J. Membrane Biol. 90, 207–217 (1986)

The Journal of
Membrane Biology

Permeability of Small Nonelectrolytes through Lipid Bilayer Membranes

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Summary. Diffusion of small nonelectrolytes through planar lipid bilayer membranes (egg phosphatidylcholine-decane) was examined by correlating the permeability coefficients of 22 solutes with their partition coefficients between water and four organic solvents. High correlations were observed with hexadecane and olive oil (r = 0.95 and 0.93), but not octanol and ether (r = 0.75 and 0.74). Permeabilities of the seven smallest molecules (mol wt < 50) (water, hydrofluoric acid, hydrochloric acid, ammonia, methylamine, formic acid and formamide) were 2- to 15-fold higher than the values predicted by the permeabilities of the larger molecules (50 < mol wt < 300). The "extra" permeabilities of the seven smallest molecules were not correlated with partition coefficients but were inversely correlated with molecular volumes. The larger solute permeabilities also decreased with increasing molecular volume, but the relationship was neither steep nor significant. The permeability pattern cannot be explained by the molecular volume dependence of partitioning into the bilayer or by the existence of transient aqueous pores. The molecular volume dependence of solute permeability suggests that the membrane barrier behaves more like a polymer than a liquid hydrocarbon. All the data are consistent with the "solubility-diffusion" model, which can explain both the hydrophobicity dependence and the molecular volume dependence of nonelectrolyte permeability.

Key Words membrane permeability · nonelectrolyte · lipid bilayer · partition coefficient · diffusion coefficient · molecular volume

Introduction

The question of how molecules cross cell membranes has been considered for nearly a century, starting with the work of Overton (1899) that resulted in "Overton's Rule," i.e., permeability coefficients correlate well with oil/water partition coefficients. In recent studies small nonelectrolytes have been used to probe the physical and chemical properties of the membrane barrier and, conversely, to determine how the barrier properties control solute permeability (e.g., Collander, 1949, 1954; Diamond & Wright, 1969; Lieb & Stein, 1969, 1971; Cohen, 1975*a*,*b*; Finkelstein, 1976*a*,*b*; Wright & Bindslev, 1976). Most investigations of nonelectrolyte permeability have focused on two questions. First, what is the rate-limiting step for translocation—entry into the bilayer or diffusion across the bilayer? Second, what is the chemical and physical nature of the rate-limiting barrier? Specifically, how hydrophobic is the rate-limiting barrier? Is the rate-limiting barrier more like an isotropic liquid or a polymer? Although these questions are difficult to answer, it is reasonable to expect that some information regarding the nature of the transport process can be gleaned by examining the exceptions to Overton's Rule.

Some very small molecules, e.g., water, formamide, and formic acid, permeate lipid bilayer membranes faster than predicted by Overton's Rule, i.e., faster than predicted by their hydrophobicities or by the behavior of other members of their homologous alkyl series (Cohen, 1975*b*; Finkelstein, 1976*a*; Walter & Gutknecht, 1984). Possible explanations for this behavior include the "soft polymer" or "mobile kink" hypotheses, which propose that very small solutes fit into holes which are abundantly available in the acyl chain region of the bilayer (Lieb & Stein, 1969, 1971; Trauble, 1971). Another possible explanation for the high permeabilities of very small molecules is that "transient aqueous pores" exist in lipid bilayers (Weaver et al., 1984).

Those solutes which have anomalously high permeability coefficients are very small (mol wt < 50) and are the most hydrophilic within an homologous series. Therefore, we decided to determine whether the high permeability coefficients are a function of either molecular size or polarity and to establish what feature(s) of the membrane might ac-

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count for their anomalous behavior. First, we examine several solvents as models for the chemical selectivity of the membrane, evaluating them by correlating permeabilities (P^m) and partition coefficients (K_p). Second, we ask whether very small solute permeabilities are predicted by these correlations by comparing the measured permeabilities of the seven smallest solutes (mol wt < 50) to the permeabilities predicted by their partition coefficients and the relationship between P^m and K_p derived from the larger solutes (50 < mol wt < 300). Third, we determine whether deviations from the correlation between hydrophobicity and permeability are a function of molecular size, first, for the smaller solutes and, second, for all the solutes. Finally, we compare our observations with expected patterns of diffusion in liquid hydrocarbons and hydrophobic polymers.

Materials and Methods

Partition Coefficients

Partition coefficients for the nonionic forms of acids and bases were determined at 25°C by means of radiotracer or chemical methods described previously (Walter & Gutknecht, 1984). Briefly, solutes were equilibrated between hexadecane and water for 24 hr. After aliquots were taken from both phases to determine solute concentrations, the original hexadecane was removed and replaced with fresh hexadecane, which was allowed to equilibrate with the original aqueous phase. This procedure, which eliminates contaminants more hydrophobic than the test solute, was repeated until a constant K_p was obtained.

In the case of ^{14}C-urea, we suspected the presence of a hydrophobic contaminant, probably CO_2 since urea breaks down in water to form CO_2 and NH_3 (Morrison & Boyd, 1973). Breakdown in water is a continuous process, so the standard procedure for eliminating hydrophobic contaminants was not adequate for urea. In some experiments the urea solution was prepared in 10 mM NaOH to convert any $^{14}CO_2$ into $^{14}CHO_3^-$ and $^{14}CO_3^{2-}$, which do not partition significantly into hexadecane. Alternatively, the equilibrated hexadecane was re-extracted into an acidic solution (10 mM HCl), which was then equilibrated with air to remove $^{14}CO_2$ before counting. Both these procedures produced 10-fold lower values of K_p, confirming the presence of a volatile, weakly acidic contaminant. Since the urea K_p is 3×10^{-7} and the CO_2 K_p is 1.5 (Simon & Gutknecht, 1980), the presence of <0.0001% $^{14}CO_2$ can produce a 10-fold elevation of the apparent K_p for urea.

When weak acid or weak base partition coefficients were to be determined chemically, an aliquot of the equilibrated hexadecane containing the test solute was re-equilibrated with either a known volume of 10 mM NaOH (for acidic solutes) or 10 mM HCl (for basic solutes) in order to re-extract the solute from the hexadecane. The chemical assay was then performed on the aqueous sample.

Analytical Methods

All solutes except HNO_3, HF, NH_3 and the primary amines were assayed radiochemically. Aqueous and hydrocarbon samples were counted in a liquid scintillation counter (Beckman LS 8000) with appropriate corrections for counting efficiency. Labeled compounds were obtained from ICN (Irvine, Calif.) or New England Nuclear (Boston, Mass.).

Nitric acid concentrations were measured by reduction of nitrate to nitrite and subsequent diazotization by sulphanilamide according to Strickland and Parsons (1972). The reducing efficiency of the cadmium-copper columns was greater than 90%.

Hydrofluoric acid concentration was determined as fluoride ion by modifying the method of Schneider (1964) for low concentrations of fluoride. The amount of fluoride was determined by cerous chloride ($CeCl_3$) titration in the presence of the metal chelator, murexid ("Royal Purple"), using as an endpoint the purple-to-orange transition when the dye binds to free cerous ion. The cerous ion forms an insoluble precipitate with fluoride but binds only weakly to murexid so that the color transition occurs after all the F^- is titrated. The titration was done in a dual beam spectrophotometer (Aminco DW-2) by following the difference between absorbance at 483 nm (murexid) and 520 nm (murexid + Ce^{3+}).

Concentrations of methylamine and ethylamine were assayed after reaction with *o*-phthalaldehyde (Roth, 1971) as modified by Benson and Hare (1975). Fluorescence was read within 10–15 min on an Aminco Fluorocolorimeter with a blue lamp and Corning Filter 7-60 for excitation and Wratten No. 3 cutoff filter for emission. Radiochemical measurements of the K_p's for methylamine and ethylamine gave values identical to the chemical measurements.

Ammonia levels were determined by the colorimetric method of Koroleff (1969) which converts ammonia to indophenol blue. After color development, absorbance was read at 650 nm using a Bausch and Lomb Spectronic 710.

Permeability Measurements

Lipid bilayer membranes were formed by the brush technique (Mueller & Rudin, 1969) from a decane solution of egg phosphatidylcholine (PC) (20 mg/ml) (Avanti Polar Lipids, Birmingham, Ala.) as described previously (Walter & Gutknecht, 1984). Membrane resistances and capacitances were determined from the current as a function of voltage and the capacitative current at constant dV/dt. When fluxes were measured with tracers, the aqueous solutions were symmetrical and contained 100 mM NaCl plus 10 mM of an appropriate buffer. Buffers were MES, PIPES, HEPES, Tricine or CAPS (Sigma Chemical Co., St. Louis, Mo.). After the membranes became optically black, tracer was injected into the *cis* compartment. The *trans* compartment was perfused continuously (1 ml/min) and the perfusate was collected at 3 or 5 min intervals for at least 30 min.

To circumvent unstirred layer limitations, membrane permeabilities were determined by the method developed for weak acids (Gutknecht & Tosteson, 1973) and modified for weak bases as described by Gutknecht and Walter (1981*c*). In brief, one-way tracer fluxes (J) were measured under symmetrical conditions at several pH's so that the concentration ratios of the permeant uncharged form [B] to the impermeant charged form [HB^+] varied over several orders of magnitude. Then the membrane permeability to B (P^m) and the unstirred layer permeability (P^{ul}) were calculated from a linearized form of the relationship

$$\frac{1}{J} = \frac{1}{P^{ul}([B] + [HB^+])} = \frac{1}{P^m[B]}. \quad (1)$$

An electrical method was developed for measuring ammonia permeability, because neither tracer nor chemical methods

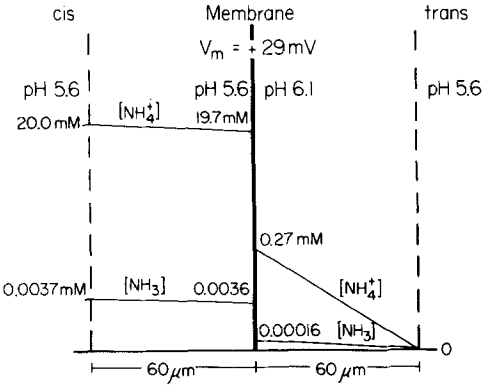

Fig. 1. Measured membrane voltage (V_m) and calculated concentration gradients caused by NH_3 diffusion across a lipid bilayer membrane and unstirred layers. The *cis* solution contains NH_4Cl (20 mM), sodium MES buffer (50 mM, pH 5.64) and NaCl (30 mM). The *trans* solution contains sodium MES buffer (1.0 mM, pH 5.64) and NaCl (100 mM). Both solutions contain the proton ionophore CCCP (carbonylcyanide *m*-chlorophenylhydrazone) (3 μM). Diffusion of NH_3 causes an increase in pH in the *trans* unstirred layer, which causes the membrane potential of +29 mV (*cis* solution = ground). Concentration gradients are not drawn to scale

were feasible in our system. The method is a simplified version of that developed by Antonenko and Yaguzhinski (1982, 1984) and is based upon the fact that net fluxes of weak acids or weak bases produce pH gradients in the unstirred layers adjacent to the membrane (Gutknecht & Tosteson, 1973). The pH gradient can be measured by adding a proton ionophore (CCCP), which causes the membrane to behave electrically as a pH electrode.

Figure 1 shows a schematic diagram of a lipid bilayer membrane and associated unstirred layers during a NH_3 permeability measurement. First, the membrane was formed in a weakly buffered (MES, 1.0 mM) solution containing CCCP (3 μM). Then the *cis* side of the membrane was perfused with a well-buffered (MES, 50 mM) solution containing CCCP plus NH_4Cl (20 mM). Diffusion of NH_3 across the membrane caused an increase in pH in the weakly buffered *trans* unstirred layer, which produced a voltage difference of 29 mV across the membrane. The size of the pH gradient was calculated by the Nernst equation.

In the *trans* unstirred layer, virtually all of the NH_3 is converted to NH_4^+, because pH $\ll$ pK. Furthermore, the flux of NH_4^+ away from the membrane is equal to the flux of protonated buffer (BH^+) toward the membrane. The BH^+ concentration difference across the *trans* unstirred layer was calculated from the total buffer concentration and the pH difference, using the Henderson-Hasselbalch equation. Then the BH^+ flux was calculated from Fick's first law, using the buffer diffusion coefficient and assuming, for simplicity, a linear gradient across the *trans* unstirred layer. Since the BH^+ flux through the unstirred layer is essentially equal to the NH_3 flux through the membrane, the membrane NH_3 permeability can be calculated from Fick's first law.

The ammonia permeability which we obtained by this method was $(1.3 \pm 0.4) \times 10^{-1}$ cm sec^{-1}, in agreement with the 0.4×10^{-1} cm sec^{-1} reported by Antonenko and Yaguzhinsky (1984), who used a high concentration of cholesterol in their egg PC-decane solution. We also verified the electrical method by comparing it with the radiotracer method (Eq. (1)), using methylamine and ethylamine as test solutes.

The primary source of error in the electrical method is the large variation in the combined unstirred layer thickness, which in our system ranges from about 95 to 145 μm (Gutknecht & Tosteson, 1973; Gutknecht & Walter, 1981c, 1982). There is also the possibility of asymmetrical unstirred layers, due to bowing of the partition, position of the membrane, etc. The radiotracer method circumvents the unstirred layer problems, because when $[HB^+]/[B]$ is large, the unstirred layer term in Eq. (1) becomes insignificant. Nevertheless, the electrical method should be useful for a variety of weak acids and weak bases which are not available in labeled form.

Results

Permeability and Partition Coefficients

If solute permeability through a lipid bilayer is governed by the same forces that determine its partitioning into nonaqueous solvents, then P^m and K_p should correlate. Figure 2 shows the relationships between membrane permeability and partition coefficients into (a) hexadecane, (b) olive oil, (c) octanol, and (d) ether. The exact values of P^m and K_p are given in Table 1. The solid lines in Fig. 2 are those predicted by the least squares fit to the P^m's and K_p's of the solutes whose molecular weights are greater than 50. Table 2 shows the statistical parameters obtained from the linear regression analyses. The relationships between log P^m and log K_p are nearly linear for all four model solvents, but the more hydrophobic solvents, hexadecane and olive oil, correlate much better (r = 0.95 and 0.93) than octanol and ether (r = 0.75 and 0.74). In all cases the correlation coefficients improve when the smaller solutes (mol wt < 50) are omitted from the analyses (Table 2), suggesting that the permeabilities of the smaller solutes are not adequately predicted by their K_p's.

The hexadecane plot (Fig. 2*a*) agrees with two previous studies which included fewer solutes and smaller ranges of hydrophobicity (Orbach & Finkelstein, 1980; Walter & Gutknecht, 1984). The only major difference is that in the present study urea (solute No. 9) falls on the regression line instead of below the line as previously reported (Finkelstein, 1976*a*). This is due to the 10-fold lower value of K_p, which we obtained by taking special precautions to eliminate a hydrophobic radiochemical contaminant in the urea solutions (*see* Materials and Methods). Otherwise, our data extend and corroborate those of Orbach and Finkelstein (1980).

Both the slopes and correlation coefficients shown in Fig. 2*a* and *c* and Table 2 differ greatly from the results of Wolosin and Ginsburg (1975) and Wolosin, Ginsburg, Lieb and Stein (1978), who also studied nonelectrolyte and weak acid permeabilities through egg PC-decane bilayers. They concluded

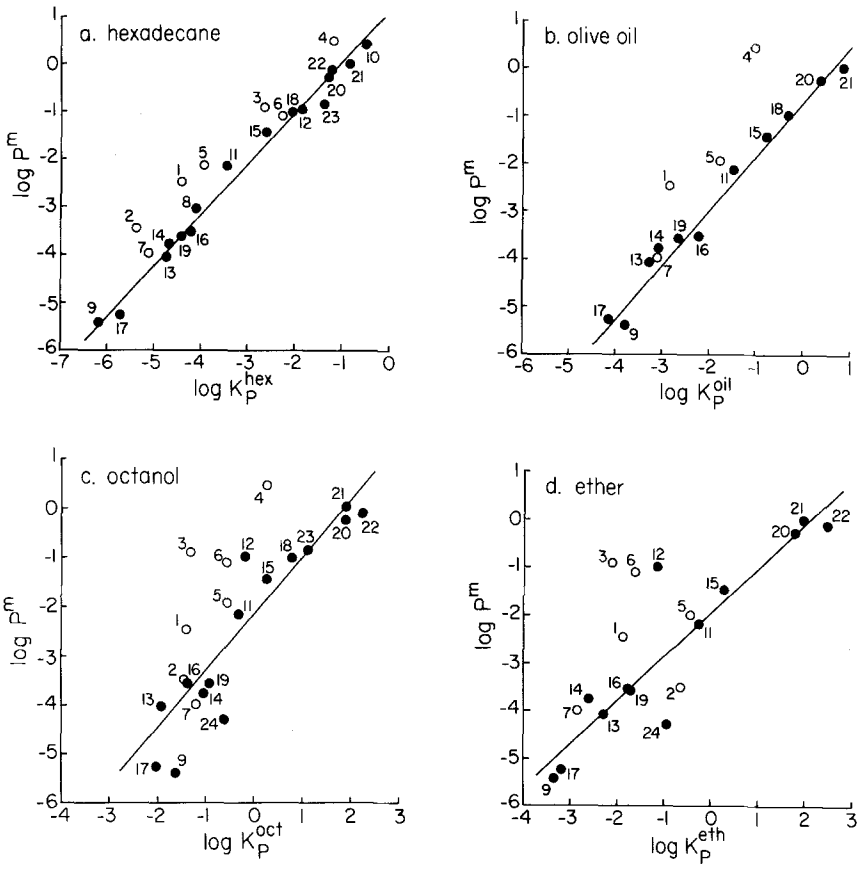

Fig. 2. Permeability coefficients (P^m) through egg PC-decane bilayers are plotted against partition coefficients (K_p) into four organic solvents. The solvents, slopes (s) and correlation coefficients (r) are: hexadecane (s = 1.06, r = 0.995), olive oil (s = 1.11, r = 0.990), octanol (s = 1.15, r = 0.841) and ether (s = 0.74, r = 0.918). Solutes are identified in Table 1. The seven smallest solutes (mol wt < 50) (open circles) were excluded from the linear regression slopes in Fig. 2. Table 2 gives the statistical parameters for all solutes and solvents

that octanol was a much better model solvent than liquid alkanes. However, their high correlation coefficient for octanol was the fortuitous result of underestimating many weak acid permeabilities, which caused the membrane barrier to appear to be more polar than pure hydrocarbon (Walter & Gutknecht, 1984).

Behavior of the Smallest Solutes

The smallest solute permeabilities all fall above the regression line, regardless of their partition coefficients which range over four orders of magnitude. For hexadecane (Fig. 2*a*) the deviations range from twofold (formamide) to 12- or 15-fold (H_2O, HF). To determine if these deviations are graded by molecular size, we plotted the differences between the observed P^m and predicted values (P^{pred}) against the respective molecular volumes (unhydrated) in a double logarithmic plot (Fig. 3*a*). The predicted values were calculated according to the P^m *vs.* K_p relationship established by the larger solutes (Fig. 2*a*). With the exception of formic acid (solute No. 5), the "extra" permeability correlates well with molecular volume (r = −0.97), and the slope is −1.7.

The deviations of the larger solutes from the line they predict also tend to be a negative function of molecular volume, but the fit is poor (r = −0.60) and the slope is rather shallow (−0.88). A similar picture emerges when the deviations from the line predicted by all the data are considered (Fig. 3*b*). The apparent improvement in linearity in Fig. 3*b* is due to the dominating effect of the small solutes (approx. one third of the total). Thus, if molecular volume is an important determinant of the permeabilites of all solutes, then the volume effect is obscured by other factors such as chemical differences, shape effects or experimental error. Volume effects are harder to document than hydrophobic effects since volume varies only 13-fold, whereas K_p varies 10^6-fold.

A similar analysis was done with the correlation between P^m and the octanol/water K_p, first for the line predicted by the larger solutes (Fig. 4*a*) and, second, for the line derived from the entire group of solutes (Fig. 4*b*). Mathematically this is equivalent to the approach taken by Wolosin et al. (1978) to fit the permeability data to two variables, the octanol/water K_p and molecular volume. The deviations observed for the small solutes correlate poorly (r = −0.49) but hint at a steep molecular volume dependence (slope = −2.6). For the larger solutes the

Table 1. Permeability coefficients (P^m) through egg PC-decane bilayers and partition coefficients (K_p) into hexadecane (hex), olive oil (oil), octanol (oct) and ether (eth). T = 25°C

Compound	P^m (cm sec^{-1})	K_p^{hex}	K_p^{oil}	K_p^{oct}	K_p^{eth}
1. water	3.4×10^{-3}[a]	4.2×10^{-5}[e]	1.4×10^{-3}[i]	4.0×10^{2}[i]	1.3×10^{2}[i]
2. hydrofluoric acid	3.1×10^{-4}[c]	4.2×10^{-6}		3.7×10^{2}[j]	2.3×10^{1}[j]
3. ammonia	1.3×10^{-1}	2.2×10^{-3}		5.2×10^{2}[j]	7.0×10^{-3}[l]
4. hydrochloric acid	2.9[b]	6.0×10^{-2}	9.0×10^{-2}[k]	1.8[j]	
5. formic acid	7.3×10^{-3}[d]	1.1×10^{-4}[d]	1.5×10^{-2}[i]	2.9×10^{1}[i]	3.6×10^{1}[j]
6. methylamine	8.0×10^{-2}	5.5×10^{-3}		2.7×10^{1}[j]	2.3×10^{2}[l]
7. formamide	1.0×10^{-4}[e]	7.9×10^{-6}[e]	7.6×10^{-4}[m]	6.2×10^{2}[n]	1.4×10^{3}[m]
8. nitric acid	9.2×10^{-4}[c]	6.9×10^{-5}			
9. urea	4.0×10^{-6}[e]	2.8×10^{-7}	1.5×10^{-4}[m]	2.6×10^{2}[n]	4.7×10^{-4}[m]
10. thiocyanic acid	2.6[f]	3.2×10^{-1}[f]			
11. acetic acid	6.9×10^{-3}[d]	5.3×10^{-4}[d]	3.0×10^{-2}[i]	4.9×10^{1}[i]	5.2×10^{1}[i]
12. ethylamine	1.2×10^{-1}	1.3×10^{-2}		6.6×10^{1}[j]	6.6×10^{2}[j]
13. ethanediol	8.8×10^{-5}[e]	1.7×10^{-5}[e]	4.9×10^{-4}[m]	1.2×10^{2}[j]	5.3×10^{3}[m]
14. acetamide	1.7×10^{-4}[e]	2.1×10^{-5}[e]	8.3×10^{-4}[m]	8.9×10^{2}[n]	2.5×10^{-3}[m]
15. propionic acid	3.5×10^{-2}[d]	2.3×10^{-3}[d]	1.5×10^{-1}[i]	1.8[i]	1.7[i]
16. 1,2-propanediol	2.8×10^{-4}[g]	6.4×10^{-5}[g]	1.7×10^{-3}[m]	4.4×10^{2}[j]	1.8×10^{-2}[m]
17. glycerol	5.4×10^{-6}[g]	2.0×10^{-6}[g]	7.0×10^{-5}[m]	1.1×10^{2}[n]	6.6×10^{-4}[m]
18. butyric acid	9.5×10^{-2}[h]	8.7×10^{-3}[h]	4.4×10^{-1}[i]	6.2[i]	
19. 1,4-butanediol	2.7×10^{-4}[g]	4.3×10^{-5}[g]	2.1×10^{-3}[m]	1.2×10^{1}[n]	1.9×10^{-2}[m]
20. benzoic acid	5.5×10^{-1}[d]	5.3×10^{-2}[d]	2.2[j]	7.4×10^{1}[j]	5.4×10^{1}[j]
21. hexanoic acid	1.1[d]	1.4×10^{1}[d]	6.8[j]	7.6×10^{1}[j]	8.6×10^{1}[j]
22. salicylic acid	7.7×10^{-1}[d]	6.0×10^{-2}[d]		1.7×10^{2}[j]	2.8×10^{2}[j]
23. codeine	1.4×10^{-1}[g]	4.2×10^{-2}[g]		1.6×10^{1}[j]	
24. lactic acid	5.0×10^{-5}[i]			2.4×10^{1}[i]	1.3×10^{-1}[i]

[a] Walter (1981)
[b] Gutknecht and Walter (1981*a*)
[c] Gutknecht and Walter (1981*b*)
[d] Walter and Gutknecht (1984)
[e] Finkelstein (1976*a*)
[f] Gutknecht and Walter (1982)
[g] Orbach and Finkelstein (1980)
[h] Walter, Hastings and Gutknecht (1982)
[i] Wolosin and Ginsburg (1975)
[j] Leo, Hansch and Elkins (1971)
[k] Macey (1948)
[l] Collander (1949)
[m] Collander (1954)
[n] Wolosin et al. (1978)

Table 2. Least squares regression analyses of membrane permeabilities (P^m) and partition coefficients (K_p) into four organic solvents, plotted according to the relation, $\log P^m = s \log K_p + b$

Model solvent	Number of solutes (n)	Correlation coefficient (r)	Slope (s)	Intercept (b)
Hexadecane	23[a]	0.950	0.95	0.94
	16[b]	0.995	1.06	1.10
Olive oil	15[a]	0.925	1.14	−0.32
	11[b]	0.990	1.11	−0.67
Octanol	22[a]	0.747	1.09	−1.78
	15[b]	0.841	1.15	−2.14
Ether	19[a]	0.735	0.74	−1.73
	13[b]	0.918	0.92	−1.90

[a] Analyses include all P^m and K_p pairs.
[b] Smallest solutes (mol wt < 50) are excluded from the analyses.

correlation is virtually nonexistent (r = −0.12), and molecular size dependence, if any, is relatively shallow (slope = −0.4). The line based upon all the solutes (Fig. 4*b*) gives a steeper slope (−2.3), but this is due once again to the dominating effect of the small solutes.

Discussion

Applicability of Overton's Rule

In general, membrane permeation follows Overton's Rule. The best model solvents for predicting permeabilities from partition coefficients are the more hydrophobic solvents, hexadecane and olive oil (Fig. 2 and Table 2). Orbach and Finkelstein (1980) also obtained a high correlation with hexadecane, but they argued that the choice of model solvents is not important because the ratios of K_p's into various organic solvents are approximately constant. For example, eight nonelectrolytes studied by Orbach and Finkelstein had K_p ratios of roughly 1:40:200 into hexadecane, olive oil and ether, respectively. However, this constant relationship breaks down if the test solutes have a wider range of polarities. For example, the ratios of K_p's into hexadecane, olive oil and octanol differ greatly among carbon dioxide (1.0:1.1:0.9), water (1:33:952), salicylic acid (1:167:2,867) and urea (1:528:91,550) (*see* Table 1 and Simon &

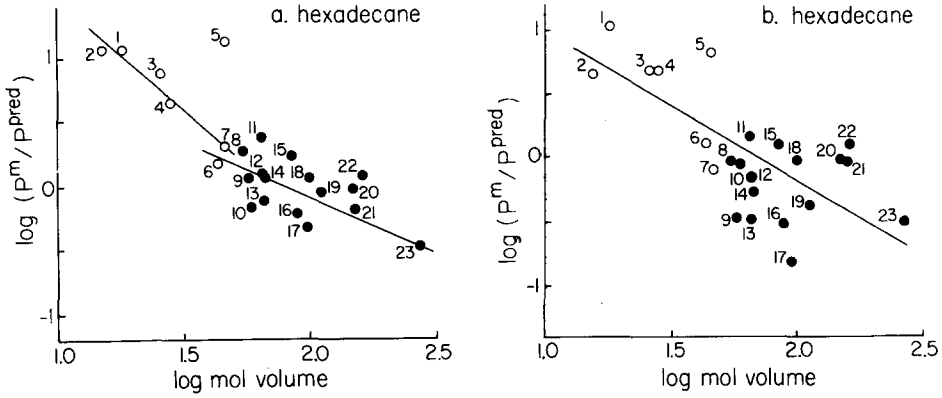

Fig. 3. The differences between measured permeabilities (P^m) and permeabilities predicted (P^{pred}) by the regression analysis of P^m *vs.* K_p^{hex} are plotted against molecular volumes in order to test whether the error observed in Fig. 2*a* is attributable to solute size. (*a*) The differences between P^m and P^{pred} from the regression line obtained from the larger solutes (mol wt > 50). The smaller (○) and larger (●) solutes are treated separately. For the smaller solutes, $s = -1.7$ and $r = -0.97$. For the larger solutes $s = -0.88$ and $r = -0.60$. (*b*) The differences between P^m and P^{pred} from the regression line derived from all the solutes ($s = -1.2$, $r = -0.67$). Formic acid (No. 5) was not included in the calculations for either *a* or *b*. The reason for its anomalously high P^m and/or low K_p is unknown. Molar volumes were calculated by the LeBas incremental method (Perry, 1963; Hayduk & Laudie, 1974)

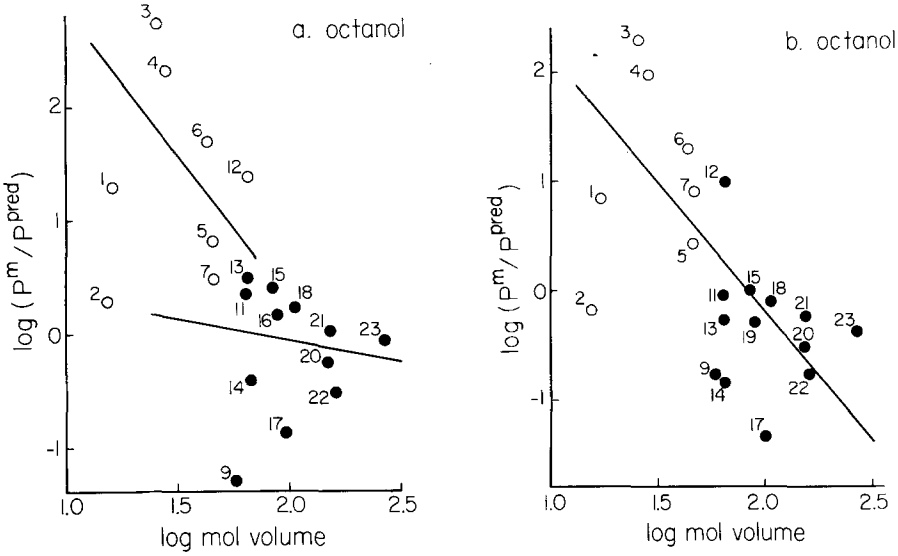

Fig. 4. Similar to Fig. 3, except that the K_p's are for octanol rather than hexadecane. (*a*) The slopes are −2.6 and −0.4, and the correlation coefficients are −0.49 and −0.12. (*b*) $s = -2.3$ and $r = -0.70$. HF (No. 2) was not included in the calculations. We suspect that the K_p for HF into octanol is based on an erroneously high value for ether, from which K_p^{oct} was calculated (*see* Leo, Hansch & Elkins, 1971)

Gutknecht, 1980). The large differences reflect primarily the differing abilities of these solutes to form hydrogen bonds with the solvents.

However, even "poor" model solvents usually give fairly high correlations between P^m and K_p (Fig. 2 and Table 2). This is because variations in the hydrophobicities of test solutes are usually due to differences in the numbers of $-CH_2-$ groups. Incremental free energies per $-CH_2-$ are similar for partitioning into most organic solvents, because the dominant factor is the gain in free energy associated with removing the $-CH_2-$ from water (Diamond & Wright, 1969). For example, for a series of monocarboxylic acids ranging from 2–6 carbons, plots of log P^m *vs.* log K_p give slopes near 1.0 and correlation coefficients >0.99 for all four model solvents shown in Table 2 (calculations based on data in Table 1). Thus, if one wishes to compare membrane permeabilities with partition coefficients into different model solvents, the test solutes must have a wide range of hydrogen bonding abilities (Stein, 1981).

Our results show also that very small solutes (mol wt < 50) permeate 2 to 15 times faster than predicted by their partition coefficients (Fig. 2*a*). The degree to which these small solutes deviate from the line predicted by the larger permeants (50 < mol wt < 300) is not a function of hydrophobicity since the small solutes have hexadecane/water K_p's ranging over four orders of magnitude (Table

1). Thus, transient aqueous pores, if they exist (Weaver et al., 1984), cannot account for the anomalously high permeabilities of the smallest solutes. However, the "extra" permeabilities do correlate with molecular volumes and show a moderately steep dependence in a log-log relationship (slope −1.7), compared to a slope of ca. −0.5 predicted by the Stokes-Einstein model for diffusion (Fig. 3*a*). Finkelstein (1976*a*) found similar size dependencies among water, formamide and acetamide in both "loose" and "tight" (high cholesterol and/or sphingomyelin) membranes. Our estimates of his slopes range from −1.5 to −2.1.

In contrast, the permeation of the larger solutes is not a steep function of size (Fig. 3*a*), as noted also by Orbach and Finkelstein (1980). We have not underestimated the importance of size by choosing hexadecane as a model solvent. The deviations from the K_p^{oct} line are greater than the deviations from the line based on hexadecane (Fig. 2*a* and *c*), but the octanol deviations correlate poorly with molecular volume ($r = -0.49$) (Fig. 4*a*).

Both the entry step and diffusion across the bilayer must be considered as possible kinetic barriers for permeation (Diamond & Wright, 1969). In the discussion which follows, we will initially assume that diffusion through the hydrocarbon interior is the rate-limiting process. This "solubility-diffusion" model is described by the expression

$$P^m = \frac{K_p^m D^m}{d} \tag{2}$$

where K_p^m and D^m are the average partition and diffusion coefficients for the solute in the membrane interior, and d is the membrane thickness (Diamond & Katz, 1974). Our discussion will focus first on the expected effects of solute molecular size on both solubility and diffusion in the membrane. Since there are no direct measurements of intramembrane diffusion coefficients as a function of solute size, we will compare the observed size dependencies with those reported for aqueous solutions, liquid hydrocarbons, and polymers.

Solubility in Bilayers Compared to Bulk Solutions

Are the deviations observed for small molecules due to differences between solubilities in bulk hydrocarbon (e.g., hexadecane) and solubilities in the bilayer? The most appropriate data for addressing this question are the solubilities of *n*-alkanes in bilayers and alkane solvents, because *n*-alkanes partition into the hydrocarbon region of the membrane rather than binding near the interface. The free energies, enthalpies and entropies of solution of small *n*-alkanes into lipid bilayers and bulk hexane were measured by Simon, Stone and Busto-Latorre (1977) and Miller, Hammond and Porter (1977). As solute size increases, the enthalpy of solution becomes more favorable in the bilayer than in hexane, because van der Waals' attractive forces are stronger in the bilayer than in isotropic liquids. Conversely, the entropy of solution becomes increasingly less favorable due to contraints on the mobility of the solute and/or changes in the mobility or packing of the phospholipids. The net effect is that the free energy of solution of a solute into a bilayer is somewhat less than that into bulk alkane solvents (Miller et al., 1977; Simon et al., 1977). However, for a series of normal alkane solutes up to the size of butane (and possibly to octane), the relative partition coefficients into bilayers and bulk alkanes are similar. Thus, we conclude that size effects on solubility are probably not important for the seven smallest molecules (mol wt < 50) in our study. Furthermore, even our larger solutes show very little size-dependent permeability (Fig. 3*a*), suggesting that size-dependent partitioning is relatively unimportant for this group of solutes in egg PC bilayers.

Diffusion in Aqueous Solutions, Liquid Hydrocarbons, and Polymers

If size-dependent solubility in the bilayer cannot explain the high permeability coefficients of tiny molecules, then an alternative explanation may be size-dependent diffusion coefficients. The bilayer interior has been described as similar to either a liquid hydrocarbon (Finkelstein, 1976*a*; Orbach & Finkelstein, 1980) or a soft polymer (e.g., Lieb & Stein, 1969, 1971; Stein, 1981). Can either of these models explain the anomalous permeabilities of tiny solutes?

Finkelstein (1976*a*) assumed that solute diffusion coefficients in the membrane interior are proportional to solute diffusion coefficients in water. However, this assumption, based on the Stokes-Einstein hydrodynamic model, does not explain the anomalously high permeabilities of water and formamide (Finkelstein, 1976*a*, 1977). The Stokes-Einstein treatment of diffusion assumes that the solvent molecules are so small that they appear as a smooth continuum to the larger solute molecules. However, when the solute is small relative to the solvent, diffusion coefficients are greater than those predicted by the bulk viscosity of the solution, e.g, hydrogen diffusion in water (Sutherland, 1905), water diffusion in hexadecane and squalane (Schatzberg, 1965), and oxygen diffusion in mixtures of oils (Subczynski & Hyde, 1984) (*see also* Evans, Tominaga & Davis, 1981).

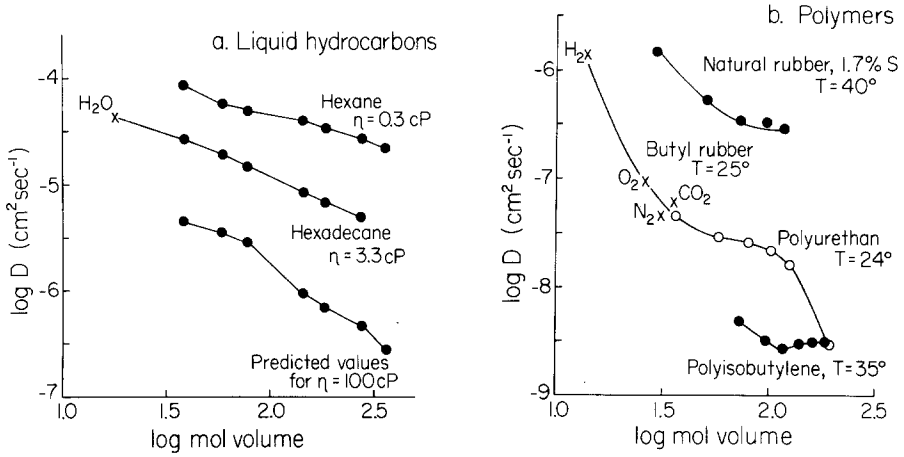

Fig. 5. Diffusion coefficients as a function of solute molar volume in (*a*) liquid hydrocarbons and (*b*) soft polymers. (*a*) Measured diffusion coefficients for water and *n*-alkanes (methane through octadecane) in hexane and hexadecane (Schatzberg, 1965; Hayduk & Ioakimidis, 1976). The lower curve in *a* was obtained by extrapolating the data of Hayduk and Ioakimidis to a viscosity of 100 cP, assumed to be an upper limit for the viscosity of a lipid bilayer. Slopes are approx. −0.6, −0.8 and −1.2. (*b*) Diffusion coefficients of several gases (x) and *n*-alkanes (●) in rubber (van Amerongen, 1964) and *n*-alkanols (○) in polyurethan (Hung & Autian, 1972). Slopes range from about 0 to −3

The published data on diffusion of water and alkanes in alkane solvents suggests that the high permeabilities of very small solutes may be explained by their high diffusion rates in long-chain liquid hydrocarbons (Schatzberg, 1965; Hayduk & Ioakimidis, 1976; Finkelstein, 1977). However, if the diffusion coefficients are plotted as a function of solute molecular volume, the relationships are approximately linear for solutes ranging in size from water to octadecane (Fig. 5*a*). However, the slopes increase from about −0.6 (hexane) to −1.2 (predicted for 100 cP alkane solvent), suggesting a steeper size dependence with increasing microscopic anisotropy. Furthermore, a graph of diffusion coefficients in benzene as a function of solute size shows that solutes smaller than the solvent (i.e., mol wt < 80) have a shallower molecular weight dependence (slope = −0.4) than larger solutes (slope = −0.7) Rossi, Bianchi & Rossi, 1958). In contrast to either of these patterns, our smallest solutes have a steeper molecular weight dependence (slope = −1.7) than our larger solutes (slope = −0.9) (Fig. 3*a*). Thus, our data cannot be explained by the solute diffusion patterns observed in liquid hydrocarbons.

Lieb and Stein and associates proposed that the membrane barrier behaves as a soft polymer (e.g., Lieb & Stein, 1969, 1971; Wolosin et al., 1978; Stein, 1981). They analyzed the dependence of permeation on solute molecular size either by plotting P^m/K_p as a function of size (similar to our Figs. 3*b* and 4*b*) or by doing multivariate regression analyses to simultaneously evaluate the model solvent and estimate the dependence of permeability on molecular size. They concluded that the bilayer interior behaves as a polymer because solute permeabilities give a steep dependence on solute molecular volume after being normalized for hydrophobicity. Figure 5*b* shows examples of size-dependent diffusion of gases and alkanes in rubber, and aliphatic alcohols in polyurethan. Nonelectrolyte permeabilities through bilayers and biological membranes have been reported to show even steeper size dependencies, e.g., planar bilayers (Wolosin & Ginsburg, 1975), liposomes (Cohen, 1975*a*), toad bladder (Bindslev & Wright, 1976), red cells, algae and other biological membranes (*see* Stein, 1981). According to Stein, the negative slopes for these membranes range from −3 (*Chara*) to −6 (beef red cells). However, most of these studies included some small polar nonelectrolytes which permeate via aqueous pores or facilitated diffusion and/or larger nonpolar molecules which were unstirred layer limited (*see* Finkelstein, 1976*b*; Sha'afi, 1981; Walter & Gutknecht, 1984). Thus, the molecular size dependencies ("mass selectivities") calculated by Stein (1981) are generally greater than the true values for the lipid bilayer region of the membrane.

Nevertheless, a comparison of Figs. 3*a* and 5*a* and *b* shows that our permeability pattern is more like that of a soft polymer than a liquid hydrocarbon. A key feature, pointed out by Bindslev and Wright (1976), is that diffusion coefficients in soft polymers show a size dependence for very small molecules which is steeper than that for medium sized molecules (Fig. 5*b*) (for other examples, *see*

van Amerongen, 1964). In contrast, diffusion of small molecules through liquid hydrocarbons shows a size dependence that either remains constant or becomes steeper as solute size increases from small to medium (Fig. 5*a* and Rossi et al., 1958). Note that the magnitude of the slope alone does not always distinguish between a soft polymer and a liquid hydrocarbon (*cf.* Fig. 5*a* and *b*).

A special transport mechanism for tiny molecules, the "mobile kink," was proposed by Träuble (1971) (*see also* Kimmich, Peters & Spohn, 1981). Träuble's model, like Lieb and Stein's, is based upon the theory of diffusion in polymers. However, Träuble presents a molecular mechanism by which tiny molecules may be "carried" in structural defects ($2gl$ kinks) which form at the membrane surface and diffuse rapidly ($D \simeq 10^{-5}$ cm^2 sec^{-1}) along the hydrocarbon chains. Träuble's model does not describe the permeation of larger molecules, which in our membranes show less size dependence than smaller molecules (Fig. 3*a*). Fettiplace and Haydon (1980) have pointed out that the degree of disorder in most bilayers is greater than that assumed in Trauble's model. As the degree of disorder increases, the hydrocarbon phase begins to resemble a fluid more than an ordered structure (Träuble, 1971). Also, as solute size increases, the "solvent" acyl chains may behave more like a continuum (Evans et al., 1981). Thus, lipid bilayers may display either polymeric (anisotropic) or liquid hydrocarbon (isotropic) behavior, depending upon solute size, lipid composition and temperature. For example, the measured diffusion coefficients for water in long-chain liquid hydrocarbons (hexadecane and squalane) are as high as the theoretical values for kink diffusion in lipid bilayers (*cf.* Schatzberg, 1965; Träuble, 1971). Thus, both the liquid hydrocarbon and mobile kink models successfully predict the high membrane permeability to water (Hanai & Haydon, 1966; Finkelstein, 1976*a*, 1977; Petersen, 1983). However, only the soft polymer model successfully predicts both the high permeability to very small molecules and the pattern of size selectivity among very small to medium sized nonelectrolytes. Further studies on larger and highly branched molecules are needed to determine which, if either, of these models provides an adequate description of membrane permeation for a wider variety of solutes.

Rate-Limiting Step

Thus far we have assumed that diffusion through the membrane interior is the rate-limiting step for permeation. Alternatively, the rate-limiting step may be entry into the membrane interior, in which case the correlation between permeability and the hexadecane/water K_p would reflect the differing activation energies required for crossing the interfacial barrier (Diamond & Wright, 1969). Most of the energy required for permeation of these generally hydrophilic solutes is needed to break hydrogen bonds that are not replaced in the interior of the membrane. These same forces also dominate the equilibrium K_p because the energy gain (or depth of energy well) upon transfer to hydrocarbon is relatively small. For example, Cohen (1975*a*) found that the activation energy for the permeation of a small solute is related to the number of hydrogen bonds the solute forms in water. However, such correlations do not distinguish between solubility-diffusion and interfacial kinetics as the rate-limiting step in permeation (Diamond & Wright, 1969).

The possibility of different rate-limiting barriers for different kinds of solutes has also been considered by several investigators. For example, Diamond and Katz (1974), Andersen (1978) and Stein (1981) have suggested that hydrophilic solutes may be rate limited by diffusion through the central core of the bilayer where polarity is minimal and the degree of disorder is high, whereas hydrophobic solutes may be rate limited by diffusion through the peripheral region of the bilayer which is more polar and more highly structured. If this idea were correct, then hydrophobic solutes might deviate from the linear relation between P^m and K_p into hexadecane (Fig. 2*a*). They do not. However, none of our solutes are strongly hydrophobic (all K_p's < 1). Fortunately, several hydrophobic weak acids (P^m's ranging from about 4–50 cm sec^{-1}, and K_p's into decane ranging from 1.2 to 23) have been studied by Dilger and McLaughlin (1979) and Benz and McLaughlin (1983). Although their experimental conditions differ slightly from ours, their P^m's fall close to the extrapolated regression line in Fig. 2*a*, which is consistent with the notion of a single rate-limiting step for both polar and nonpolar solutes.

Conclusions

Overton's Rule describes nonionic solute permeability through lipid bilayer (egg PC-decane) membranes over at least a millionfold range of permeabilities and partition coefficients. The model solvents that give the best predictions for permeability are the most hydrophobic solvents, i.e., hexadecane and olive oil. Among the solutes tested, the only exceptions to Overton's Rule are the very small molecules (mol wt < 50), whose permeabilities are 2- to 15-fold higher than predicted by the relationship established for the larger molecules (50

< mol wt < 300). The "extra" permeability of the smallest molecules is an inverse function of molecular volume but is not a function of hydrophobicity. Deviations of larger molecules from the main pattern do not show a strong volume dependence. The molecular size dependence of solute permeability suggests that the membrane barrier behaves more like a soft polymer than a liquid hydrocarbon. All of our results are consistent with the "solubility-diffusion" model, which can account for both the hydrophobicity dependence and the molecular volume dependence of nonelectrolyte permeability.

We thank Dr. Sidney Simon for helpful advice and discussions and Dr. William Thompson for suggesting the residual error plots in Figs. 3 and 4. This work was supported by National Institutes of Health grant GM 28844.

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Received 31 July 1985; revised 6 December 1985