J. Membrane Biol. 7, 275–284 (1972)

Water Permeability of Bilayer Lipid Membranes: Sterol-Lipid Interaction

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Received 24 June 1971

Summary. Bilayer lipid membranes were generated in an aqueous medium from synthetic, egg or plant phosphatidyl choline (PC) or from plant monogalactosyl diglyceride (MG). The water permeability of the black membranes was determined by measuring the net volume flux produced by a NaCl gradient. The osmotic permeability coefficient, P_{os}, was markedly affected by the number of double bonds in the fatty acid conjugates of the lipids: the greater the degree of unsaturation, the higher the value of P_{os}. The temperature dependence of P_{os} of the lipid membranes was studied over a range of 29 to 40 °C. The experimental activation energy, E_a, estimated from the linear plots of log (P_{os}) *versus* $1/T$, was significantly higher for MG membranes (17 kcal/mole) than for the various PC membranes (11 to 13 kcal/mole), probably owing to hydrogen bonding between MG and water molecules. In comparison with PC membranes, the membranes generated from PC and cholesterol (1:1 molar ratio) had lower P_{os} but similar E_a values. Likewise, either stigmasterol or β-sitosterol decreased P_{os} of MG membranes, while E_a was not affected by the sterols. MG-cholesterol membranes were specifically characterized by a unique value of E_a (-36 kcal/mole) thus indicating temperature dependent structural changes.

Lipid bilayer membranes are similar to cellular membranes with respect to thickness, electrical capacitance, interfacial tension (Tien & Diana, 1968) permeability to urea, glycerol and mannitol (Vreeman, 1966), as well as water permeability and activation energy of water permeation (Hanai & Haydon, 1966; Henn & Thompson, 1969; Price & Thompson, 1969) as affected by vasopressin (Graziani & Livne, 1971). The similarity in water permeability characteristics may indicate a common mechanism of water transport in biological and artificial bilayer membranes.

The range of values of water permeability found in various biological systems (0.37 to 400 μ/sec according to Dick, 1959) is much wider than that measured in thin lipid membranes. This apparent discrepancy does not necessarily indicate that the artificial lipid membrane is a poor model for water permeation. It may rather reflect the relatively limited variety of lipids used for the study of water permeation through thin lipid membranes. Most of the studies were performed with egg lecithin, with or without

cholesterol. Indeed, thin films made from mixed lipids of ox brain are considerably less permeable to water than are lecithin membranes (Finkelstein & Cass, 1967).

Phosphatidyl choline and cholesterol are major constituents in membranes of animal cells, such as red blood cells (van Deenen & de Gier, 1964). In green plants, however, glycolipids (particularly galactolipids) are more abundant than phospholipids (Benson, 1964; Allen, 1966). The sterols found in membranous fractions of a plant tissue are primarily β-sitosterol and stigmasterol, together with campesterol and cholesterol (Grunwalt, 1970). It is thus of interest to increase the variety of lipids and sterols in studying water permeability in a model system.

The purpose of this investigation was to examine the water permeability of thin lipid membrane as affected by: (a) the polar-end group of the lipid, as well as the degree of unsaturation of the fatty acid conjugates, and (b) the sterol moiety added to the membrane-generating solution. It is shown that each of these factors may modify to varying extents the net flux of water across a thin lipid membrane and/or the temperature dependence of the flux.

Materials and Methods

Experimental System

The experimental design for measuring osmotic water flux across thin lipid membranes was essentially as described by Price and Thompson (1969), except that the compartment bearing the membrane was constructed of polyethylene tubing (1 mm internal diameter), as used by Cass and Finkelstein (1967). Lipid solutions (1 %, w/v) were made in *n*-decane. When included, sterols were added at a sterol to lipid molar ratio of 1:1. The membrane was generated in a medium of 0.3 molal NaCl at 37 ± 0.1 °C. To generate a membrane, a minute droplet of the lipid solution was layered with a syringe on the end of the polyethylene tubing. The membrane usually turned black within 6 to 8 min as viewed with the aid of binoculars ($\times 40$ magnification). The NaCl concentration of the open compartment was then raised to 0.6 molal. Water flux was determined at 1-min intervals by measuring the volume of solution which needed to be replaced in the closed compartment in order to restore the membrane to its flat state. A Hamilton syringe (# 701) attached to the closed compartment and equipped with a calibrated micrometer allowed measurement of volume changes of 0.005 ± 0.001 µliter.

The linear plot of the change in volume as a function of time was used to compute the permeability constants. Calculation of the filtration coefficient, L_p, is based on the terminology of Kedem and Katchalsky (1958) as presented by Price and Thompson (1969):

$$L_p = \frac{J_t}{\nu \mathrm{RTA}(\phi M - \phi' M')} \tag{1}$$

where, J_t is total volume flow; ν is number of moles of ions given by one mole of electrolyte; R is universal gas constant; T is absolute temperature; A is membrane surface area; M is molal solute concentration; ϕ is molal osmotic coefficient. (ϕM and $\phi' M'$ are related to the open and the closed compartments, respectively).

L_p is expressed in units of length, time^{-1} · pressure^{-1}. It may be converted to a coefficient of permeability, P_{os}, according to Dick (1966).

$$P_{os} = L_p \frac{RT}{\bar{V}_w} \tag{2}$$

where $\bar{V}_w$ is the partial molar volume of water. P_{os} has the units of length · time^{-1}. Equating $\nu = 2$, and substituting the expression of L_p from Eq. (1) into Eq. (2), the following is obtained:

$$P_{os} = \frac{J_t}{2A(\phi M - \phi' M')\bar{V}_w}. \tag{3}$$

Following the application of NaCl gradient, the water flux was first measured at 37 °C and then the temperature was modified to determine the temperature-dependent fluxes over the range of 29 to 40 °C. The same membrane was used to record P_{os} at several different temperatures.

Electrical properties of galactolipid and lecithin black membranes were studied in a system as described by Vreeman (1966). The resistance of the membranes was 10^6 to 10^8 Ω cm^2, the capacitance was 0.38 to 0.56 μF/cm^2, and the calculated thickness 44 to 60 Å. The breakdown voltage of the membranes was 140 to 200 mV. These parameters are typical for bimolecular, or bilayer, membranes (Mueller *et al.*, 1962; Henn & Thompson, 1969).

Lipids

Phosphatidyl choline (PC)[1] and monogalactosyl diglyceride (MG) were extracted from leavens of greenhouse-grown tobacco plants (*Nicotiana rustica*), and the lipids were purified according to the procedures described by Allen *et al.* (1966). Fractions obtained by column chromatography of the crude extract on DEAE-cellulose (BioRad, Richmond, Calif.) were purified first on a column of silicic acid (BioRad, Richmond, Calif.), and then by TLC on preparative silica gel plates (Merck, Darmstadt). The mixture of solvents for TLC consisted of chloroform-methanol water (65:25:4, v/v/v).

To evaluate its purity, a fraction of MG was hydrolyzed according to Eichenberger *et al.* (1966), and the sugar concentration in the hydrolyzate was determined by Somogi's method (1930). Phosphate content was analyzed after ashing and hydrolysis according to the procedure described by Ames (1966). The molar ratios of sugar to lipid and of phosphate to lipid were 0.95 and 0.02, respectively.

Egg PC was prepared according to Pangborn (1951). Synthetic PC (dipalmitoyl) was purchased from Sigma-Israel (Ramat Gan, Israel). The fatty acid composition of the lipids was analyzed by gas-liquid chromatography, after saponification of the lipids and methylation of the fatty acids with BCl_3 in methanol, as described by Kuiper (1970). Peaks were identified with the aid of standard methyl esters of the various fatty acids (Sigma, St. Louis, Missouri). A Packard gas chromatograph model 7400, provided with a flame ionization detector, was used for analysis. The column was composed of 15% diethylene glycol succinate as a stationary phase on chromosorb as a support. The fatty acid composition of MG and of the various PC preparations used in this study is summarized in Table 1. Stock solutions of the lipids in chloroform were stored under nitrogen at −18 °C.

β-sitosterol and stigmasterol were purchased from Sigma-Israel, (Ramat Gan, Israel). Cholesterol was obtained from Riedel-de-Haen (Seelze-Hannover, Germany).

1 Abbreviations used: PC, phosphatidyl choline (lecithin); MG, monogalactosyl diglyceride; TLC, thin-layer chromatography; DEAE cellulose, diethylaminoethyl cellulose.

Table 1. Fatty acid composition of lipids used to generate bilayer membranes

Lipid	Percentage of total fatty acid of each lipid				
	16/0[a]	18/0	18/1	18/2	18/3
PC synthetic	100	—	—	—	—
PC egg	28.5	14.2	40.0	16.8	0.5
PC plant	25.0	2.9	6.1	30.4	35.6
MG plant	14.4	2.6	11.2	6.4	65.4

[a] Number of C atoms and number of double bonds, respectively.

All three sterols were crystallized twice from ethanolic solutions. The melting points of the recrystallized sterols conformed to known values. Furthermore, each of the sterols appeared as a single spot in TLC with silica gel plates using any one of the following solvents: benzene, methanol, chloroform or chloroform-methanol-water (65:25:4, v/v/v).

Results

The water permeability of bilayer PC membranes was studied to allow comparison with previous reports. Fig. 1 shows the temperature dependence of water permeability of such membranes at the range of 29 to 39 °C. The plot of log P_{os} *versus* $1/T$ is linear, as shown by Price and Thompson (1969). Fig. 1 shows that membranes made from a mixture of PC and cholesterol (1:1 molar ratio) have a lower rate of water flux, thus confirming the data given by Finkelstein and Cass (1967). The slope of the temperature dependence curve is not affected by cholesterol.

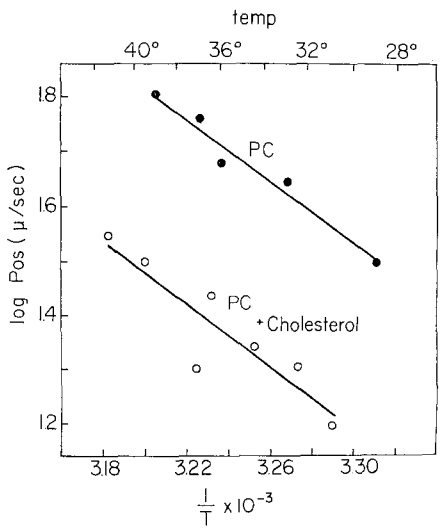

Fig. 1. Temperature dependence of P_{os} of PC and of PC + cholesterol membranes. Log P_{os} is plotted *versus* the reciprocal of absolute temperature

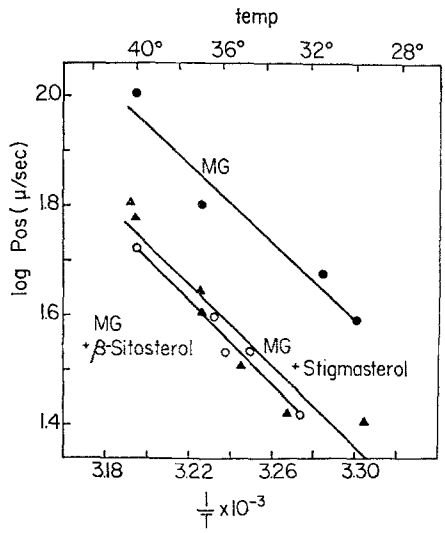

Fig. 2. Temperature dependence of P_{os} of MG (closed circle) and of MG + β-sitosterol (open circle) or MG + stigmasterol (triangle). Log P_{os} is plotted against the reciprocal of absolute temperature

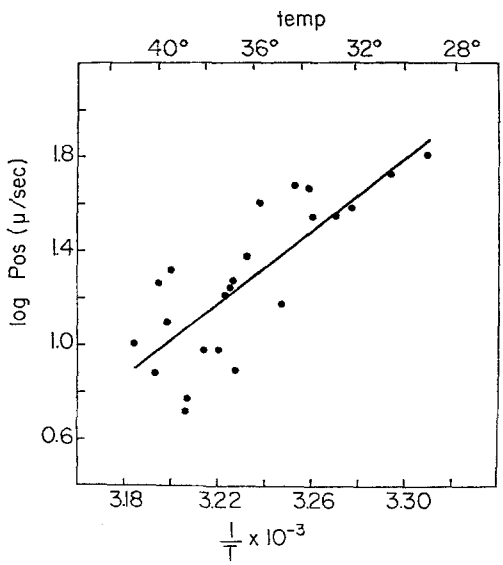

Fig. 3. Temperature dependence of P_{os} of MG + cholesterol

Fig. 2 presents the temperature dependence of water permeability of MG membranes as affected by stigmasterol and by β-sitosterol. Again, the sterols reduce the rate of water flux, but the curves are parallel. However, when cholesterol is mixed with MG, the temperature dependence of water permeability of the membrane differs markedly (Fig.3). Despite the consider-

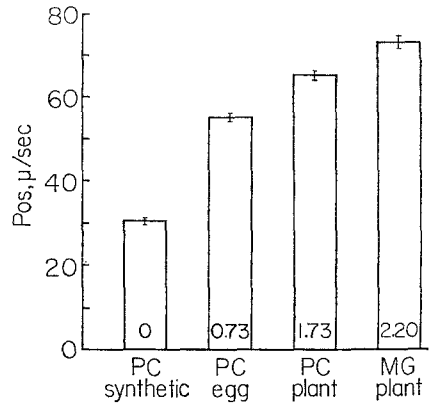

Fig. 4. Values of P_{os} at 37 °C of the lipids studied. The figure in each bar indicates the average number of double bonds per fatty acid conjugate. The vertical lines on top of the bars = SE

able variation the results presented are consistent with several experiments, all showing the same unique slope as the one illustrated.

To compare quantitatively the water permeability of membranes of the various lipids, values of P_{so} at 37 °C were deduced from the temperature dependence curves.

Fig. 4 shows that the water permeability of thin lipid films is affected by the fatty acid composition of the lipids. As an index for comparing the lipids, the average number of double bonds per fatty acid conjugate was calculated from Table 1, and the values are included in the histogram. MG membranes are more water permeable than the PC membranes, but this could be attributed to either the different polar group or to the higher degree of unsaturation in MG.

Table 2. Effect of sterols on water permeability of bilayer membranes of galacto and phospholipids

Membrane components	P_{os} (37 ± 0.1 °C) (μ/sec)	E_a (cal/mole)
MG	73.1 ± 1.6[a]	17,030 ± 120
MG + β-sitosterol	41.9 ± 1.1	17,550 ± 40
MG + stigmasterol	45.1 ± 1.1	17,390 ± 90
MG + cholesterol	17.4 ± 2.7	−36,300 ± 2,000
PC synthetic	31.5 ± 1.1	13,750 ± 300
PC plant	64.1 ± 1.1	10,760 ± 330
PC egg	55.3 ± 1.3	12,960 ± 50
PC egg + cholesterol	25.4 ± 1.5	13,350 ± 100

[a] Mean ± SE.

The experimental activation energy, E_a, may be calculated from the slopes in Figs. 1–3, assuming that the linear plots obey an Arrhenius equation. Values of E_a and of P_{os} (at 37 °C) deduced from temperature dependence curves are given in Table 2. Membranes of the various PC species yielded similar but not identical values of E_a. These, in turn, were significantly lower than the value of E_a for water permeation through MG membranes (17 kcal/mole). The membranes generated from the specific combination of MG + cholesterol were uniquely characterized by a particularly high, but negative, "energy of activation". While P_{os} of MG is reduced by stigmasterol and by β-sitosterol, E_a was not modified by these sterols.

Discussion

The water permeability of thin lipid membranes is clearly affected by the number of double bonds in the fatty acid conjugates of the lipids. The higher the degree of unsaturation, the greater the water permeability (Fig. 4). This conclusion substantiates a similar observation of Cass and Finkelstein (1967), who compared the water permeability of untreated and saturated egg lecithin membranes. It is of interest that the permeability of lecithin liposomes to glucose and other polyols increases concomitantly with an increased degree of unsaturation of the fatty acid residues in the lecithin molecules (Demel *et al.*, 1968; de Gier *et al.*, 1968; Chen *et al.*, 1971).

Träuble (1970) suggested the operation of conformational isomers ("kinks") which represent small mobile free volumes in the hydrocarbon phase of the membrane. The "kinks" could harbor water or larger molecules. In terms of this theory, the increased degree of unsaturation increases the concentration of the "kinks" and thus enables a greater permeability of the lipid membrane.

Despite the difference in fatty acid composition of the lecithin preparations (Table 1), the temperature dependence of the water permeability of the lecithin membranes is similar and unaffected by the addition of cholesterol (Table 2). This observation is in agreement with published data (Price & Thompson, 1969). The value for E_a, the experimental energy of activation (about 12 kcal/mole), is close to the value predicted for the solubility-diffusion model for permeation through lipid membranes (Zwolinski *et al.*, 1949; Hanai & Haydon, 1966; Price & Thompson, 1969; Reeves & Dowben, 1970). According to this model, E_a for water permeation is the sum of the activation energy of diffusion (3 to 4.5 kcal/mole) and the enthalpy of the partition coefficient of water between the solution and the

membrane (8 to 9 kcal/mole) (for references *see* Price & Thompson, 1969). E_a for water permeation through thin membranes made from MG, MG and stigmasterol or MG and β-sitosterol is more than 17 kcal/mole. Thus, if the diffusion-solubility model is assumed to account for the permeability of MG membranes, the estimated value of either energy of activation of diffusion or the enthalpy of partition coefficient must be modified accordingly. Since the degree of unsaturation of PC membranes did not affect E_a, the observed difference in E_a between MG and PC membranes is related to the hydrophylic, rather than to the hydrophobic moiety in the bilayer structure. The hydrophylic portion of the lipid bilayer faces the exterior of the membrane (Bangham, 1968; Levine & Wilkins, 1971). On this basis, and since E_a of water diffusion was similar in different lipids (Schatzberg, 1965) it seems likely that the value assigned to the enthalpy of the partition coefficient, has to be increased for MG membranes by 4 to 5 kcal/mole. Whether the similarity between this increase and the energy of the hydrogen bonding (i.e., between hydroxyl groups of MG and water molecules) is coincidental, is an open question.

The temperature dependence of P_{os} predicted from the pore model is also too low in magnitude to account for the value of E_a observed for the MG membranes. In terms of the small pore model an upper limit for E_a was estimated to be 15.5 kcal/mole, as the sum of the enthalpy of vaporization (10.5 kcal/mole) and the transport activation energy (5 kcal/mole) (Price & Thompson, 1969). Assuming that the pore is ice-like, E_a would be 16 kcal/mole, as estimated for the self diffusion of water in single ice crystals (Itagaki, 1964). An adjustment for hydrogen bonding between the permeating molecules and MG, as proposed above for the diffusion-solubility model, would also justify a higher upper limit for E_a for the pore model.

The interaction of MG with sterols is characterized by an interesting specificity. Both β-sitosterol and stigmasterol reduced P_{os} but did not affect its temperature dependence. However, cholesterol not only modified the magnitude of the temperature dependence of P_{os} but also reversed its slope. The same preparation of cholesterol interacted with PC in our system in the "usual" manner: reducing P_{os} but having no affect on E_a. Stigmasterol and β-sitosterol differ from cholesterol by an additional ethyl group (C-24). Thus, the side chain of the sterol molecule is apparently involved in the sterol-MG interaction. The unique temperature dependence of water permeation through thin MG+cholesterol membranes is yet not understood. One attractive explanation (Ora Kedem, *personal communication*) is based on the formation of an hypothetical polymeric complex of [cholesterol: MG; $(H_2O)_n$], in which the number of bound H_2O molecules is negatively

temperature dependent, and this number in turn affects the permeability of the membrane to water.

On the basis of conductance measurements, Redwood and Haydon (1969) concluded that black films containing cholesterol exhibit composition changes which are temperature dependent: with increasing temperature, the mole fraction of cholesterol in the membrane is apparently decreasing, even though a constant composition of membrane-generating solution was used. To minimize possible changes in the amount of cholesterol in the membrane due to temperature shifts, particular care was taken in the present study to generate a membrane at 37 °C and to maintain it for about 1/2 hr at 37 °C prior to further temperature changes. A marked effect of the cholesterol/lecithin ratio on the osmotic permeability coefficient of the membrane was observed by Finkelstein and Cass (1967). Since membranes generated from lipids solutions with or without sterol showed rather similar E_a values (Table 2), it is unlikely that substantial changes took place in the membrane composition on changing the temperature in our system. MG-cholesterol/membranes, however, behaved differently and temperature-dependent composition changes are indeed possible in this case.

By using a variety of lipids and sterols, this study demonstrates a wider range of water permeabilities of thin membranes than previously reported. This illustrates the usefulness of the thin membrane model system in facilitating the study of permeability of biological membranes.

Valuable discussions with Professor Ora Kedem and with Dr. Pieter J. C. Kuiper are appreciated. We wish to thank Professor F. de Körösy and Mrs. M. Taboch for guidance in the electrical measurements, and Mr. Y. Noll for capably constructing the water flux apparatus. Thanks are also extended to Mrs. Cynthia Bellon for editing the manuscript.

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