

Water and Nonelectrolyte Permeability of Lipid Bilayer Membranes

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ABSTRACT Both the permeability coefficients (P_d 's) through lipid bilayer membranes of varying composition (lecithin [L], lecithin:cholesterol [LC], and sphingomyelin:cholesterol [SC]) and the n -hexadecane:water partition coefficients (K_{hc} 's) of H_2O and seven nonelectrolytes (1,6 hexanediol, 1,4 butanediol, n -butyramide, isobutyramide, acetamide, formamide, and urea) were measured. For a given membrane composition, P_d/DK_{hc} (where D is the diffusion constant in water) is the same for most of the molecules tested. There is no extraordinary dependence of P_d on molecular weight; thus, given P_d (acetamide), P_d (1,6 hexanediol) is correctly predicted from the K_{hc} and D values for the two molecules. The major exceptions are H_2O , whose value of P_d/DK_{hc} is about 10-fold larger, and urea, whose value is about 5-fold smaller than the general average. In a "tight" membrane such as SC, $P_d(n\text{-butyramide})/P_d(\text{isobutyramide}) = 2.5$; thus this bilayer manifests the same sort of discrimination between branched and straight chain molecules as occurs in many plasma membranes. Although the *absolute* values of the P_d 's change by more than a factor of 100 in going from the tightest membrane (SC) to the loosest (L), the *relative* values remain approximately constant. The general conclusion of this study is that H_2O and nonelectrolytes cross lipid bilayer membranes by a solubility-diffusion mechanism, and that the bilayer interior is much more like an oil (à la Overton) than a rubber-like polymer (à la Lieb and Stein).

INTRODUCTION

To account for nonelectrolyte permeability,¹ physiologists have generally accepted Collander and Bärlund's view of the plasma membrane as a "lipoidal sieve" (see Höber, 1945). This concept was developed to reconcile the permeability *increase* occurring in homologous series of lipophilic molecules (such as alcohols and weak acids) upon addition of each methyl group, with the permeability *decrease* of small hydrophilic molecules (such as urea, acetamide, and ethylene glycol) with increasing molecular weight. The plasma membrane was viewed as a lipoidal matrix (nowadays a lipid bilayer), through which molecules pass via a solubility-diffusion mechanism, and in which are sprinkled aqueous pores that select among nonelectrolytes according to size.

Recently, this "accepted" view has been challenged by Lieb and Stein (1971),

¹ I exclude in these considerations obviously specialized transport processes that, for example, discriminate between L and D amino acids or recognize subtle structural differences among sugars.

who attempt to do away entirely with the pore concept by viewing the lipoidal region not as a viscous fluid such as olive oil, but rather as a polymeric network such as rubber. Because the diffusion coefficient in such a polymer can show a striking dependence on molecular weight, they argue that the decrease in permeability with size seen among polar nonelectrolytes is a consequence of *this* rather than of the presence of separate aqueous pathways.

Artificial lipid bilayer membranes offer the possibility of directly measuring the nonelectrolyte permeability of the structure that is the lipoidal backbone of cell membranes. But in spite of numerous investigations of ion permeability (by electrical means) and several studies of water permeability, little attention has been paid to nonelectrolyte permeability. Technical problems associated with either too small or too large permeability coefficients have restricted the amount of usable data and obscured their interpretation.

I undertook the present investigation for two reasons. The first, the subject of this paper, concerns the issues raised above. How well does the solubility-diffusion model explain nonelectrolyte permeability of lipid bilayers? Is the bilayer interior a liquid hydrocarbon milieu, or is it a rubber-like polymeric structure? Studies of water permeability indicate the former (Finkelstein and Cass, 1968), but other permeability data are needed to ensure that water is not a fortuitous exception. The second reason concerns the action of antidiuretic hormone on water permeability and is considered in the succeeding paper.

MATERIALS AND METHODS

Permeability Measurements

Membranes were formed by the brush technique of Mueller et al. (1963) at either $25^{\circ}\text{C} \pm 2^{\circ}$ or $14.5^{\circ}\text{C} \pm 0.5^{\circ}$ across a 0.8-mm^2 hole in a $125\text{-}\mu\text{m}$ thick Teflon partition separating two Lucite chambers. Magnetic fleas stirred both chambers continuously during the course of an experiment. Each chamber contained 3.0 ml of unbuffered ($\text{pH} \approx 5.6$) 0.1 M NaCl. The procedure for measuring the permeability coefficient (P_d) of a molecule was that described by Holz and Finkelstein (1970); in some experiments, both THO and a ^{14}C -labeled solute were used, so that P_d 's for both water and solute were obtained on the same membrane.

An unstirred layer thickness of $122\ \mu\text{m}$ was calculated from the observed value of $8.2 \times 10^{-4}\ \text{cm/s}$ for P_d (*n*-butanol), on the assumption that butanol is so permeant that its flux is completely determined by the unstirred layers on the two sides of the membrane (Holz and Finkelstein, 1970). This was used to correct all measured P_d values; the correction was generally trivial (<5%) and never exceeded 40%.

Three different membrane-forming solutions were used: 2% lecithin in decane (L), 2% lecithin + 4% cholesterol in decane (LC), and 2% sphingomyelin + 4% cholesterol in decane (SC). Egg lecithin was obtained from Sylvana Chemical Company (Orange, N. J.); bovine sphingomyelin from Analabs, Inc. (North Haven, Conn.); cholesterol from Eastman Kodak Co. (Rochester, N. Y.) and twice recrystallized from ethanol; *n*-decane (99.9%) from Chemical Samples Co., Columbus, Ohio.

Partition Coefficient Measurements

The partition coefficient (K_{hc}) of a given solute between *n*-hexadecane and water was determined by static equilibration. 200 μl of water containing from 5 to 20 μCi of labeled

solute were placed on the bottom of a small (capacity ~ 2 ml) screw-cap bottle, and 500 μl of *n*-hexadecane were carefully layered on top of this. The length of the hexadecane phase was about 0.75 cm. The bottle was then tightly capped, and diffusion allowed to take its course at room temperature ($25^\circ\text{C} \pm 2^\circ$). Duplicate bottles were run in all experiments. After about 24 h, three 25- μl samples were taken from the hexadecane phase and added to 15 ml of counting fluid containing 25 μl of H_2O . 25- μl samples of a 100–200-fold dilution of the aqueous phase were added to 15 ml of counting fluid containing 25 μl of "cold" *n*-hexadecane. All samples were then counted on a Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The ratio of counts per milliliter in the hexadecane phase to counts per milliliter in the aqueous phase gives K_{hc} .

For the diffusion distance (~ 0.75 cm) and the diffusion constants of the solutes ($\sim 10^{-5}$ cm^2/s), 24 h is sufficient for equilibrium to be achieved; indeed, samples taken after 48 and 72 h agreed with those taken after 24 h.

Since the solutes studied partition very poorly into hydrocarbon, spuriously high values of K_{hc} can result from small percentages of lipophilic radioactive impurities. Two procedures checked for this. After taking the 25- μl samples from the hexadecane phase for counting, (a) we removed the remainder of the hexadecane and replaced it with a fresh 0.5 ml. This was repeated every 24 h until constant values were obtained for the counts in this phase. (b) The hexadecane that was removed was relayered on top of 150 μl of H_2O and then sampled 24 h later. If a lipophilic contaminant was present, a significant number of counts would remain in the hydrocarbon phase; if absent, all of the radioactivity should repartition back into water.² Although these procedures detect lipophilic contaminants, they do not detect hydrophilic contaminants much more lipophilic than the solutes investigated. The best indication that K_{hc} values are not grossly in error is the internal consistency among homologues (see Discussion).

The small amount of lipophilic contamination in [^{14}C]formamide was removed after one replacement of the hexadecane phase. [1,4- ^{14}C]butanediol and [1,6- ^{14}C]hexanediol had contaminations of several percent; these were removed by numerous extractions into tetradecane (accomplished by vigorous shaking and then centrifugation to separate the phases) until a constant level of counts was achieved in the hydrocarbon phase. This "purified" material was then used in both permeability and partition experiments.

The same aqueous stock solutions of radioactive materials were used for both permeability and partition experiments. [^{14}C]acetamide, *n*-butanol, and THO were from New England Nuclear Corp. (Boston, Mass.); [^{14}C]*n*-butyramide, isobutyramide, and 1,4 butanediol were from American Radiochemical Corp. (Sanford, Fla.); [^{14}C]formamide was from California Bionuclear Corp., Sun Valley, Calif.; [^{14}C]urea was from Schwarz Bio Research, Inc., Orangeburg, N. Y. [1,6- ^{14}C]hexanediol was from ICN Pharmaceuticals Inc., Irvine, Calif. *n*-Hexadecane was spectroquality from Matheson, Coleman, and Bell (East Rutherford, N. J.).

RESULTS

Table I summarizes our findings. Column 1 lists the molecules investigated in the order of decreasing values of K_{hc} , which are given in column 2. They span a range from 1,6 hexanediol ($K_{hc} = 5.4 \times 10^{-4}$), the most lipophilic, to urea ($K_{hc} = 3.5 \times 10^{-6}$), the least lipophilic. (Even the most lipophilic, hexanediol, is very

² Even when there was clearly no contaminant present, approximately 5% of the counts remained in the hydrocarbon phase. Since there was very little cold carrier with the tracers, this may represent association with small amounts of polar impurities in the hexadecane.

hydrophilic.) Column 3 gives their diffusion constants (D) in water (at 25°C). Columns 4, 5, and 6 list the values for P_d on lecithin membranes (L), lecithin:cholesterol membranes (LC), and sphingomyelin:cholesterol membranes (SC), respectively. (For L membranes, P_d 's were not measured for hexanediol, *n*-butyramide, and isobutyramide, because unstirred layer corrections were too large; for SC membranes, P_d was not measured for urea, because its value was too small.) Column 7 gives P_d (*n*-butyramide) and P_d (H₂O) on SC membranes at 14.5°C. Columns 8, 9, and 10 list values of $P_d/(DK_{hc}/\Delta x)$ for L, LC, and SC membranes, where Δx , the thickness of the hydrocarbon interior of the membrane, is taken as 50 Å.

TABLE I
PERMEABILITY COEFFICIENTS (P_d 's) OF WATER AND NONELECTROLYTES THROUGH LIPID BILAYER MEMBRANES OF VARYING COMPOSITION

Molecule	$10^6 K_{hc}$	$10^5 D$	$10^7 P_d$				$P_d(L)/$ $(DK_{hc}/\Delta x)$	$P_d(LC)/$ $(DK_{hc}/\Delta x)$	$P_d(SC)/$ $(DK_{hc}/\Delta x)$
			L	LC	SC	SC, 14.5°			
		<i>cm²/s</i>							
		<i>cm/s</i>							
1,6 Hexanediol	540	0.9*	—	2,250	450	—	—	0.023	0.0046
Isobutyramide	370	1.02‡	—	1,980	118	—	—	0.026	0.0016
<i>n</i> -Butyramide	360	1.07‡	—	3,000	288	51	—	0.039	0.0038
1,4 Butanediol	43	1.0*	2,600	200	30	—	0.30	0.023	0.0035
H ₂ O	42§	2.44	22,000¶	5,730	810	210	1.08	0.28	0.040
Acetamide	21	1.32‡	1,650	196	21	—	0.30	0.035	0.0038
Formamide	7.9	1.7‡	1,030	269	25	—	0.38	0.10	0.0093
Urea	3.5	1.38**	40‡‡	6.1	—	—	0.042	0.006	—

* Estimated from the diffusion coefficient of 1.0×10^{-5} cm²/s for butanol.

‡ Gary-Boho and Weber (1969).

§ Schatzberg (1965).

|| Wang et al. (1953).

¶ Calculated from 4.2×10^{-3} cry/s at 36°C (Finkelstein and Cass, 1967) and Q_{10} 's for the solubility and diffusion constant of H₂O in hexadecane of 1.54 and 1.19, respectively (Schatzberg, 1965).

** Gosting and Akeley (1952).

‡‡ Vreeman (1966) and Gallucci et al. (1971).

The standard deviation for any K_{hc} value in column 2 is less than 20% and the standard deviation for any P_d value in columns 4–7 is less than 35%.

DISCUSSION

Partition Coefficients

It has long been fashionable to worry about which organic solvent (and polarity) is the best model for the lipoidal region of a particular cell membrane (Collander, 1954). These solvents have ranged from isobutanol (the most polar) to olive oil (the least polar). I have never understood the point of this. If the lipoidal region of the plasma membrane is a lipid bilayer, then clearly the appropriate model solvent is hydrocarbon. For artificial bilayers this is obviously so. I chose *n*-hexadecane as the particular hydrocarbon, because its chain length is comparable to that of the fatty acid residues in most phospholipids, and it is conveniently available. A more "realistic" choice would be a mixture of hydrocarbons from C₁₄ to C₂₀ with varying degrees of unsaturation, plus some cholestane for good measure. It is very doubtful that the results would be materially different.

Indeed, olive oil would serve almost as well (and taste much better); values of K are about 40-fold higher in olive oil than in *n*-hexadecane.

In both the amide and diol series, K_{hc} increases about 3.5-fold with each additional CH_2 group. The free energy change per CH_2 of about 800 calories agrees with studies of hydrocarbon solubilities in water (Tanford, 1973), giving us confidence that K_{hc} values, even for such hydrophilic solutes as formamide, are not seriously in error. (The 20% difference between K (*n*-butyramide) and K (isobutyramide) in olive oil (Wright and Pietras, 1974) was not observed in *n*-hexadecane; this discrepancy may be real or simply reflect the accuracy of the determinations.)

Permeability Coefficients

VALIDITY OF OVERTON'S RULE The general conclusion for lipid bilayers is the same as that of Overton and Collander for cell membranes: molecules cross the membrane by a solubility-diffusion mechanism, and their permeability coefficients are, in general, directly proportional to the product of their partition coefficients and diffusion constants in bulk liquid hydrocarbon.³ Thus, for a given membrane composition, $P_d/(DK_{hc}/\Delta x)$ is (with the exceptions to be discussed shortly) the same (within a factor of 2) for all molecules (see columns 8, 9, and 10 of Table I). There is no extraordinary dependence of permeability on molecular weight, such as assumed by Lieb and Stein (1971).⁴ On the contrary, given P_d (acetamide), P_d (1,6 hexanediol) is correctly predicted from the K_{hc} and D values for the two molecules. (Much larger molecular weight species probably have abnormally low P_d 's [see footnote 7], but this is not relevant to the question raised by Lieb and Stein, as their theory, if valid, should be particularly applicable to the molecules under consideration.)

QUALIFICATIONS OF OVERTON'S RULE

Urea P_d (urea) is abnormally small (by about a factor of 5). This cannot be attributed simply to its small partition coefficient, since Galluci et al. (1971) noted the same anomaly for thiourea, whose partition coefficient (in olive oil) almost equals that of acetamide, but whose P_d is only 1/30th that of acetamide. The

³ With the exception of H_2O , diffusion constants in hydrocarbon have not been determined for the molecules used in this study. I have therefore taken diffusion constants in water as a reasonable approximation.

⁴ Wolosin and Ginsburg (1975) claim to have measured P_d 's on lecithin bilayers for the un-ionized forms of a number of weak acids and to find a strong dependence of these values on molecular weight, but the values they report for these molecules, whose partition coefficients into hydrocarbon are, according to them, all greater than 1, cannot be correct. For example, their value of 2.38×10^{-4} cm/s for P_d of un-ionized acetic acid on lecithin membranes is similar to my value of 1.65×10^{-4} cm/s for P_d (acetamide) on the same type membrane. Yet the partition coefficient of un-ionized acetic acid into hydrocarbon is 10^5 times larger than that of acetamide. (The *measured* value for P_d of butanol, whose K_{hc} of about 0.1 [Aveyard and Mitchell, 1969] is at least 10-fold less than any of the molecules studied by Wolosin and Ginsburg [1975], was the same [8.2×10^{-4} cm/s] on both LC and SC, and was the only molecule tested that did not show at least a fivefold reduction of P_d in going from LC to SC. Clearly the observed P_d was a measure of unstirred layer thickness [122 μm], as pointed out previously [Holz and Finkelstein, 1970].)

double amide group is probably the culprit. Its strong hydrophilicity may make the interfacial kinetics rate limiting. That is, partition equilibrium is not attained across the interface for the ureas, as it is for the other molecules.⁵

Water $P_a(\text{H}_2\text{O})$ is abnormally large (by about a factor of 3 in L membranes and a factor of 10 in LC and SC membranes). The characteristic (relevant to permeability) that distinguishes H_2O from the other molecules studied is *size*. The cylindrical radius of H_2O (from CPK molecular models) is 1.5 Å, whereas that of formamide is 2.07 Å, and that of acetamide is 2.38 Å (Sha'afi et al., 1971). Consequently, the microdomain seen by the water molecule, as it partitions into the bilayer and diffuses through it, may be different from that seen by the other molecules of this study. Interestingly, $P_a(\text{formamide})$, the next smallest molecule, is also abnormally large, but only by about a factor of 3 (in LC and SC membranes).⁶ Possibly Träuble's (1971) "kinks" (mobile structural defects formed by conformational changes in the hydrocarbon chain) are particularly relevant in describing the movement of small molecules such as water and formamide through bilayers. If indeed molecular size accounts for the relatively large P_a values of water and formamide, only these molecules (of those studied) show any size-dependent effect such as postulated by Lieb and Stein (1971).

Isobutyramide $P_a(\text{isobutyramide})$ is smaller than $P_a(n\text{-butyramide})$, and this difference is more pronounced in tight membranes. Thus, $P_a(n\text{-butyramide})/P_a(\text{isobutyramide}) = 1.5$ in LC membranes and 2.5 in SC membranes. (Clearly, from column 10 of Table I, $P_a(\text{isobutyramide})$ is abnormally low rather than $P_a(n\text{-butyramide})$ being abnormally large.) Cell membranes also discriminate between straight chain and branched isomers; in fact, the value of 2.5 for $P_a(n\text{-butyramide})/P_a(\text{isobutyramide})$ in SC membranes is comparable to the value of 2.8 in human red cells (Sha'afi et al., 1971) and 3.4 in toad bladder (Wright and Pietras, 1974). Interestingly, lengthening the carbon chain from two (acetamide) to four (*n*-butyramide), or even to six (1,6 hexanediol), creates no unusual discrimination pattern, but introducing a single branch has a significant effect. If straight chain compounds enter and diffuse through the membrane in an extended conformation with their long axis parallel to the hydrocarbon chains of the phospholipids, this would explain why $P_a/(DK_{hc}/\Delta x)$ is constant for these molecules and smaller for branched ones, since the cross-sectional areas of the straight chain compounds are all approximately the same, whereas those of the branched isomers are larger.⁷

⁵ It is possible that partition equilibrium is not attained for these other molecules either. If, however, their interfacial kinetics *are* rate limiting, our results show that these rates are directly proportional to K_{hc} . In that case, the interfacial kinetics for urea are abnormally slow compared to those of the other molecules tested.

⁶ Poznansky et al. (1976) find $P_a(\text{formamide}) > P_a(\text{propionamide})$ on plain lecithin bilayers, thus making formamide even more anomalous. Neither our results nor those of Galluci et al. (1971) confirm this. (The discrepancy between the data of Poznansky et al. and those of Galluci et al. and ours, is their much smaller values for $P_a(\text{other amides})$.)

⁷ This suggests that molecules of greater molecular weight and larger cross-sectional area will have disproportionately small values of $P_a/(DK_{hc}/\Delta x)$, particularly in tight membranes such as SC. This has been confirmed for codeine.

EFFECT OF LIPID COMPOSITION AND TEMPERATURE ON PERMEABILITY P_d 's in lecithin bilayers (L) are about eightfold larger than in lecithin:cholesterol bilayers (LC), which in turn are about eightfold larger than in sphingomyelin:cholesterol bilayers (SC); P_d 's in SC bilayers are about fivefold larger at 25 than at 14.5°C. Thus, the permeability coefficient of a given molecule can vary over a several 100-fold range, as a function of phospholipid composition, cholesterol content, and temperature; undoubtedly, even larger ranges could be spanned with a greater variety of lipid mixtures and temperatures.

The effects of cholesterol and temperature on the water permeability of bilayer membranes have been noted earlier (Finkelstein and Cass, 1967; Price and Thompson, 1969; Redwood and Haydon, 1969). (The permeability decrease occurring in SC membranes between 25 and 14.5°C is about twice that occurring in lecithin:cholesterol membranes for a 10°C decrement [Price and Thompson, 1969; Redwood and Haydon, 1969]. Possibly a phase transition exists near 15° in SC membranes.) The smaller P_d values in SC membranes compared to those in LC membranes is consistent with a previous observation of a smaller $P_d(\text{H}_2\text{O})$ in sphingomyelin than in lecithin membranes (Finkelstein and Cass, 1968). Brockhoff (1974) has predicted that sphingomyelin membranes should be less permeable than lecithin membranes, because of a belt of hydrogen bonds formed by the 3-hydroxy groups of the sphingosines. However, the longer mean chain length and the greater degree of saturation of bovine sphingomyelin than of egg lecithin may, in themselves, account for the lower permeability.

In considering the effect of cholesterol, sphingomyelin vs. lecithin, or temperature reduction, the question arises: Do lower values of P_d result from lower diffusion constants in the membrane or smaller partition coefficients into the membrane? Cholesterol, for example, promotes "packing" of the lipid chains (Lecuyer and Dervichian, 1969). This could lead to lower permeabilities, through either an increased viscosity of the membrane interior (i.e. a reduction in diffusion constants) or a tightening of the membrane surface to penetration and solute accommodation (i.e. a reduction in partition coefficients). Probably both effects operate, but it is difficult to sort them out experimentally. (Studies such as those by Katz and Diamond [1974] on partition coefficients into lecithin liposomes are irrelevant to this problem. The partition coefficients needed are those into the hydrocarbon region of the bilayer; the partition coefficients determined by Katz and Diamond [1974] for polar nonelectrolytes relate to accumulation of these molecules in the polar regions near the head groups.)

SUMMARY The amount of space I have devoted to the "exceptions" to Overton's rule is disproportionate. Actually, the magnitude of these exceptions is relatively small. The main point is that the *relative* permeabilities of molecules up to the size of hexanediol are governed by the product of partition coefficients and free diffusion constants in bulk hydrocarbon. The *absolute values* of the permeability coefficients are scaled by the lipid composition. Even the anomalies (with the exception of the effect of branching), if one chooses to focus on them, remain relatively constant as the absolute values of P_d change. Thus, the ratio of $P_d(\text{H}_2\text{O})$ (which is anomalously high) to $P_d(n\text{-butyramide})$ changes only by about

a factor of 2 in going from LC at 25° to SC at 14.5°, whereas the absolute values of these P_d 's change by a factor of 27 and 60, respectively.

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