potentials were observed, thus making significant electroosmotic contributions most unlikely.

spectively.) Fig. 4 summarizes the results obtained on four different membranes made from the same lipid solution as in the above experiment with NaCl as the solute. The reproducibility of P_f from membrane to membrane was good with no systematic dependence of its value on area or Δc_s.

TABLE I

VALUES OF P_f OBTAINED ON SEVERAL MEMBRANES FORMED FROM A PARTICULAR LIPID SOLUTION

Film No.	Area	$\Delta c_s \times 2 \times \phi$	Flux	P_f
	mm²		*μl/min*	*cm/sec*
1	0.54	0.513	0.00322	1.07×10^{-3}
		1.020	0.00488	0.82×10^{-3}
2	0.54	0.368	0.00113	0.52×10^{-3}
3	0.54	0.478	0.00253	0.91×10^{-3}
4	1.02	0.242	0.00213	0.80×10^{-3}
		0.690	0.0070	0.92×10^{-3}
5	1.02	0.285	0.00466	1.48×10^{-3}
6	1.02	0.375	0.00527	1.27×10^{-3}

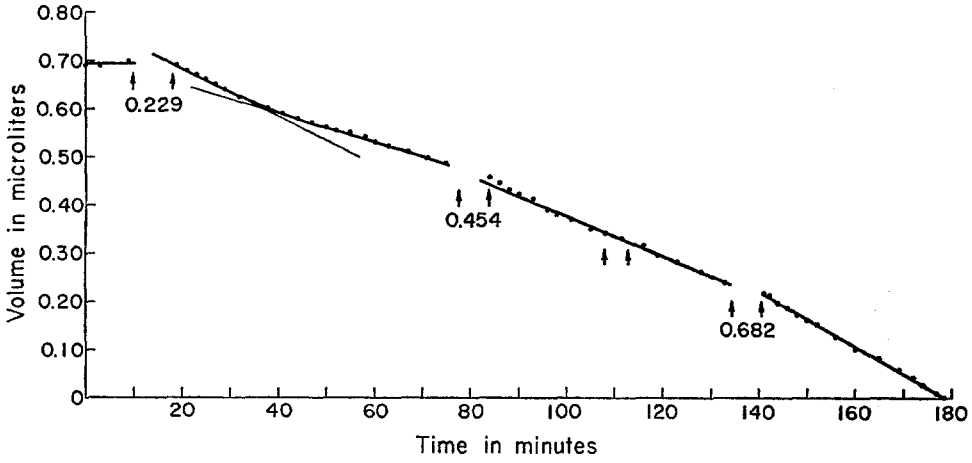

FIGURE 5. Volume flow of water across a single membrane for three different concentrations of glucose. The pairs of arrows mark the time during which the glucose concentration was changed. (The unlabeled pair designates a period of stirring.) Note the change in slope with a constant Δc_s of 0.229 M. From the four slopes in the figure and equation (5 *a*), we obtain successive values with time for P_f of 1.98, 1.35, 0.78, and 0.74 $\times$ 10^{-3} cm/sec. Area of film = 1.02 mm²; temperature = 36 ± 0.02°C.

It should be pointed out, however, that not all lipid solutions gave such reproducibility. Table I summarizes the results from membranes formed with another solution; i.e., with lipids from the brain of a different ox. Again, despite the spread in values of P_f, there is no systematic dependence on area or

Δc_s . We wish to emphasize, however, that in a given experiment the volume vs. time relationship was as linear as that shown in Fig. 3, and that for variations of Δc_s across the *same membrane*, P_f remained constant. Thus, it appears that the composition and/or structure of the black film that finally forms from the initial thick film that is painted across the aperture can vary. This is not unexpected, however, considering the variety of lipids in the mixture and the

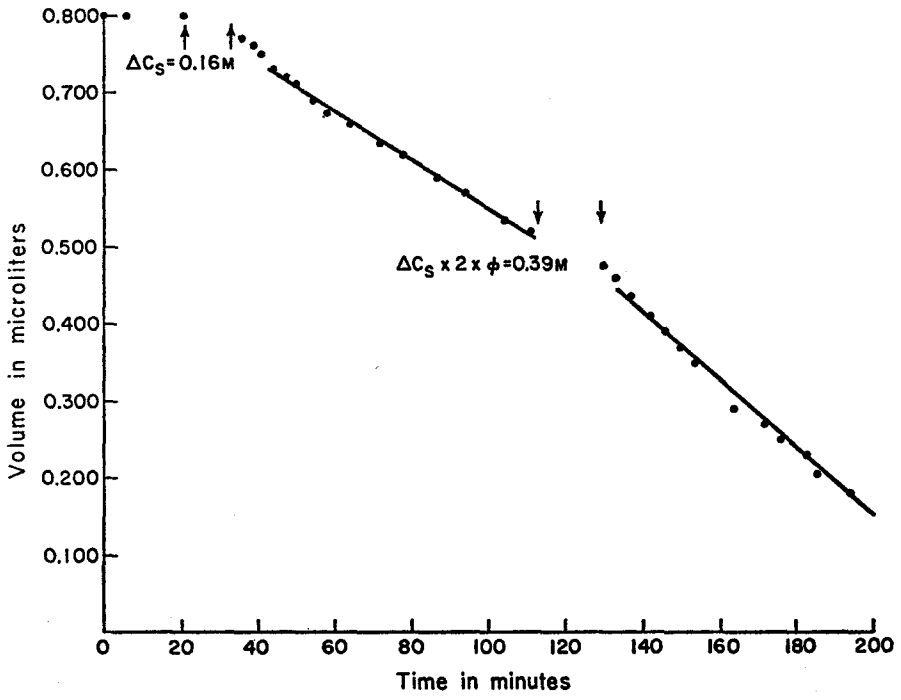

FIGURE 6. Effect of sucrose on the subsequent osmotic response of the membrane to NaCl. The first pair of arrows marks the introduction of a 0.16 M Δc_s of sucrose; the second pair of arrows marks the introduction of an additional 0.21 M Δc_s of NaCl ($\Delta c_s \times 2 \times \phi = 0.39$) to produce a total osmolarity difference across the membrane of 0.55 M. Note that although the osmotic gradient has more than tripled upon the addition of NaCl, the rate of flow has increased by a factor of only 1.4. (See text.) Area of film = 1.02 mm²; temperature = 36 ± 0.02°C.

very small number of the molecules ($\sim 10^{13}$) from the original thick film that end up in the final thin structure. In general, the values for P_f that we have obtained with mixed lipid films formed from five different solutions have ranged from 0.75 to 1.2 $\times$ 10^{-3} cm/sec, the most extreme values being a minimum of 0.52 $\times$ 10^{-3} and a maximum of 2.05 $\times$ 10^{-3} cm/sec.

2. GLUCOSE AND SUCROSE AS SOLUTES The results obtained with either glucose or sucrose as the solute were much more complex than those reported for the NaCl experiments, where a linear volume vs. time relationship (of 6 hr

duration or longer) and a simple additive effect of NaCl concentration difference on the flow rate were observed. Fig. 5 presents results typically obtained *on a single membrane* when glucose was used as the solute. Several points should be noted: first, the rate of movement of water is not constant, but decreases with time over several hours; second, the initial value of P_f (in this

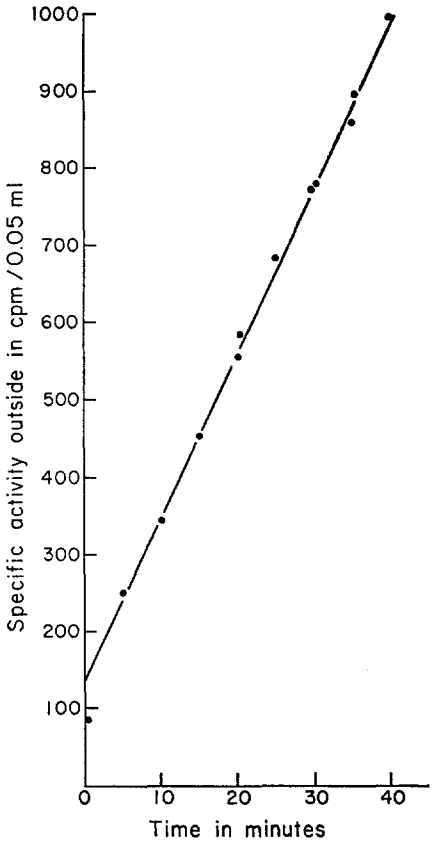

FIGURE 7. Results of THO diffusion experiment for a single membrane. Specific activity of the inside compartment was 32×10^4 cpm/0.05 ml; outside volume = 3.95 ml; area of film = 0.62 mm². From these data and the slope of the line, P_d is found from equation (7) to be 0.81×10^{-3} cm/sec. (This experiment was performed using the second design (see Methods).) Temperature = 36 ± 1°C.

case, 1.98×10^{-3} cm/sec) is significantly larger than any value obtained on films formed from this membrane solution in the NaCl experiments; third, the decreasing trend of P_f continues during successive increases of Δc_s of glucose and is not affected by stirring of the outer solution. Similar results were obtained with sucrose.[6]

In addition, both sucrose and glucose affected the P_f value obtained upon subsequent addition of NaCl (Fig. 6). From the initial rate of flow established by a 0.16 M Δc_s of sucrose, a value of $P_f = 1.87 \times 10^{-3}$ cm/sec is calculated, which is larger than any value obtained with NaCl on membranes from this

[6] In some experiments the rate of water movement was constant, but of a higher magnitude than predicted from NaCl data for the same lipid solution.

same lipid solution. A 0.21 M NaCl Δc_s was then introduced and the new rate of flow determined. Subtracting the previous rate in sucrose from the new rate in sucrose + NaCl, an apparent P_f in NaCl of 0.267×10^{-3} cm/sec is obtained; this value is twofold less than any other obtained on membranes from this particular lipid solution.

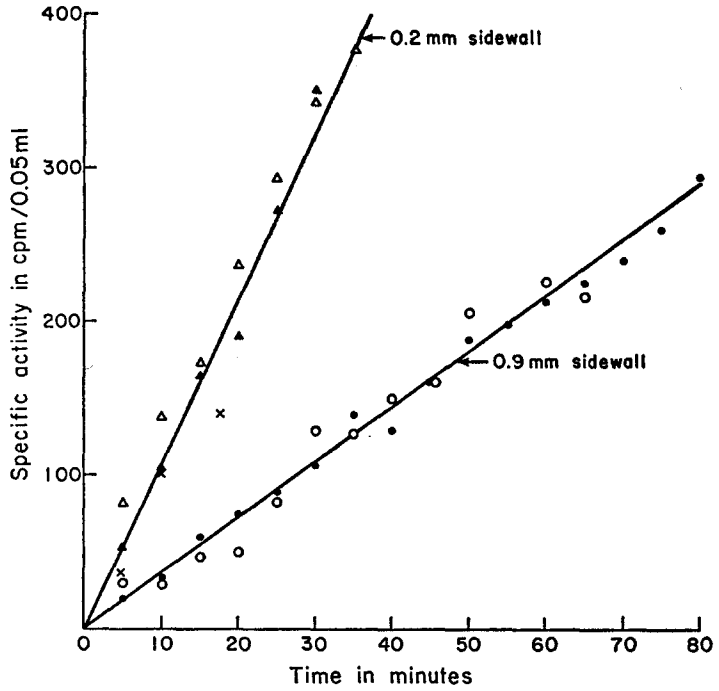

FIGURE 8. The effect of sidewall thickness on the rate of THO diffusion. The experiments were performed using the first design. The points designated by ×, ▲, and △ were obtained from three different membranes formed on a hole in a 0.2 mm thick sidewall; the points designated open circles and filled circles were obtained from two different membranes formed on a hole in a 0.9 mm thick sidewall. (All points have been normalized for a membrane area of 0.55 mm^2; specific activity of the inside compartment of 5.84×10^6 cpm/ml; and volume of the outside compartment of 6.0 ml.) Temperature = 36 ± 1°C.

3. UREA AS SOLUTE As with NaCl, a given Δc_s of urea produced a constant flow of solvent over a period of several hours. The values of P_f obtained with urea on membranes from a given lipid solution were not significantly different from the values obtained with NaCl. Thus, the reflection coefficient for urea is essentially 1; i.e., these films are quite impermeable to urea.

B. *Tagged Water Flux*

The results of a typical tagged water experiment *on a single membrane* are given in Fig. 7. From the slope of the straight line, the area of the film, the volume

on the "cold" side, and the specific activity on the "hot" side, P_d is immediately calculated (see section III).

With the early design (using a 0.2 mm thick sidewall), values obtained for P_d (in units of 10^{-3} cm/sec) from six membranes formed from three different lipid solutions were: 0.77, 0.61, 0.63, 0.48, 0.61, 0.51. The mean value was $P_d = 0.62 \times 10^{-3}$ cm/sec. With the second design (using a 0.125 mm thick plate), seven membranes from a single membrane solution gave an average P_d of $1.06 \pm 0.26 \times 10^{-3}$ cm/sec.

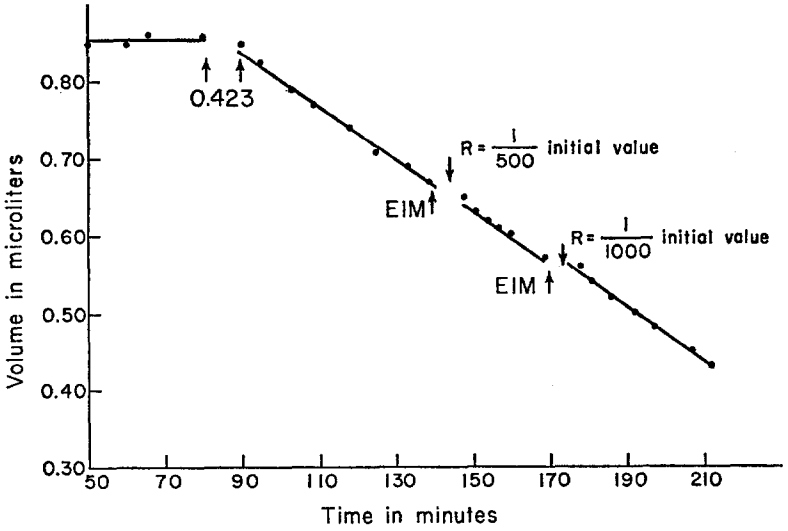

FIGURE 9. The effect of EIM on the osmotic flux of water. The first pair of arrows marks the time during which the NaCl concentration was changed to make $\Delta c_s \times 2 \times \phi = 0.423$ M. At each of the arrows marked EIM, 0.5 ml of unpurified EIM solution was introduced; the final protein concentration was 120 μg/ml. Area of film = 1.02 mm²; temperature = 36 ± 0.02°C.

The difference in values obtained with the two designs suggests that the thickness of the sidewall (or plate) and degree of stirring can affect the measured P_d. The following two experiments confirm these facts. When the sidewall thickness was changed from 0.2 to 0.9 mm in the first design, the measured P_d fell from 0.62 to 0.22 $\times 10^{-3}$ cm/sec (Fig. 8). In addition, when stirring on the hot side was stopped in the second design, P_d fell from 1.06 to 0.48×10^{-3} cm/sec. It is apparent from these two kinds of experiments that the measured value of P_d is highly dependent on the thickness of the barrier supporting the membrane and the rate of stirring.

C. *The Effect of "EIM" on P_f and P_d*

Mueller and Rudin (1963) have reported the existence of an as yet unpurified protein called "EIM" (excitability-inducing material) obtained from the cul-

ture fluid of *Aerobacter cloacae* grown on pure egg white. This material, when present on one side of the film in concentrations of 100 μg protein/ml or less, lowers the electrical resistance of these lipid membranes by a factor of 500-1000 within a period of several minutes. The drop in resistance can be attributed to an increased permeability of the membrane to cations. Although the nature of this material and its mechanism of action remain unknown, we felt it would be of interest to see whether a substance that produces such profound effects on the electrical properties of thin lipid films also affects their water permeability. Fig. 9 shows the result of an osmotic experiment in which EIM was introduced midway through its course. Also indicated in the same figure is the electrical resistance, as determined in a separate experiment, of a membrane formed from the same lipid solution when subjected to EIM at the same concentration. We see that while the electrical resistance of the membrane decreased after the introduction of EIM and eventually reached a value 500- to 1000-fold less than the original film resistance, the osmotic rate of water movement underwent no observable change. A similar result was found in the tagged water experiments.

V. DISCUSSION

A. *Edge Effects*

The fact that the flux of water, in either the THO or osmotic experiments, was directly proportional to the area of the hole on which the film was formed, indicates that the values of P_d and P_f obtained are properties of the black film per se, rather than of the boundary region between the film and the supporting structure. (By bulging the film to change its area, Hanai and Haydon (1966) obtained similar results.)

B. *Comparison of P_f and P_d*

The osmotic permeability coefficient, P_f, has been found to be severalfold greater than the tagged water permeability coefficient, P_d, for various types of cells (Prescott and Zeuthen, 1953; Paganelli and Solomon, 1957; Nevis, 1958). Since such a result is also obtained in artificial membranes of a porous nature (e.g., cellulose, collodion) (Renkin, 1954; Robbins and Mauro, 1960), the conclusion has been drawn that water traverses plasma membranes through a few aqueous pores or channels, and that the bulk of the membrane consists of lipoid regions impermeable to water (Danielli, 1954). This view is consistent with the classical observations that plasma membranes are permselective to "water-soluble" molecules on the basis of size, while being readily permeable to large "lipoid-soluble" molecules (Overton, 1895). The origin of these pores is postulated by some to be the result of penetration of the bimolecular lipid matrix by proteins (Danielli, 1954), while others have sug-

gested that globular lipid micelles within the bimolecular leaflet could give rise to aqueous pores (Lucy, 1964). It is relevant, therefore, to consider carefully the water permeability data obtained by us and by others on artificial phospholipid films.

The first point to note is that the permeability coefficients (either P_f or P_d) which we obtained on films made from white matter lipids + DL-α-tocopherol fall within the range of values reported for plasma membranes, and, in fact, lie at the high end of this range. (Similar values of P_f have been reported by Hanai and Haydon (1966) on films of egg lecithin + cholesterol and decane; even higher values have been obtained by Huang and Thompson (1966) on membranes formed from egg lecithin and tetradecane dissolved in chloroform-methanol.) This is remarkable in view of the fact that the electrical resistance

TABLE II

COMPARISON OF P_d AND P_f

	P_d	P_f	Ratio	Reference
	cm/sec	*cm/sec*		
	$0.62 \pm 0.13 \times 10^{-3}$*	$0.77\text{–}1.14 \times 10^{-3}$‡		Present work
One lipid solution	$1.06 \pm 0.26 \times 10^{-3}$§ (7 membranes)	$1.14 \pm 0.06 \times 10^{-3}$ (5 membranes)	1.1	Present work
	0.23×10^{-3}	$0.88\text{–}1.99 \times 10^{-3}$	4–8	Hanai et al. (1965)
	0.44×10^{-3}	$1.70\text{–}10.4 \times 10^{-3}$	4–24	Huang and Thompson (1966)

* Obtained using first design with 0.2 mm thick sidewall (see Methods).
‡ Lowest and highest average values obtained from four different lipid solutions.
§ Obtained using second design (see Methods).

of these films ($\sim 10^7 \Omega$ cm^2) is 10^3-10^6 times higher than those values reported for plasma membranes. In addition, we have seen that the introduction into the system of a protein which lowered the electrical resistance of the membrane by a factor of 500-1000 had no observable effect on water permeation. Thus, it appears that the water permeability of plasma membranes is (in magnitude) compatible with a general property of thin lipid films, while ionic permeability is a reflection of certain modifications of this basic structure.

The second point to note is that there was no significant difference between P_f and P_d in the type of films we studied. This is in contrast to the results reported by Hanai et al. (1965) and Huang and Thompson (1966), the former reporting ratios of P_f to P_d of approximately 4 to 8 and the latter obtaining ratios of from 4 to 24, depending upon the particular preparation of egg lecithin used. In Table II are shown the values of P_f and P_d obtained by these other workers, along with our own data. Two features of the table are relevant to this discrepancy: first, the values of P_d reported by Hanai et al. and Huang and Thompson are one-fourth to one-half of our own values; and second,

while P_f in Huang and Thompson's experiments varies by a factor of 6 from one lecithin batch to another, P_d remains constant. The question arises whether these differences reflect properties of the various types of films used, or whether they can be attributed to features of the experimental design.

One obvious possible difficulty in the tagged water experiments is the existence of unstirred or stagnant layers on the two sides of the film,[7] a possibility raised by Hanai et al. (1966) and discussed, but dismissed, by Huang and Thompson (1966). The experiments in Fig. 8 were performed to determine whether such an effect could account for the differences in our observations from those of the above authors. Comparison of the values of P_d obtained with membranes formed on the 0.125, 0.2, and 0.9 mm thick partitions (1.06, 0.62, and 0.22 $\times$ 10^{-3} cm/sec, respectively), with stirring in both bulk phases, shows that additional resistance to tagged water movement is introduced as the barrier thickness is increased. This must be attributed to insufficient mixing adjacent to the membrane interfaces (at least for the two thicker partitions) despite the fact that the stirring produced rapid oscillations of the membrane. The region most likely to be unstirred is the cylinder of fluid immediately behind the membrane (see Fig. 2). It is therefore instructive to calculate the $P_{d_{H_2O}}$ for a cylinder of water of varying lengths. Taking the free diffusion coefficient, D_{H_2O}, of water at 36°C as 3 $\times$ 10^{-5} cm²/sec (Wang et al., 1953) and applying Fick's law, we have:

$$P_{d_{H_2O}} = \frac{D_{H_2O}}{l} \tag{8}$$

where l is the length of the cylindrical channel. We thus obtain:

l	$P_{d_{H_2O}}$
mm	*cm/sec*
0.9	0.33×10^{-3}
0.5	0.6×10^{-3}
0.2	1.5×10^{-3}

It is apparent, that even if the membrane offered no resistance to water, i.e.

[7] This is a much more serious problem a priori in the tagged water experiments than in the osmotic ones for two reasons: first, for the rates of flow involved in our experiments, any plausible thickness of unstirred layer (<2 mm) will diminish P_d significantly more than P_f (Dainty, 1963); second, because significant density gradients arise in the osmotic experiments as pure water moves across the membrane, convective stirring may be expected to assist in maintaining boundary conditions (such an effect is, of course, inoperative or trivial in the tagged water measurements). Finally, the invariance of P_f with Δc_s and the constancy of $\frac{dV}{dt}$ over long periods of time for a given Δc_s are experimental facts incompatible with the development of significant unstirred layers during the course of the osmotic measurements. (Note that even with the high flow rate of 0.01 μl/min across a 1 mm² area film, it would take a minimum of 100 min to develop an unstirred layer of 1 mm, which would cause a reduction of less than 20% in P_f.)

$P_d = \infty$, the largest *observable* value of P_d would be 0.6×10^{-3} cm/sec, if there existed an unstirred layer of 0.5 mm, which is the thickness of the membrane support used by Huang and Thompson.

In view of this calculation it is pertinent to inquire whether the average value of $P_f(1 \times 10^{-3}$ cm/sec) obtained by us is inconsistent with our determination of $P_d(0.6 \times 10^{-3}$ cm/sec) using a 0.2 mm thick sidewall. For diffusion barriers in series we have:

$$\frac{1}{P_{d_{obs}}} = \frac{1}{P_{d_{H_2O}}} + \frac{1}{P_d} \tag{9}$$

where P_d is the actual diffusion permeability coefficient to water of the membrane and $P_{d_{obs}}$ is the experimentally observed value.
Substituting we have:

$$\frac{1}{0.62 \times 10^{-3}} = \frac{1}{1.5 \times 10^{-3}} + \frac{1}{P_d}$$

$$P_d = 1.06 \times 10^{-3} \text{ cm/sec}$$

which is the same as P_f. (If we substitute this value of P_d into (9) along with $P_{d_{H_2O}}$ for a 0.9 mm thick sidewall, we get $P_{obs} \approx 0.25 \times 10^{-3}$ which is the result obtained experimentally.)

Finally, in row 2 of Table II are given the values of P_f and P_d for membranes formed from a single lipid solution. P_d in this case was determined using the second design which employs the thinnest partition (0.125 mm) and vigorous, continuous stirring. Clearly, within experimental error, $P_f = P_d$.

From the above considerations we conclude that in our films, i.e. those made from a Folch extract of white matter + DL-α-tocopherol, $P_f = P_d$ (to at least within 20%), and that in the films used by other investigators, there is *no* evidence to indicate that P_f is significantly greater than P_d. We wish to emphasize that we do not question the values of P_f obtained by these workers, but feel that their values for P_d are much too low because of unstirred layer effects.[8] (We have, in fact, measured P_f on membranes formed from a preparation of egg lecithin + tetradecane dissolved in chloroform-methanol[9] and obtained the high value of $P_f \approx 6 \times 10^{-3}$ cm/sec, which is consistent with Huang and Thompson's values. For such membranes, it would be very difficult to establish the equality of P_d and P_f, inasmuch as an unstirred layer of only 50 μ would have a P_d of the same magnitude, so that the observed P_d would be only 3×10^{-3} cm/sec even if the actual P_d of the film equaled P_f.) *Thus, insofar as ratios of P_f to P_d greater than 1 indicate the existence of aqueous pores* (Prescott and Zeuthen, 1953; Ussing, 1954), *we can conclude that there is no such evidence for their existence in synthetic thin lipid membranes.*

[8] Hanai et al. (1966) have reached a similar conclusion regarding their determination of P_d.

[9] We thank T. Thompson for kindly supplying us with the lecithin sample.

The above finding differs from the results reported on plasma membranes of several types of cells, where it has been generally found that $\frac{P_f}{P_d} > 1$. This is of some interest, since, as pointed out earlier, the values of P_f and P_d obtained on the thin lipid films lie within the range of values reported for plasma membranes. Before invoking in explanation structural differences between plasma membranes and synthetic thin lipid films, however, one should be confident that the findings for plasma membranes are correct. As vigorously pointed out by Dainty (1963), the values of P_d in the plasma membrane literature are open to serious question because of the possible existence of significant unstirred layers. From our own experience with stirring problems in these relatively accessible artificial systems, we feel that serious consideration should be given to this very real possibility. Thus, for plasma membranes, $\frac{P_f}{P_d}$ may actually equal 1.

C. *Mechanism of Osmotic Water Transport in Thin Lipid Films*

The fact that $P_f = P_d$ implies that the same mechanism operates to drive water across the thin lipid membranes when there is a difference in chemical potential of water between the two sides produced by an impermeant species, as operates to move isotopically labeled water in the absence of an osmotic gradient. (Considering that the flux of labeled water in one direction means an equal and opposite flux of unlabeled water, then the above statement is equivalent to saying that whether the chemical potential difference of water is created by its own isotope or by an impermeant species, the mechanism of water transport across the membrane is the same.) Equality of P_f and P_d would be expected, to a first approximation, for a liquid membrane in which the activity coefficient of water in the liquid composing the membrane is constant throughout. In both flow processes, water traverses the membrane by dissolving in the membrane on one side, diffusing through the bulk liquid portion of the membrane, and then redissolving in solution on the other side. The kinetics of such a system have been discussed by Longsworth (1933) with particular reference to the liquid guaiacol membranes of Osterhout and Stanley (1932). In a recent paper, Hanai and Haydon (1966) have shown that by substituting into the above model reported values of the partition coefficient of water in bulk hydrocarbons and the diffusion coefficient of water in an organic phase obtained from an empirical formula, the rate of water movement across their films was predicted within a factor of 2.

Other possibilities which must be considered for such thin films as these are that water traverses them via fluctuating defects in their structure, or that water molecules possessing sufficient energy can "shoot" through the barrier even in the absence of a defect. In these mechanisms, as distinct from the solu-

bility mechanism, there is no water that can be considered dissolved within the membrane phase.

In order to distinguish one mechanism from others, information is required on the magnitude and nature of the energy barriers involved in water (and solute) transport through these bilayer films. Temperature studies, which are presently in progress, along with investigation of the dependence of P_f on lipid composition are experiments pertinent to these important points. It is perhaps worth noting that in the retardation of evaporation of water by fatty acid monolayers, Archer and La Mer (1955) conclude that the most significant barrier to evaporation is at the polar ends of the film, rather than in the long hydrocarbon chains. It is also interesting that these monolayers reduce the rate of water evaporation by a factor of approximately 10^4, and that the lipid films studied reduce the rate of water transport (as compared to a "barrier" of water of the same thickness as the film) by the same factor.

D. *Interaction of Membrane with Sugars*

The results obtained in the glucose and sucrose experiments indicate that these molecules are in some way interacting with the membrane and modifying its properties.[10] It is noteworthy, however, that this anomalous behavior of sugars is not unprecedented. Dainty and Ginzburg (1964) have reported effects of sucrose on the permeability of *Nitella* to water and urea. Also, Baer (1956), Reinwein (1961), and LeFevre et al. (1964) have shown that glucose and sucrose are partitioned into an organic phase if phospholipids are present. Further exploration of the interaction of sugars with thin lipid films may be useful in elucidating the nature of the "carrier" system implicated in monosaccharide transport in some cells (LeFevre and McGinniss, 1960).

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REFERENCES

1. ARCHER, R. J., and V. J. LA MER. 1955. The rate of evaporation of water through fatty acid monolayers. *J. Phys. Chem.* **59**:200.
2. BAER, E. 1956. The synthesis of glycerol phosphatides. *Can. J. Biochem. Physiol.* **34**:288.
3. BRAY, G. E. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279.
4. DAINTY, J. 1963. Water relations of plant cells. *Advan. Botan. Res.* **1**:279.
5. DAINTY, J., and B. Z. GINZBURG. 1964. The permeability of the cell membranes

[10] For the lecithin-cholesterol-decane films, Hanai and Haydon (1966) found no such effect, obtaining essentially the same results with sucrose as with NaCl and urea.

of *Nitella translucens* to urea, and the effect of high concentrations of sucrose on this permeability. *Biochim. Biophys. Acta.* **79**:112.

6. DANIELLI, J. F. 1954. Morphological and molecular aspects of active transport. *Symp. Soc. Exptl. Biol.* **8**:502.
7. HANAI, T., and D. A. HAYDON. 1966. The permeability to water of bimolecular lipid membranes. *J. Theoret. Biol.* **11**:370.
8. HANAI, T., D. A. HAYDON, and W. R. REDWOOD. 1966. The water permeability of artificial bimolecular leaflets: A comparison of radiotracer and osmotic methods. *Ann. N. Y. Acad. Sci.* **137**:731.
9. HANAI, T., D. A. HAYDON, and J. TAYLOR. 1965. Some further experiments on bimolecular lipid membranes. *J. Gen. Physiol.* **48** (5, Pt. 2): 59.
10. HARNED, H. S., and B. B. OWEN. 1958. The Physical Chemistry of Electrolytic Solutions. Reinhold Publishing Corp., New York. 3rd edition. 492.
11. HUANG, C., and T. E. THOMPSON. 1966. Properties of lipid bilayer membranes separating two aqueous phases: Water permeability. *J. Mol. Biol.* **15**:539.
12. HUANG, C., L. WHEELDON, and T. E. THOMPSON. 1964. The properties of lipid bilayer membranes separating two aqueous phases: Formation of a membrane of simple composition. *J. Mol. Biol.* **8**:148.
13. LEFEVRE, P. G., K. I. HABICH, H. S. HESS, and M. R. HUDSON. 1964. Phospholipid-sugar complexes in relation to cell membrane monosaccharide transport. *Science.* **143**:955.
14. LEFEVRE, P. G., and G. F. MCGINNISS. 1960. Tracer exchange *vs.* net uptake of glucose through human red cell surface. *J. Gen. Physiol.* **44**:87.
15. LONGSWORTH, L. 1933. The theory of diffusion in cell models. *J. Gen. Physiol.* **17**:211.
16. LUCKÉ, B., H. K. HARTLINE, and M. MCCUTCHEON. 1931. Further studies on the kinetics of osmosis in living cells. *J. Gen. Physiol.* **14**:405.
17. LUCY, J. A. 1964. Globular lipid micelles and cell membranes. *J. Theoret. Biol.* **7**:360.
18. MUELLER, P., and D. O. RUDIN. 1963. Induced excitability in reconstituted cell membrane structure. *J. Theoret. Biol.* **4**:268.
19. MUELLER, P., D. O. RUDIN, H. TI TIEN, and W. C. WESCOTT. 1962. Reconstitution of excitable cell membrane structure *in vitro*. *Circulation.* **26**:1167.
20. MUELLER, P., D. O. RUDIN, H. TI TIEN, and W. C. WESCOTT. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* **67**:534.
21. NEVIS, A. H. 1958. Water transport in invertebrate peripheral nerve fibers. *J. Gen. Physiol.* **41**:927.
22. OSTERHOUT, W. J. V., and W. M. STANLEY. 1932. The accumulation of electrolytes. V. Models showing accumulation and a steady state. *J. Gen. Physiol.* **15**:667.
23. OVERTON, E. 1895. Uber die osmotischen Eigenschaften der lebenden Pflanzen und Tierzelle. *Vierteljahreschr. Naturforsch. Ges. Zuerich.* **40**:159.
24. PAGANELLI, C. V., and A. K. SOLOMON. 1957. The rate of exchange of tritiated water across the human red cell membrane. *J. Gen. Physiol.* **41**:259.

25. PRESCOTT, D. M., and E. ZEUTHEN. 1953. Comparison of water diffusion and water filtration across cell surfaces. *Acta Physiol. Scand.* **28**:77.

26. REINWEIN, D. 1961. Quoted by Park, C. R. *In* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. Academic Press, Inc., New York. 453.

27. RENKIN E. M. 1954. Filtration, diffusion, and molecular sieving through porous cellulose membranes. *J. Gen. Physiol.* **38**:225.

28. ROBBINS, E., and A. MAURO. 1960. Experimental study of the independence of diffusion and hydrodynamic permeability coefficients in collodion membranes. *J. Gen. Physiol.* **43**:523.

29. SCATCHARD, G., W. J. HAMER, and S. E. WOOD. 1938. Isotonic solutions. I. The chemical potential of water in aqueous solutions of sodium chloride, potassium chloride, sulfuric acid, sucrose, urea and glycerol at 25°. *J. Am. Chem. Soc.* **60**:3061.

30. SIDEL, V. W., and A. K. SOLOMON. 1957. Entrance of water into human red cells under an osmotic pressure gradient. *J. Gen. Physiol.* **41**:243.

31. USSING, H. H. 1954. Ion transport across biological membranes. *In* Ion Transport Across Membranes. Hans T. Clarke, editor. Academic Press, Inc., New York. 3.

32. WANG, J. H., C. V. ROBINSON, and I. S. EDELMAN. 1953. Self-diffusion and structure of liquid water. III. Measurement of the self-diffusion of liquid water with H^2, H^3, and O^{18} as tracers. *J. Am. Chem. Soc.* **75**:466.