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Effect of Temperature on Nonelectrolyte Permeation across the Toad Urinary Bladder

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Summary. The permeability of the toad urinary bladder to 22 nonelectrolytes was obtained from measurements of radioactive tracer fluxes. The permeability coefficients (P's), after suitable corrections for unstirred layers, were proportional to the olive oil/water partition coefficients for the majority of the molecules ($P \alpha K_{oil}^{1.3}$). In the absence of chain branching, inductive effects, and intramolecular hydrogen bonding effects, a hydroxyl group reduced P an average 500-fold and a methylene group increased P an average four fold. Branched chain solutes were less permeable than their straight chain isomers, and small solutes, polar *and* nonpolar, exhibited higher rates of permeation than expected from the relationship between P and K_{oil}. (Over the molecular size range 18–175 cc/mole $P \alpha$(Molecular Volume)$^{-2.7}$.) The high rates of permeation of small molecules are consistent with diffusion through a highly organized lipid structure. Large polar solutes, e.g., sucrose, appear to pass across the epithelium via an extracellular shunt pathway. The apparent activation energies (E_a) for the permeation of 16 select molecules were obtained from permeability measurements over the temperature range 2–32 °C. Linear Arrhenius plots (i.e., log P/T^{-1}) were obtained for all molecules after unstirred layer corrections. In the absence of these corrections "phase transitions" were seen for molecules with very high P's ($P > 300 \times 10^{-7}$ cm/sec), but these are simply due to diffusion limited permeation. E_a increased by 2.5–3.6 kcals/mole with the introduction of each additional methylene group into a molecule, and decreased by up to 9 kcals/mole for the addition of a hydroxyl group. Qualitatively similar results were obtained in preliminary studies of olive oil/water partition coefficients. Arrhenius plots of the toad bladder conductance over the temperature range 2–32 °C yield apparent activation energies of 4–5 kcals/mole which is identical to that found previously for "leaky" epithelia.

To gain insight into mechanisms of nonelectrolyte permeation across biological membranes we have focused our attention on the effect of temperature on the permeation of a highly select group of molecules across the toad urinary bladder. We have chosen the toad urinary bladder for three reasons: (1) earlier experiments demonstrated that the selectivity of this membrane towards nonelectrolytes was much greater than most

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other epithelia and single cells (Wright & Pietras, 1974); (2) permeation via the extracellular route (i.e., tight junctions and lateral intracellular channels) is of minor importance in this epithelium (Wright & Pietras, 1974); and (3) it is relatively simple to estimate the permeability of this epithelium by direct methods employing radioactive tracers. The timing of our study appears most appropriate in view of concurrent studies on the effect of temperature on amide permeation across red blood cell membranes (Galey, Owen & Solomon, 1973), on solute permeation across liposome membranes (Cohen, 1975*a*, 1975*b*), and on the partition of solutes into liposome membranes (Katz & Diamond, 1974*a*, 1974*b*; Diamond & Katz, 1974; Dix, Diamond & Kivelson, 1974).

Prior to a detailed examination of the temperature coefficients we characterized the properties of the toad urinary bladder by measuring the permeability coefficients (P's) of 22 molecules. We confirm a common observation that the sequence of solute permeation across membranes is largely controlled by the intermolecular forces in the aqueous phase, whereas the range of nonelectrolyte selectivity is determined by interactions of the solutes with the membranes. Small molecules, both polar *and* nonpolar, permeate across the bladder more rapidly than expected on the basis of the general correlation between permeability coefficients and molecular structure. Evidence is presented to suggest that this anomalous behavior of small molecules is due to the high rate of diffusion of small molecules though the hydrocarbon interior of the membrane rather than through aqueous pores or via "carriers". The actual diffusion of molecules in membranes resembles diffusion in hydrophobic polymers. On the other hand, large polar molecules, e.g., sucrose, seem to permeate across the bladder via a shunt pathway (*see also* Wright & Pietras, 1974).

The apparent activation energies for permeation through the epithelium were obtained for 16 select molecules. The molecules were chosen so that we could obtain specific information about: (1) the contributions made by methylene ($-CH_2-$) and hydroxyl ($-OH$) groups to permeation, (2) the anomalous high permeation of the small molecules, and (3) the mechanism and route of permeation of large polar molecules. We find that the apparent activation energies increase with increasing chain length in two homologous series of molecules, the monohydroxyalcohols and dihydroxyalcohols, and that the activation energies actually *decrease* with the addition of hydroxyl groups to a molecule. These results are similar to those we obtained in preliminary studies of the partition of solutes between olive oil and water. The interpretation of these results is reserved for the following paper (Wright & Bindslev, 1976). Temperature experiments lend

further support to our hypothesis that small molecules permeate through the hydrocarbon interior of plasma membranes more readily than larger molecules, and that large polar molecules permeate via a shunt pathway. Preliminary accounts of some of these results have been presented (Bindslev & Wright, 1974, 1975).

Materials and Methods

The experimental approach was similar to that used previously for the rabbit gallbladder by Smulders & Wright (1971). Sheets of urinary bladders from the toad *Bufo marinus* were mounted between lucite half chambers. The area of the window between the half chambers was 0.47 cm^2, and each half chamber was connected to a glass reservoir (*see* Fig. 1, Smulders & Wright, 1971). Saline was recirculated through the chambers and the reservoirs by gas lifts (100% O_2) and the temperature of the saline was controlled by circulating water through the outer jackets of the reservoirs. Constant temperature circulators were used to control the temperature and pump the water through the water jackets. The temperature of the saline in the flux chambers, which was varied between 2 and 32 °C, was monitored continuously using a Yellow Springs telethermometer (Model 42SC) with a calibrated hypodermic probe. To improve mixing and minimize the thickness of the unstirred layers next to the epithelium, the saline in each chamber was vigorously agitated by magnetically driven stirring bars.

Transmural electrical potentials (p.d.'s) and conductances were continuously recorded as described previously (Wright, Barry & Diamond, 1971; and Bindslev, Tormey, Pietras & Wright, 1974), and we only used bladders with a resistance greater than 1,750 $\Omega\,cm^2$. The mean resistance of the bladders in this series of experiments was 3,760 $\Omega\,cm^2$, with a range from 1,750 to 12,200 $\Omega\,cm^2$. The initial p.d. was about 40 mV, serosa positive.

Permeability coefficients were obtained by the radioactive tracer techniques described elsewhere (Smulders & Wright, 1971; Bindslev *et al.*, 1974; Wright & Pietras, 1974). P's are expressed in cm/sec, and are corrected for the presence of unstirred layers using the relation

$$1/P_{ob} = 1/P + \delta/D$$

where P_{ob} is the value observed experimentally, δ is the effective thickness of the unstirred layers (the sum of the mucosal, cytoplasmic and serosal unstirred layers), and D is the free solution diffusion coefficient. δ was determined by the flux of benzyl alcohol at 22 °C, where it is assumed that the flux of this solute across the bladder is limited by the unstirred layers, i.e., $1/P \ll \delta/D$. The validity of this assumption is supported by the observation that the apparent activation energy for permeation of benzyl alcohol over the temperature range 22° to 32 °C was 4 kcals/mole, i.e., close to that expected for diffusion in free solution, but was 26 kcals/mole below 18 °C. Taking $D_{\text{benzyl alcohol}}$ to be 0.82×10^{-5} cm^2/sec, the benzyl alcohol P_{ob} at 22 °C [$5181 \pm 20(2) \times 10^{-7}$ cm/sec] corresponds to an unstirred layer of 160 μm. This value is close to that obtained by Pietras & Wright (1975) using this and an independent method.

Variations in δ/D with temperature were obtained from the temperature coefficients of diffusion in aqueous solution – the validity of this was proved in an earlier study on the gall bladder where δ/D was measured experimentally at each temperature (Smulders & Wright, 1971). At 22 °C the unstirred layer correction amounts to less than 20% for solutes with P's less than $1{,}300 \times 10^{-7}$ cm/sec, but the correction becomes increasingly more important for solutes with higher permeabilities (*see* Fig. 5). However, reliable estimates of these high P's can be obtained at lower temperatures owing to the fact that the apparent activation energies for permeation are much higher than for diffusion in aqueous solution. We have taken advantage of this to obtain accurate estimates of the P's for monohydroxyalcohols.

In calculating P's we have taken the area of the window between the flux chambers as the area of the exposed urinary bladder. This appears reasonable as the capacitance of the bladders mounted in our chambers was 0.5–0.7 $\mu F/cm^2$ (*see also* Bindslev *et al.*, 1974). For comparison with the results obtained by others we note that the wet weight of the exposed membrane was 2.5 mg/cm^2 – about a factor of 6 less than that obtained in sac preparations.

Solutions

The saline used contained 104.5 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and was buffered to pH 7.3 with 2.5 mM Na_2HPO_4/NaH_2PO_4. The nonelectrolytes were added to the saline to give a final concentration of 5 mM. Radioactive isotopes were used as supplied by the manufacturers (New England Nuclear, Boston, Mass.; Amersham/Searle Corp., Arlington Heights, Illinois; International Chemical and Nuclear Co., Irvine, California; American Radiochemical Corp., Sanford, Florida; Dhom Products Ltd., Los Angeles, California; and Cal Atomic, San Diego, California).

Partition Coefficients

Bulk phase olive oil/water partition coefficients were measured for a few of the solutes by shaking about 5 μC of the isotopes with a mixture of oil and water for 24 hr in a constant temperature water bath, and, after separating the two phases, estimating the activity in each phase by scintillation counting. The K's obtained at 22 °C were very similar to those obtained by Collander (1954) and Pietras and Wright (1975). The temperature coefficients for the partition coefficients were obtained by measuring the K's in constant temperature rooms set at 10, 22 and 37 °C.

The experimental procedure

As soon as the membrane resistance and p.d. stabilized, 0.5–1 hr after mounting the bladder in the flux chamber, radioactive isotope was added to the solution on one side of the tissue and aliquots were taken from both the "hot" and "cold" solutions at regular intervals. The unidirectional fluxes reached a steady state within 20 min and remained constant at any given temperature for at least 6 hr. The unidirectional fluxes and their temperature coefficients were independent of the direction of the flux. On changing the temperature the unidirectional fluxes reached a new steady state within 30 min. At each temperature the value of the flux was taken as the average flux obtained in two consecutive 30 min periods after the system reached the steady state. The fluxes were calculated from the change in total counts on the "cold" side of the membrane and the total counts on the "hot" side during each flux period. In most experiments fluxes were measured at six temperatures between 2 and 32 °C, and the sequence of the temperature changes was varied in a random fashion. The temperature coefficients were independent of the sequence of the temperature changes. An experiment was terminated if there was an unexpected drop in the membrane resistance. Two experiments with different solutes were carried out simultaneously on membranes from the same toad to minimize variations from one animal to another.

To conserve isotope, minimize loss of isotope from the "hot" side, minimize the backflux, and to obtain the greatest accuracy, we used a small area of membrane (0.47 cm^2), and, depending on the absolute permeability of the particular solute, we varied the volume of the saline on each side of the membrane from 5 to 15 ml and varied the specific activity of the isotope. In no experiment lasting over six hours did the loss of isotope from the "hot" side exceed 30%

of the initial amount, and at no time did the ratio of the counts in the "cold" side to the counts in the "hot" side exceed 0.15.

Tritiated water fluxes were obtained in experiments where we also measured the fluxes of ^{14}C isotopes using double label techniques. All radioactive samples were assayed using a Beckman LS 250 scintillation counter equipped with an automatic quench correction system. The spillover of ^{14}C counts into the ^{3}H channel was fixed at a ratio of 1 to 5, and to reduce the influence of the ^{14}C spillover we arranged that the counting rate in the ^{3}H channel was about five times higher than in the ^{14}C channel.

The experimental errors in this series of experiments, conducted between May and January, were expressed as the standard error of the mean with the number of estimates in parenthesis.

Results and Discussion

Permeability Coefficients

The P's obtained at 22°C in this and the previous series of experiments (Wright & Pietras, 1974) are listed in Table 1. In all but one of the ten cases where molecules were tested in both series there was good agreement. The reason for the higher 1,4-butanediol permeability in the present series, a factor of three higher, is not yet clear. The P's are presented under four main categories:

The Main Pattern. Fig. 1 shows log P plotted against log K_{oil} where it can be seen that for the majority of solutes P was directly proportional to K_{oil}. The slope of the regression line was 1.3 and the regression coefficient was 0.96. The accepted interpretation of this correlation is that both P's and K's are controlled by the balance of solute/water and solute/lipid intermolecular forces (*see* Diamond & Wright, 1969*a*, 1969*b* for reviews). Solute/water intermolecular forces are generally much stronger than solute/lipid forces, and so the selectivity of both P's and K's are governed by interactions between the solutes and water. The major attractive intermolecular forces in the aqueous phase are hydrogen bonds, and the greater the number and strength of the hydrogen bonds that a solute can make with water, the lower the P and K (*see also* Stein, 1967). The addition of one $-OH$ group (two H bonds) to *n*-butanol or *n*-propanol reduced P approximately 500-fold in the toad bladder and K_{oil} 150-fold.

Another important class of intermolecular forces accounts for the increase in P or K observed on the addition of successive $-CH_2$-groups to a molecule. The addition of one $-CH_2$-group to *n*-propanol increases P (in the toad bladder) and K_{oil} 9- and 4-fold respectively. These increases are due, at least in part, to the decrease in entropy in the aqueous phase

Table 1. Nonelectrolyte P's at 22 °C[a]

Compound	MV (cc/mole)	K_{oil}	$P \times 10^7$ (cm/sec)
1. sucrose	215.6	1×10^{-6}	1.1 ± 0.7 (2)
2. mannitol	122.3	1.2×10^{-6}	0.9 ± 0.3 (5)
3. erythritol	98.4	3×10^{-5}	1.2 ± 1 (3)
4. glycerol	73	7×10^{-5}	0.7 ± 0.2 (4)
5. urea	45.5	1.5×10^{-4}	6.1 ± 0.3 (2)
6. ethyleneglycol	56	4.9×10^{-4}	8.4 ± 1.8 (4)
7. water	18	7×10^{-4}	1095 ± 61 (8)
8. acetamide	59.2	8.3×10^{-4}	13.7 ± 0.7 (2)
9. 1,3-propanediol	72.3	1.3×10^{-3}	4.2 ± 0.5 (3)
10. 1,2-propanediol	73.4	2×10^{-3}	9.2 ± 2.4 (3)
11. 1,4-butanediol	88.6	2.1×10^{-3}	45 ± 7 (6)
12. nicotinamide	87.2	5×10^{-3}	12 ± 1 (8)
13. 1,6-hexanediol	123	6.8×10^{-3}	72 ± 2 (12)
14. methanol	40.5	7.8×10^{-3}	990[b]
15. *n*-butyramide	84.4	1.7×10^{-2}	54 ± 2 (24)
16. iso-butyramide	84.4	1.4×10^{-2}	16 ± 1 (5)
17. 1,7-heptanediol	139	3.1×10^{-2}	309 ± 54 (4)
18. ethanol	58.4	3.2×10^{-2}	960[b]
19. antipyrine	175.1	3.2×10^{-2}	100 ± 10 (4)
20. caffeine	157.9	3.3×10^{-2}	290 ± 10 (6)
21. *n*-propanol	77.1	1×10^{-1}	2241 ± 183 (3)
22. *n*-butanol	91.5	3.9×10^{-1}	$20{,}000 \pm 4{,}000$ (4)

[a] Molar volumes (MV) were estimated from the molecular weights and densities. Olive oil/water partition coefficients were taken from Collander (1954) except for 1,2-propanediol, 1,3-propanediol, 1,7-heptanediol, *n*-propanol, *n*-butanol, nicotinamide, *n*-butyramide and isobutyramide which were obtained directly in our laboratory. The P's for *n*-butyramide, iso-butyramide, 1,6-hexanediol, caffeine, antipyrine and nicotinamide were obtained in an earlier series of experiments carried out under similar experimental conditions (Wright & Pietras, 1974).

[b] The P's for methanol and ethanol at 22 °C were estimated from the values obtained over the temperature range 2–15 °C. For further details *see* the text.

which "pushes" the solute out of water into the lipid, and in part due to the increase in the attractive forces (van der Waal's forces) between the solute and the lipid that pull the solute into the lipid. The decrease in entropy in the aqueous phase is accounted for by the ability of hydrocarbons to produce stabilized H-bonded water clusters (so called "icebergs"). (*See* Diamond & Wright 1969*a*, 1969*b*.)

Branched Chain Molecules. The toad bladder, as well as most other biological membranes, sharply discriminates between branched and straight chain isomers (*see* Wright & Diamond, 1969; Diamond & Wright, 1969*a*, 1969*b*; Sha'afi, Gary-Bobo & Solomon, 1971; and Wright &

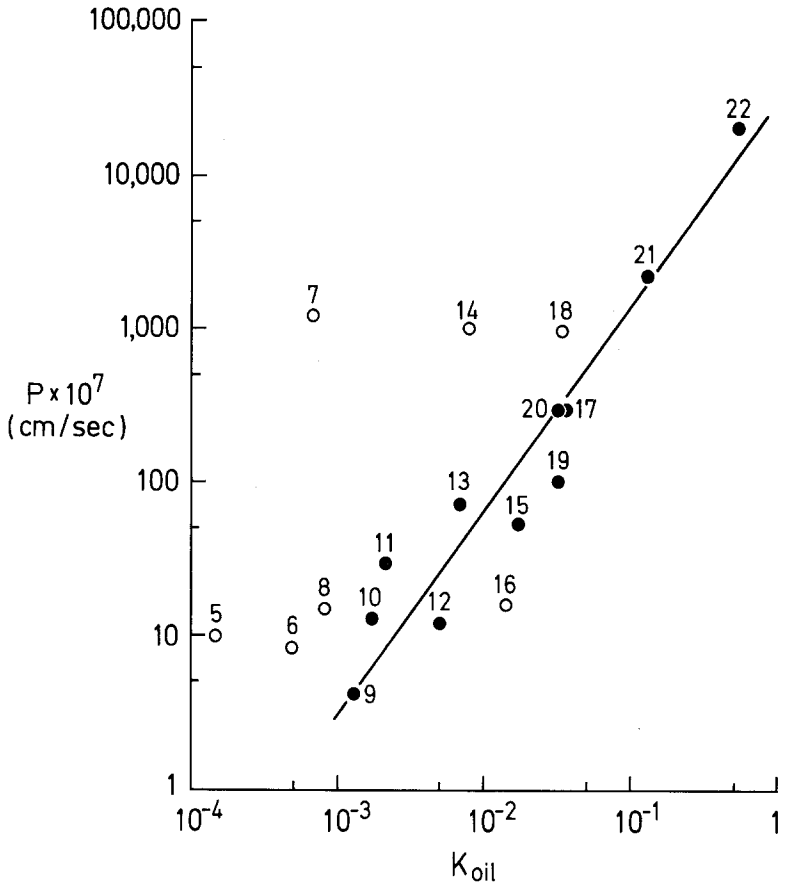

Fig. 1. The correlation between P and K_{oil}. The P's and K's were taken from Table 1. The line of identity was drawn to fit all points except the small molecules [water (7), acetamide (8), urea (5), methanol (14), ethanol (18), and ethylene glycol (6)] and the branched compound iso-butyramide. The slope of the regression line was 1.3 and the regression coefficient was 0.96. All numbers refer to the compounds listed in Table 1. Note the low position of iso-butyramide (16) relative to *n*-butyramide (15)

Pietras, 1974). This effect is illustrated for the toad bladder in Fig. 1 by the low position of iso-butyramide relative to *n*-butyramide. The P ratio is 0.30, whereas the ratio of the olive oil partition coefficients is 0.82 (*see* Table 1). The higher discrimination between the branched and straight chain compounds in the membrane is interpreted to mean that the membrane lipids are in a highly ordered configuration. It should be noted that antidiuretic hormone reduces the discrimination between the branched and straight chain isomers and this suggests that the hormone "melts" the membrane lipids (Pietras & Wright, 1975).

Small Molecules. In biological membranes small polar molecules are generally more highly permeant than predicted from the general relation between P and K. In any given series of homologous molecules (polyhydroxyalcohols, amides, ureas) the first and second members are more permeant than the third or fourth member of the series. This phenomenon is also seen in the toad urinary bladder, Fig. 1, where the points for urea (5), acetamide (8), ethylene glycol (6) and water (7) lie high relative to the regression line describing the general relation between P and K_{oil}. The urea and water P's are between 2 and 3 orders of magnitude higher than predicted. The dominant effect controlling permeation of the small molecules appears to be size – the larger the molecular volume the closer the permeability is to the general relation between P and K_{oil}.

One of three explanations is usually offered to explain these observations: (1) permeation of small polar molecules occurs through aqueous "pores", i.e., they bypass the high resistance pathway through the membrane lipid; (2) permeation of these molecules occurs via "carriers", i.e., specific molecules in the membrane facilitate the translocation of the molecules through the lipid; and (3) the membrane is homogeneous, and molecular sieving is simply due to the packing of the membrane lipids.

In a attempt to distinguish between these three alternative explanations, we studied the permeation of small *nonpolar* molecules across the bladder. The results obtained for the monohydroxyalcohols are given in Tables 1 and 2 and in Fig. 1. These show that methanol and ethanol also violate Overton's rules, i.e., despite the fact that both the P's and K's for the alcohols are about 2 orders of magnitude greater than those for the diols and ureas, the P's for methanol and ethanol are greater than expected. At room temperature $P_{methanol}$ (14) is more than an order of magnitude greater than expected from the general relationship between P and K_{oil} (Fig. 1), and at 2°C, where the results are much more accurate because of minor unstirred layer corrections, $P_{methanol}$ and $P_{ethanol}$ are both significantly greater than $P_{propanol}$ (Table 2). Anomalous permeation of small polar

Table 2. Alcohol P's at 2°C

	$P \times 10^7$ cm/sec
methanol	139 ± 19 (6)
ethanol	118 ± 12 (2)
n-propanol	74 ± 8 (4)
n-butanol	524 ± 63 (5)

molecules has also been observed in "undoped" liposome membranes (Cohen, 1975*b*; Poznansky, Tong & Solomon, 1975), which are most certainly devoid of pores. We may then conclude that the anomalous high permeation of small molecules is a more general phenomenon and is restricted to neither small polar solutes nor to biological membranes.

According to the pore theory the difference between the observed P's and those expected from the general relationship between P and K_{oil} should be related to the size of molecules and the size of the pores by the Renkin equation, i.e.,

$$P_s/P_w = \frac{(1-s/r)^2[1-2.104(s/r)+2.09(s/r)^3-0.95(s/r)^5]}{(1-w/r)^2[1-2.104(w/r)+2.09(w/r)^3-0.95(w/r)^5]}$$

where s and w are the radius of the solute and water respectively, r is the radius of the pore, and P_s and P_w are the permeability coefficients for solute and water respectively (Renkin, 1954). The P's for the permeation of small solutes through the "pores" of the toad bladder have been estimated from the data in Fig. 1 and these are given in Table 3. Plots of log P_w/P_s against the molecular radii cannot be fitted by a single value for a pore radius. For example, a pore radius of between 2.5 and 3.0 Å can account for the water, urea, acetamide and ethylene glycol results, but this leads to an underestimate of the permeation of methanol and ethanol through pores by over an order of magnitude (Fig. 2). We conclude that "pores" will not account for the anomalous permeation of the small nonpolar solutes.

Table 3. Contributions of lipid and pores to the permeation of small molecules

Molecule	P	P_{lipid} (cm/sec × 10^7)	P_{pore}	Radius Å
water	1095	2	1093	1.5
methanol	990	50	940	1.97
ethanol	960	300	660	2.27
ethylene-glycol	8.4	1.3	7.1	2.38
urea	10.1	0.3	9.8	2.41
acetamide	13.7	2.8	10.9	2.53

Permeability coefficients for the toad urinary bladder were taken from Table 1, P_{lipid} was obtained from Fig. 1 assuming that the permeation of these molecules through the lipid of the toad bladder membranes is described by the general relationship between P and K_{oil}, i.e., $P \propto K^{1.3}$, and P_{pore} is the difference between permeation via the lipid and that actually measured, i.e., $P_{pore} = P - P_{lipid}$. The molecular radii are cylindrical radii taken from Soll (1967) and Sha'afi *et al.* (1971).

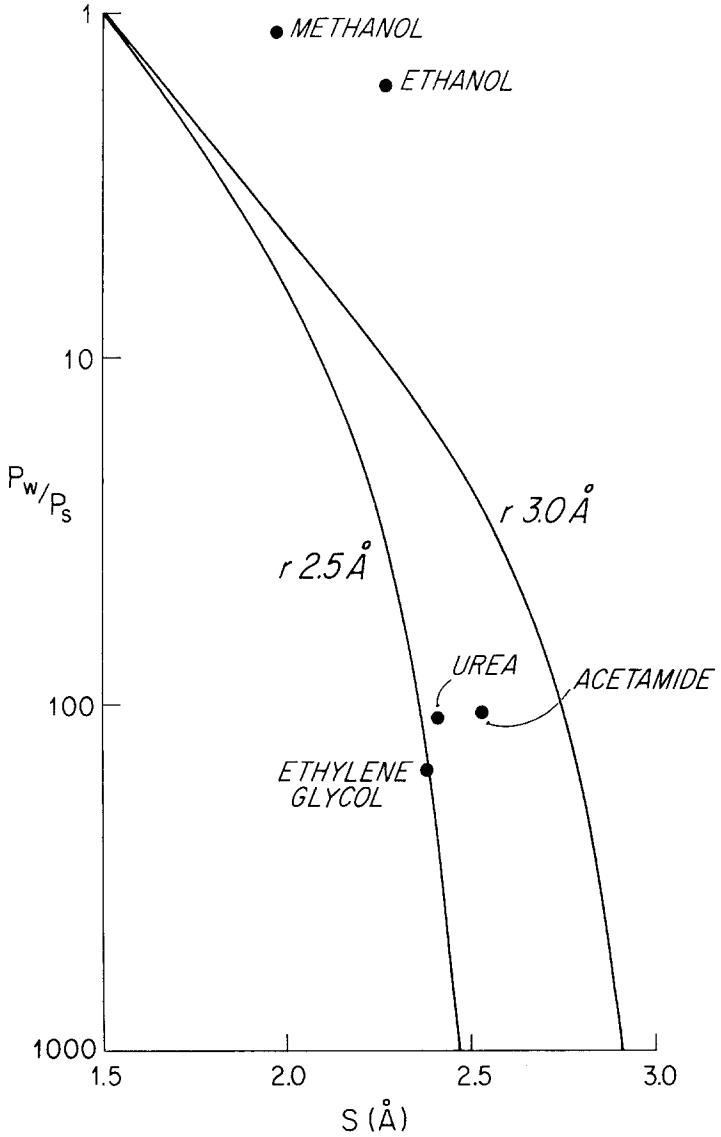

Fig. 2. Renkin plots for the permeation of small molecules through "pores." Plots of log P_w/P_s against molecular radii (s) are shown for pores of 2.5 and 3.0 Å radius, and the actual data points for the small polar and nonpolar molecules are shown. Note that although pores of between 2.5 and 3.0 Å may be able to account for the anomalous high permeation of the small polar molecules, the same pores cannot account for the anomalous permeation of methanol and ethanol

Water. The magnitude of the water permeability coefficient in the toad bladder (Table 1) is very similar in magnitude to that reported for lipid bilayer membranes (House, 1974; Table 4.2), but is substantially less than that for erythrocytes (*see* House, 1974; Table 5.1). The diffusional water

permeability (P_d) obtained in this study is about one third of the osmotic water permeability (P_f) reported for toad bladder by Hays and Leaf (1962). This inequality between P_d and P_f is usually taken as strong evidence for the presence of pores in toad bladder, but, in the presence of rather thick unstirred layers adjacent to both sides of epithelium (*see* page 267), and the errors inherent in both the P_f and P_d measurements (*see* for example Wright, Smulders & Tormey, 1972), this conclusion is unjustified at the present time.[1]

Membrane Diffusion Coefficients. Further insight into the sieving effect of the membrane lipids on permeation can be obtained from plots of membrane diffusion coefficients (D_m) against molecular volume (MV). Estimates of D_m are obtained from the ratio $P/K_{oil}{}^{1.3}$ since it may be shown that to a first approximation $P = K_m D_m / x_m$, where K_m and D_m are membrane partition coefficients and diffusion coefficients respectively, and x_m is the thickness of the membrane (*see also* Lieb & Stein, 1969, 1971).[2] The plot of log D_m against log MV, Fig. 3, shows that D_m is proportional to $MV^{-2.7}$. Similar results were obtained for *Chara* (Lieb & Stein, 1969, 1971), liposomes (Cohen & Bangham, 1972; Cohen, 1975*b*), and hydrophobic polymers (Stein & Nir, 1971).

Despite the resemblance between diffusion in the hydrophobic polymers and biological membranes, it should be noted that the relations between D_m and molecular size are based on a narrow range of molecular size. In this study, for example, the molecular volumes range from only 18 to 175 cc/mole, with just five molecules having a MV greater than 100 cc/mole. In the case of *Chara*, however, Collander (1954) obtained P's for a much wider range of molecular size (MW 18–480), and careful analysis of his data (Collander, 1954; Smulders & Wright, 1971) shows that the relationship between P and molecular size is much steeper for small molecules than for larger molecules: $P \alpha MW^{-3.7}$ with molecular weights less than 70, but $P \alpha MW^{-1.2}$ with molecular weights greater

1 Even when unstirred layer phenomena have been properly taken into account P_f may still be greater than P_d in biological membranes. One possible explanation is that osmotic gradients induce "pore" formation in the membrane (*see* Taupin, Dvolaitzky & Sauterey, 1975). This appears unlikely in the toad urinary bladder because the osmotic gradients used to estimate P_f were much lower than the gradients required to produce changes in ion and nonelectrolyte permeability (Bindslev *et al.*, 1974).

2 The validity of using the ratio P/K to estimate membrane diffusion coefficients has been established for polymer membranes by direct comparison of the diffusion coefficients obtained in this manner with those obtained by more direct methods (*see* Meares, 1965). The approach is also justified by the good correlation between P and K_{oil} (Fig. 1) and the fact that D's are usually a smooth function of molecular volume (Horowitz & Fenichel, 1964).

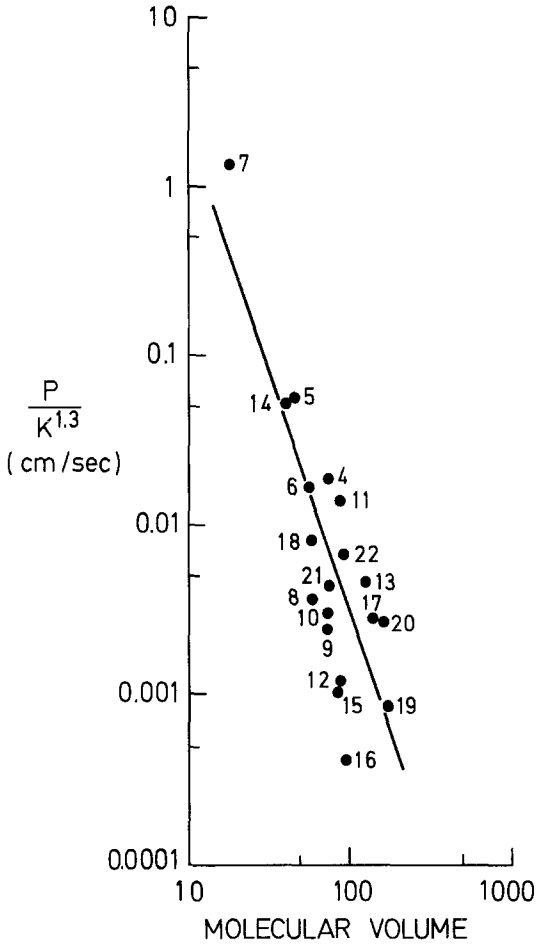

Fig. 3. Membrane diffusion coefficients as a function of molecular volume. The log of the relative diffusion coefficients ($D_m = P/K_{oil}^{1.3}$) on the ordinate was plotted against the log of the molecular volume on the abscissa. The regression line was drawn through the points (slope $= -2.7$, $r = 0.77$). All numbers refer to the compounds listed in Table 1

than 70. From the data of Barrer and Skirrow (1948) and Aitken and Barrer (1955) it may also be concluded that diffusion in soft polymers shows the same molecular size effect, i.e., $D \alpha MW^{-1.1}$ when $MW < 70$ and $D \alpha MW^{-0.5}$ when $MW > 70$. Therefore the *correct* conclusion to draw is that diffusion of low molecular weight compounds in membranes shows a steeper relationship to size than larger molecules, and that this is similar to that observed for diffusion in hydrophobic polymers.

In summary, the results obtained for toad bladder show that for molecules with K_{oil} in the range 7×10^{-5} to 3.9×10^{-1} and molecular

volumes in the range 18–175 cc/mole, permeabilities are proportional to $K_{oil}^{1.3} MV^{-2.7}$.

In our view, carrier mechanisms are unlikely to explain the high rates of permeation of small molecules across biological membranes, as this would require the presence of either a very nonselective carrier, or a large number of distinct carrier systems, to explain the high rates of permeation of such diverse molecules as amides, alcohols (mono and polyhydroxy), ureas and water. [For an alternative view *see* Levine, Franki & Hays (1973) who interpret some observations to indicate the presence of carriers for amides and ureas.]

On the basis of our observations and further evidence to be discussed below and in the following paper, we suggest that it is an intrinsic property of the membrane lipids, namely their highly ordered configuration, that accounts for the anomalous high permeation of small molecules across the toad bladder.

Large Polar Molecules. P's for sucrose, mannitol and erythritol, are given in Table 1 and the apparent activation energies (E_a) in Table 4. All the E_a's lie between 9 and 11 kcals/mole, which supports the evidence presented earlier (Wright & Pietras, 1974) that suggests that large polar molecules (sucrose, mannitol, erythritol and perhaps even glycerol) permeate across the toad urinary bladder via a shunt pathway. Namely: (1) the P's for sucrose and mannitol are about three to four orders of magnitude greater than expected from the general relationship between P and K_{oil}; (2) the relationship between P and MV is less steep for larger than for smaller solutes (Wright & Pietras, 1974; Fig. 2); (3) ADH does not effect the P's for sucrose and mannitol although the P's for all other solutes are significantly increased (Pietras & Wright, 1975), and (4) c-AMP dramatically increased the permeability of the larger polar solutes, but not other solutes, and this may be related to the action of c-AMP on the muscle of the sub-mucosa of the bladder (Pietras & Wright, 1975).

As yet the anatomical location of the pathway used by these large polar solutes has not been identified. It is possible, although unlikely, that these molecules pass through a shunt caused by damage at the edge of the tissue. This is unlikely because: (1) Reuss & Finn (1974) found no current sink at the edge of the tissue in voltage scan experiments on bladders with electrical resistances comparable to those in this study. They did, however, estimate that 40% of the total bladder conductance was through an extracellular shunt. Even if it is assumed that most of the bladder conductance is through a free solution shunt, we estimate that $P_{sucrose}$ is

about 5000 times larger than the expected permeability of the shunt; (2) there is no correlation between the permeability of large molecules and the conductance of the bladder, e.g. in seven experiments where the conductance of the bladders ranged from 1.1×10^{-4} to 5.3×10^{-4} mhos/cm^2 there was no correlation between P_{mannitol} and the conductance[3].

Temperature Coefficients

To obtain further information about the mechanisms of nonelectrolyte permeation across the urinary bladder we measured the effect of temperature on a group of 16 selected molecules. The temperature coefficients are expressed as apparent activation energies (E_a in kcal/mole) which were obtained from Arrhenius plots of $\log P$ against $1/T$. Fig. 4 shows the results of one experiment with 1,7-hepatanediol where P was measured at six temperatures ranging from 2 to 32 °C. There was a linear relationship between $\log P$ and $1/T$ and the slope corresponds to an E_a of 19 cal/mole. In all experiments of this type we obtained linear Arrhenius plots, and the apparent activation energies obtained are summarized in Table 4.

"*Phase Transitions*" *and Unstirred Layers.* Plots of $\log P$ against $1/T$ are only linear for molecules with P's greater than 300×10^{-7} cm/sec *after* the unstirred layer corrections. Without the corrections, breaks in the curves were obtained in experiments with butanol, propanol, water and benzyl alcohol. This effect is illustrated for *n*-butanol in Fig. 5. In the absence of the unstirred layer correction there was a break in the curve above 15 °C. Above 22 °C the slope (3–4 kcal/mole) is that expected for diffusion in free aqueous solution, and below 15 °C the slope corresponds to an E_a of 19.9 kcal/mole. The curvature at 15 °C is not a phase transition in the membrane lipids because: (1) the "*transition temperature*" appears to increase with smaller absolute values of P, e.g., with water the "*transition temperature*" is 28 °C; (2) the transition temperature is not seen with solutes where $P < 300 \times 10^{-7}$ cm/sec; and (3) taking account of the

3 Pietras and Wright (*unpublished observations*) have obtained certain correlations between P's and the conductance of the toad bladder. The permeability of small polar molecules increased with increasing membrane conductance (for urea and acetamide regression coefficients of 0.86 and 0.95, and slopes of 0.85 and 0.44, respectively), whereas the permeability of lipophilic molecules decreased with increasing membrane conductance (for 1,7-heptanediol a regression coefficient of 0.95 and a slope of −0.58). Although the origin of these correlations are obscure, we found that phloretin abolished the relationship between urea permeation and conductance.

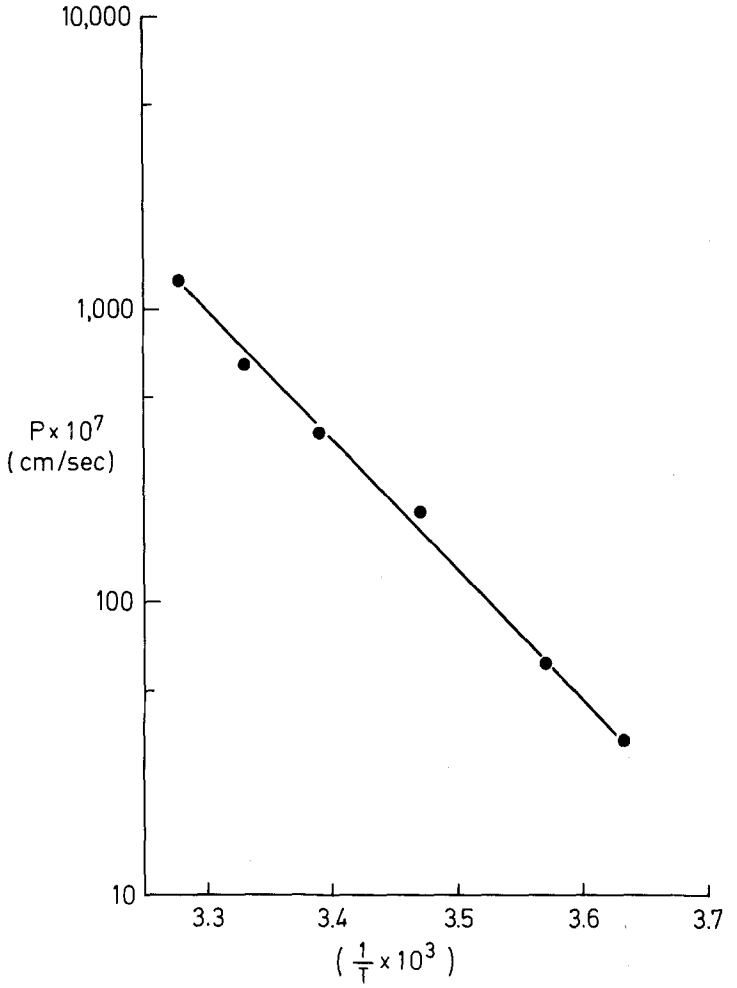

Fig. 4. $P_{\text{heptanediol}}$ as a function of temperature. The results of one experiment where the heptanediol fluxes were measured over the temperature range 2–32 °C. Log P was plotted against $1/T$. The regression line ($r=0.99$) yields an E_a of 19.2 kcal/mole

unstirred layers eliminates the apparent transition (*see* Fig. 5). It is possible that inadequate unstirred layer corrections account for the transitions observed for permeation processes in frog skin and rabbit gall bladder (Grigera & Cereijido, 1971; van Os & Slegers, 1973; van Os, de Jong, & Slegers, 1974). Clearly, diffusion limitation has to be taken into account when temperature coefficients decrease with increasing temperature.

E_a and Molecular Size. E_a increases with chain length as shown in Fig. 6 for two homologous series of compounds. In the case of the terminal

Table 4. Temperature coefficients for nonelectrolyte permeation

Compound	E_a(kcal/mole)	Compound	E_a(kcal/mole)
methanol	15.8±1.5 (3)	urea	5.2±2.2 (2)
ethanol	16.9±0.8 (2)	acetamide	9.8±0.7 (2)
n-propanol	23.9±0.7 (3)	water	15.5±0.4 (8)
n-butanol	26.0±4.1 (4)		
		glycerol	9.6±2.5 (3)
ethylene glycol	11.3±1.1 (4)	erythritol	10.6±1.1 (3)
1,2-propanediol	19.6±1.4 (2)	mannitol	8.2±0.5 (3)
1,3-propanediol	14.6±0.3 (3)	sucrose	9.7±0.6 (2)
1,4-butanediol	18.0±1.4 (7)		
1,7-heptanediol	23.6±2.1 (4)		

E_a was obtained from plots of log $P/1/T$ for individual experiments. All slopes were linear over the temperature range tested (2–32 °C for all compounds except methanol and ethanol which were tested over the range 2–15 °C). In all cases regression lines were fitted to the 5–6 points obtained in each experiment (3 points in the case of methanol and ethanol). The regression coefficients were greater than 0.96 in 35 experiments, and between 0.90 and 0.95 in 7 other experiments. Low regression coefficients, between 0.7 and 0.9, were generally obtained with solutes having low P's.

diols each successive addition of a $-CH_2$-group resulted in a smooth increase in E_a of 2.5 kcal/mole per $-CH_2-$. Although E_a was higher for the corresponding monohydroxyalcohols (*see* below), E_a also increased with each additional $-CH_2$-group by 3.6 kcal/mole. Galey *et al.* (1973) reported a similar phenomenon for the permeation of amides across human red blood cell membranes, i.e., E_a increased from 12 to 38 kcal/mole on going up the series from formamide to valeramide[4], i.e., 6.5 kcal/mole per $-CH_2$-group. An increase in E_a with increasing molecular size also appears to occur in bovine red blood cells, arbacia eggs, and ascites tumor cells (*see* Stein, 1967). On the other hand in liposomes, permeation studies (Cohen, 1975*a*) show little effect of size on E_a, but partition studies (Katz & Diamond, 1974*b*) show that E_a actually decreases with increasing size, methanol, ethanol and propanol.

E_a and H *Bonding Ability.* Observations that P's are inversely proportional to the H bonding ability of the solute (page 269) suggest that solute

4 Galey *et al.* (1973) did not in fact claim that E_a increased as a smooth function of chain length, but instead claimed a discontinuity between the lower hydrophilic members of the series (formamide and acetamide) and the larger lipophilic members (propionamide, butyramide, and valeramide). However, their interpretation is not entirely convincing owing to the large error in their measurements, particularly with formamide (12±5 kcal/mole), and the smooth increase in E_a from acetamide to valeramide. Their large errors are due, at least in part, to technical difficulties and the fact that they only measured P's at 20, 25, and 30 °C.

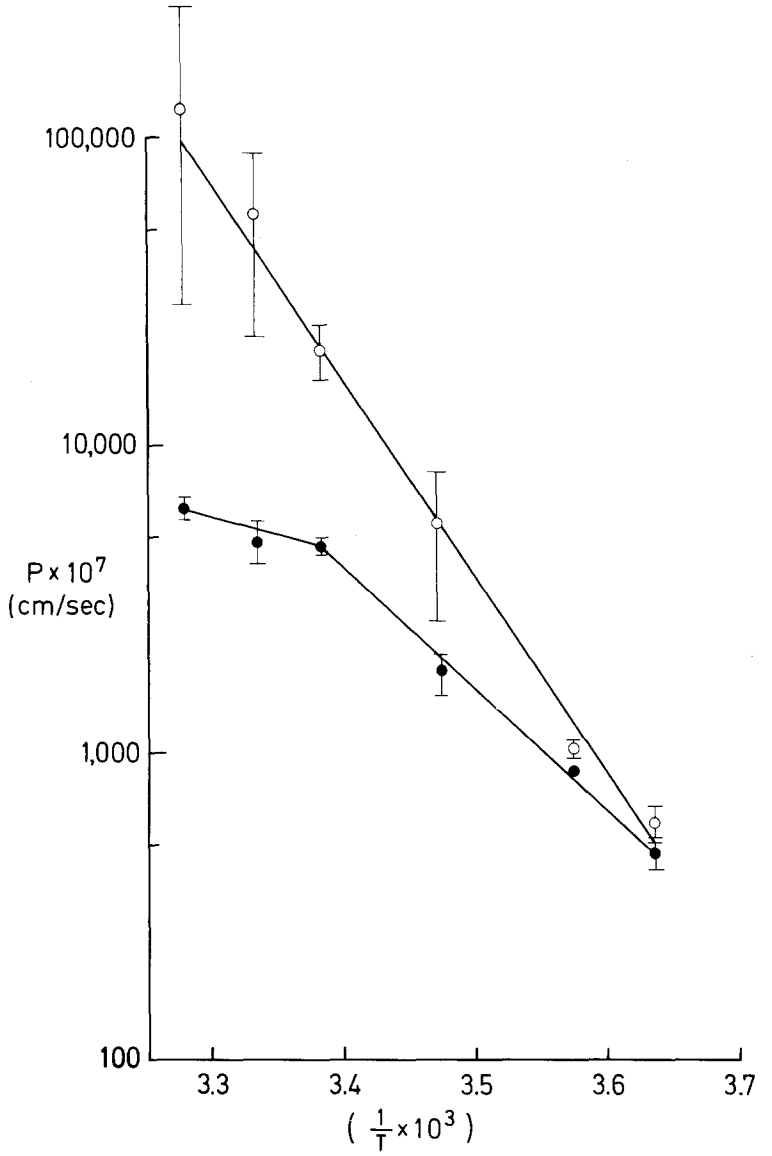

Fig. 5. Effects of unstirred layers on the plots of log P_{butanol} *vs.* $1/T$. In the absence of unstirred layer corrections (●) there was a break in the curve at 14 °C, but when the unstirred layers are taken into account (○) no break occurs in the curve. The mean values from 4 experiments are shown. The large SEM for corrected P's at high temperatures is due to the delicate sensitivity when permeation is close to diffusion limited. Similar plots of log P_{water} *vs.* $1/T$ show that the unstirred layer correction only becomes important at 28 °C and above

dehydration is a major step in the permeation process. If dehydration is a rate limiting step, it is anticipated that the apparent activation energies for permeation should increase with the number and strength of the H bonds that a solute can make with water, i.e., the stronger the bonds between

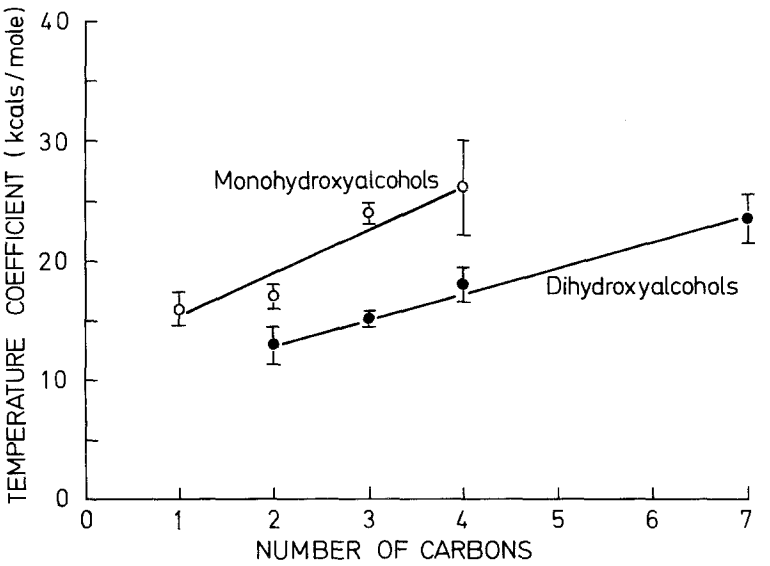

Fig. 6. Temperature coefficients (E_a cal/mole) as a function of chain length. The results for two homologous series, the mono- and di-hydroxyalcohols, are shown

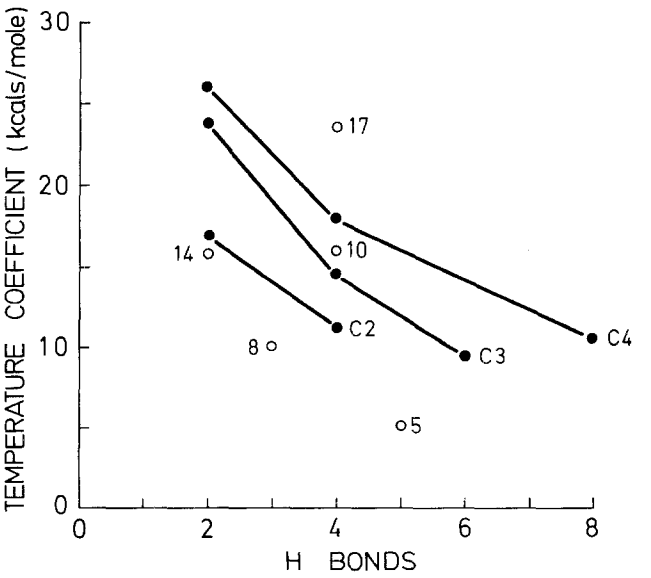

Fig. 7. Temperature coefficients (E_a) as a function of the ability of the molecule to form H bonds with water. It is assumed that each $-OH$ group can form two H bonds, acetamide (8) three and urea (5) five bonds. C2, C3, and C4 refer to the two, three and four carbon alcohols, respectively. Also included are the points for 1,7-heptanediol (17), 1,2-propanediol (10), and methanol (14). Note that the position of 1,2-propanediol (10) is intermediate between 1,3-propanediol and 1,4-butanediol, and that the positions of urea and acetamide are consistent with their relative ability to form H bonds and their lack of methylene groups

solute and water the greater the energy required to tear the solute out of water and transfer it to the hydrocarbon interior of the membrane. Fig. 7 shows a plot of E_a against the number of H bonds made by each molecule.

Although there is considerable variation in E_a amongst molecules making the same number of H bonds, largely due to variations in chain length (*see* Fig. 6), the graph shows that E_a actually *decreases* with the ability of the molecules to form H bonds. This is particularly clear when molecules of the same chain length are compared: e.g., four carbon compounds, *n*-butanol (26) *vs.* 1,4-butanediol (18) *vs.* erythritol (10.6); three carbon compounds, *n*-propanol (23.9) *vs.* 1,3-propanediol (14.6) *vs.* glycerol (9.6); and two carbon compounds, ethanol (16.9) *vs.* ethylene glycol (11.3). On the average, E_a decreased by 5.6 ± 1.1 (6) kcal/mole per $-$OH group. Intramolecular H bonding accounts for some of the variation as judged by the comparison of 1,3-propanediol (14.6) *vs.* 1,2-propanediol (19), and the fact that the increments in E_a on adding additional $-$OH groups to 1,4-butanediol, 1,3-propanediol and ethanol are smaller than average. In the absence of intramolecular H bonding we estimate that the average effect of one hydroxyl group is close to 9 kcal/mole (*see* Fig. 6).

It should be noted that the addition of an $-$OH group to ethanol to give ethylene glycol produced a decrease in E_a that is not significantly different from that seen for the addition of an $-$OH group to larger molecules where intramolecular H bonding is also possible. This is consistent with our view that the small molecules permeate through the lipid of the membrane rather than through pores or carriers.

In bovine red cells there is some evidence to show that E_a *increases* with the number of $-$OH groups on a molecule, e.g., propanol (5) *vs.* 1,3-propanediol (19.5) *vs.* glycerol (24.5) (Jacobs, Glassman, & Stewart, 1935, *cited* by Stein, 1967). This is more evident in the case of permeation and partition in liposome membranes (Cohen, 1975*a*: and Katz & Diamond, 1974*b*, respectively) where E_a increased by 1–2 kcal/mole per H bond. Consequently the rate limiting step governing permeation may not be identical from one membrane to another (*see* Wright & Bindslev, 1976).

Finally, the lack of variation in E_a amongst the fully hydroxylated compounds (glycerol, erythritol, mannitol and sucrose) may be interpreted as showing that the increase in H bonding ability is offset by the increase in size of the molecules. A more likely alternative explanation, however, is that the large polar molecules permeate across the toad urinary bladder via a separate shunt pathway (*see* pp. 277–278). Nevertheless, the fact that the apparent activation energies for these molecules (10 kcal/mole) are substantially larger than those for diffusion of these molecules in free

solution (4–5 kcal/mole) indicates some degree of interaction between these solutes and the membrane.

E_a's for Water, Urea and Acetamide Permeation. The results obtained for toad urinary bladder in this study are similar to those reported previously by Hays and Leaf (1962) [E_a urea 4.1 kcal/mole] and Hays, Franki and Soberman (1971) [E_a water 11.7 kcal/mole]. In addition, the values for urea and acetamide (5.2 and 9.8 kcal/mole respectively) are comparable to those reported for permeation across the rabbit gall bladder (Smulders & Wright, 1971) and human red cell membrane (Galey *et al.*, 1973). The explanation for the apparent low values obtained for urea and acetamide E_a's in toad bladder is simply a direct consequence of the fact that these two molecules are highly polar solutes lacking a number of hydrophobic methylene groups (*see* pp. 279–283, Figs. 6 and 7).

Although the absolute water permeability of toad bladder is comparable to that in other cells and tissues (*see* House, 1974, Tables 5–1 and 9–1), the activation energy for diffusional water permeation is the highest reported (15.5 *vs.* 3–6 kcal/mole, *see* House, 1974, Table 5–6). Larger values have been obtained for hydraulic water flow than diffusional water flow, e.g., greater than 11 kcal/mole for sea urchin eggs and chicken erythrocytes (*see* House, 1974, Table 5–6).

Finally, it should be noted that both the magnitude of the water permeability and its temperature dependence in toad bladder are very similar to those reported for water permeation across bilayer lipid membranes (*see* Redwood and Haydon, 1969; Price and Thompson, 1969). This is consistent with our view that it is not necessary to invoke the presence of membrane "pores" to explain water permeation across the bladder.

E_a and Bulk Phase Water/Oil Partition Coefficients. In view of the close correlation between P's and bulk phase water/olive oil partition coefficients (Fig. 1 and p. 269), we thought it would be informative to measure the temperature coefficient of the partition process, particularly since these are not readily available from the literature. The preliminary results obtained for four solutes partitioned between olive oil and water over the temperature range 10 to 37 °C are summarized in Table 5. In all four cases E_a was less than 6 kcal/mole, in agreement with the results obtained for decane/water (Johnson & Bangham, 1969) and octanol/water (Klein, Moore, & Smith, 1971). It is noteworthy also that the absolute values of the partition coefficients for *n*-propanol and *n*-butanol between olive oil

Table 5. Olive oil water partition coefficients and their temperature coefficients

	K_{oil}	E_a (kcal/mole)
n-propanol	0.10	3.8
1,3-propanediol	0.0013	2.2
n-butanol	0.39	5.8
1,7-heptanediol	0.031	2.8

K_{oil} at 22 °C, and apparent activation energies (E_a) obtained from plots of log $K_{oil}/1/T$ over the temperature range 10–37 °C. Olive oil is mainly composed of a mixture of glycerides of oleic acid (84%), palmitic acid (9%) and linoleic acid (4%). No correction is made for the variation in density of oil with temperature as the ratio of the oil and water densities is constant over the temperature range used in these experiments.

and water are similar to those found recently for partition between dimyristoyl lecithin and water (Katz & Diamond, 1974*b*), and that the E_a for *n*-propanol distribution between dimyrsitoyl lecithin and water, above the phase transition temperature, was 1.48 kcal/mole compared with 3.8 kcal/mole for olive oil.

E_a increased with chain length (*n*-propanol *vs.* *n*-butanol and 1,3-propanediol *vs.* 1,7-heptanediol) and decreased with increasing number of hydroxyl groups on the molecule (*n*-propanol *vs.* 1,3-propanediol), i.e., the effects were similar but smaller than those observed for permeation across the toad urinary bladder. In the lecithin/water system E_a for partition *decreased* with increasing chain length and *increased* with increasing H bonding ability at temperatures above the phase transition point.

E_a for Bladder Conductance. Arrhenius plots of toad urinary bladder conductance over the temperature range 2–32 °C yield apparent activation energies of 4–5 kcal/mole. This is indistinguishable from the temperature dependence of the conductance of the saline. There was no difference between high resistance (greater than 8,000 ohms cm^2 at 22 °C) and low resistance bladders (lower than 2,500 ohms cm^2 at 22 °C). These results are similar to those obtained for a low resistance epithelium – the rabbit gall bladder (*see* discussion in Barry, Diamond & Wright, 1971).

Using the value for urinary bladder conductivity and the temperature dependence of the conductivity, we have estimated that the apparent membrane viscosity and viscous activation energy for the plasma membranes of the urinary bladder are 93 cP and 4.3 kcal/mole respectively (Bindslev & Wright, 1975). These values are close to that reported for the red cell membrane by Rudy and Gitler (1972) and Solomon (1974).

Conclusion

The good correlation between P's and K's suggests that permeation of nonelectrolytes across the toad urinary bladder epithelium is accounted for by solubility/diffusion processes in the lipid of the plasma membranes. The discrimination between branched chain isomers, and the high rate of permeation of small molecules are described in terms of the highly ordered configuration of the lipids in the membrane, i.e., straight chain isomers and small molecules encounter less steric hindrance in crossing the parallel array of membrane phospholipids than more bulky molecules. Strong evidence in support of this interpretation is (1) the correlation between diffusion in membranes and in polymers, (2) the anomalous high rate of permeation of both hydrophilic *and* lipophilic small molecules, and (3) changes in P and E_a produced by the addition of hydroxyl groups to small molecules are similar to those produced in much larger molecules. The contributions of methylene groups and hydroxyl groups are analyzed in more detail in the following paper, where we also present a view of permeation across the plasma membranes of the toad bladder.

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