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Routes of Nonelectrolyte Permeation across Epithelial Membranes

Ernest M. Wright and Richard J. Pietras

Department of Physiology, University of California Medical Center, Los Angeles, California 90024

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Summary. Radioactive tracer techniques were used to study the permeability of three epithelial membranes, the toad urinary bladder, frog choroid plexus, and rabbit gallbladder, to 16 nonelectrolytes. The general patterns of nonelectrolyte permeation were similar for all three membranes, and similar to those in other biological membranes. The permeability of lipophilic solutes was roughly proportional to their bulk phase oil/water partition coefficients, but the slope was greater in the toad bladder than in the gallbladder and plexus. Branched nonelectrolytes were less permeable than their straight-chain isomers in both the urinary bladder and gallbladder, but not in the choroid plexus. Small polar solutes permeated more rapidly than expected, and in the urinary bladder and gallbladder the permeation of urea and acetamide, but not water was inhibited by phloretin. This agent also increased 1,7-heptanediol permeability in the urinary bladder but in the gallbladder there was a marked inhibition. In all three epithelia a separate pathway exists for the permeation of large polar solutes, but quantitatively this is least important in the toad bladder. It is concluded that variations in passive nonelectrolyte permeation across epithelia are due to (*i*) variations in the composition and configuration of membrane lipids, (*ii*) the presence or absence of pathways for small solutes, and (*iii*) the absence or presence of pathways for larger polar solutes. We also conclude that there are at least two effects of phloretin on the permeation of nonelectrolytes across biological membranes, and that there are variations in each effect from one membrane to another.

Simple epithelial membranes, such as those of the intestine, gallbladder, choroid plexus, renal tubule, and toad urinary bladder, are composed of a sheet of cells held together at the apical surface by tight junctions *(zonula occludens)*. These junctions are formed by the fusion of the plasma membranes of adjacent cells completely around the perimeter of each cell (*see* Weinstein & McNutt, 1972). The tight junctions thereby form a barrier to the passage of large molecules between adjacent cells. In recent years much attention has been focused on the role of tight junctions in the regulation of the diffusion of small ions and nonelectrolytes across epithelial mem-

branes. Much of this attention has arisen from the fact that the electrical resistance of these epithelia ranged from less than 10 $\Omega\,cm^2$ (proximal tubule) to more than 20,000 $\Omega\,cm^2$ (urinary bladder). In the best-studied, low resistance epithelium, the gallbladder, Frömter (1972) has elegantly shown that 96% of the transepithelial current bypasses the cells, and that the anatomical location of the shunt was the tight junctions; i.e., the tight junctions in the gallbladder are not tight for small ions. It is now commonly assumed that the major difference between the high and low resistance epithelia is the magnitude of the shunt across the so-called tight junctions (*see* Frömter & Diamond, 1972).

To further characterize membranes of high and low resistance epithelia we decided to make a detailed comparison of nonelectrolyte permeability in three epithelia – the rabbit gallbladder (32 $\Omega\,cm^2$), the toad urinary bladder (3,000 $\Omega\,cm^2$), and the frog choroid plexus (200 $\Omega\,cm^2$). The stimulus to undertake this study was the observation that the membrane, or membranes, controlling nonelectrolyte permeation across the gallbladder was poorly selective relative to single cell membranes (Smulders & Wright, 1971). This suggested that either the plasma membranes of the gallbladder epithelium are much more hydrophilic than single cell membranes or that nonelectrolytes cross the epithelium via the highly hydrated tight junction route. To gain insight into this problem, we have measured the permeability of 16 selected compounds in the three epithelia in order to evaluate (*i*) the nature of the route for lipophilic solutes, (*ii*) the extent of the polar pathway for the permeation of small polar solutes, and (*iii*) the significance of shunt pathways for large polar solutes. Although the selectivity of nonelectrolyte permeation across the gallbladder, choroid plexus and intestine has been measured previously by an osmotic method (Smyth & Wright, 1966; Diamond & Wright, 1969*a*; Wright & Diamond, 1969*a*, *b*; Wright & Prather, 1970; Hingson & Diamond, 1972), it is difficult to extract information about the absolute magnitude of permeability coefficients from such data owing to uncertainties about unstirred layer effects and the hydraulic water permeabilities of the membranes. Furthermore, it is not possible to extend this particular method to high resistance epithelia such as the toad urinary bladder.

The toad urinary bladder was chosen to represent high resistance epithelial membranes mainly because of the simple anatomy of the tissue. Nonelectrolyte permeability of the urinary bladder has been studied to some extent by Leaf and Hays (1962). The frog choroid plexus was included in this study since recent electrophysiological experiments in our laboratory (Wright, 1972*a*) suggested that the tight junctions are on the major route

of ion permeation across this tissue. Anatomical studies (Castel, Sahar & Erlij, 1972) support this interpretation.

The results show that the general pattern of nonelectrolyte permeability is very similar in all three epithelia, but there are striking differences in detail from one tissue to another. From these differences it is concluded that variations in the permeability of both the tight junctions and the plasma membranes may contribute to differences in nonelectrolyte permeability between high and low resistance epithelia.

Materials and Methods

The approach to this investigation was very similar to that used previously for the rabbit gallbladder (Smulders & Wright, 1971). Permeability coefficients were determined by the use of radioactive tracer techniques and the results were corrected for unstirred layer effects by the relation

$$\frac{1}{P}=\frac{1}{P_m}+\frac{\delta}{D}$$

where P is the permeability coefficient determined experimentally, P_m is the true membrane permeability coefficient, δ the sum of the unstirred layers on each face of the epithelium, and D the free solution diffusion coefficients. The thickness of the unstirred layers was estimated from the time course of the build-up of streaming potentials or diffusion potentials as described previously for the gallbladder.

Toad Urinary Bladders

The urinary bladders from toads *(Bufo marinus)* were mounted between Lucite flux chambers. The area of the window between the two halves of the chamber was 1.3 cm^2 and each half contained 16 ml of saline which was continuously oxygenated and vigorously agitated by magnetic stirring bars. The average electrical resistance of these preparations was 2,600 $\Omega\,cm^2$ with a range of 1,500 to 6,000 $\Omega\,cm^2$.The thickness of the unstirred layers in these chambers under our stirring conditions amounted to 200 μ (Pietras & Wright, 1974)[1].

Bullfrog Choroid Plexuses

Nonelectrolyte fluxes across the bullfrog choroid plexus *(Rana catesbeiana)* were measured as described previously for ions (Wright, 1972*a*) and amino acids (Wright, 1972*b*). The thickness of the unstirred layers, as estimated from streaming potentials (Wright & Prather, 1970), was about 900 μ, and the average resistance of these preparations was about 200 $\Omega\,cm^2$.

1 Pietras, R. J., Wright, E. M. 1974. Effects of ADH on nonelectrolyte permeation across the toad urinary bladder. *(In preparation)*

Rabbit Gallbladders

In the experiments carried out during this investigation the gallbladders were mounted as flat sheets between Lucite flux chambers. The area of the window between the half-chambers varied between 0.1 and 1.3 cm^2. Each half-chamber contained 16 ml of saline and was continuously oxygenated and stirred vigorously with magnetic stirring bars. The thickness of the unstirred layers under these conditions amounted to 365 μ (Weidner & Wright, 1974)[2], and the average resistance was 32 Ω cm^2. *P*'s for sucrose, urea, acetamide, 1,4-butanediol and 1,7-heptanediol were in the same range as reported previously (Smulders & Wright, 1971).

The composition of the amphibian saline used in these experiments was (mM) 104.5 NaCl, 2 KCl, 1 $MgSO_4$ and 1 $CaCl_2$ buffered to pH 7.3 with 2.125 mM Na_2HPO_4/0.375 mM NaH_2PO_4; and in rabbit experiments, 148 NaCl, 6 KCl, 0.25 $CaCl_2$ buffered at pH 7.3 with 2.125 mM Na_2HPO_4/0.375 mM NaH_2PO_4. All experiments were carried out at room temperature, 22 to 23 °C. Permeability coefficients were corrected for unstirred layer effects and were expressed in cm/sec. In some experiments phloretin (dissolved in ethanol) was added to the saline solutions to give a final phloretin concentration of 0.5 mM.

All ^{14}C-labeled compounds were obtained from the sources indicated previously, except that *n*-butyramide, iso-butyramide and 1,4-butanediol were obtained from American Radiochemical Corp., Florida. Tritiated water was obtained from Calatomic, California. All isotopes were used as supplied by the manufacturer. Olive oil partition coefficients for the molecules used in this study are included in Table 1.

For the purpose of discussion, molecules with olive oil partition coefficients significantly greater than water are considered as lipophilic molecules, and those with partition coefficients significantly less than water are considered as hydrophilic molecules.

Results

The nonelectrolyte permeability coefficients obtained for the toad urinary bladder, frog choroid plexus and rabbit gallbladder are summarized in Table 1. The *P*'s are ranked according to the olive oil partition coefficients (K_{oil}). Before considering the results in detail it should be noted that the spread in *P*'s from the least permeable (sucrose) to the most permeable (water) was about 20 times greater for the urinary bladder than either the choroid plexus or the gallbladder.

Permeation of Lipophilic Molecules

The *P*'s for the lipophilic solutes are plotted against K_{oil} in Fig. 1. This shows that the *P*'s are roughly proportional to K_{oil} for all three epithelial membranes. However, in the urinary bladder the slope of the regression line (1) was significantly greater than slopes for gallbladder and choroid plexus (0.2).

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2 Weidner, G., Wright, E. M. 1974. The role of the lateral intercellular spaces in the control of ion permeation across the rabbit gallbladder. *(In preparation)*

Table 1. Permeability of epithelia to nonelectrolytes

Nonelectrolyte	K_{oil}	Rabbit gallbladder	Frog choroid plexus (cm/sec × 10^7)	Toad urinary bladder (cm/sec × 10^7)
sucrose	1×10^{-6}	40	16	1.4
mannitol	1.2×10^{-6}	—	21	1.9*
erythritol	3×10^{-5}	140	49*	3.0
glycerol	7×10^{-5}	180	69	2.3
urea	1.5×10^{-4}	890	120	14
water	7×10^{-4}	2020	680	1300
acetamide	8.3×10^{-4}	700	125	16
1,2-propanediol	1.7×10^{-3}	370	98	18
1,4-butanediol	2.1×10^{-3}	340	96	16
nicotinamide	5×10^{-3}	380	171*	12
1,6-hexanediol	6.8×10^{-3}	640	—	72
n-butyramide	1.7×10^{-2}	500	166	54
iso-butyramide	1.4×10^{-2}	320	136	16
1,7-heptanediol	3.1×10^{-2}	680	283	280
antipyrine	3.2×10^{-2}	760	242	100
caffeine	3.3×10^{-2}	760	432	290

The values of the permeability coefficients for the gallbladder were taken from Smulders & Wright (1971) except for *n*-butyramide, iso-butyramide and water which were estimated during the present series of experiments. The *P* values are quoted as the means estimated from an average of 30 estimates. The standard errors of the means were less than 10% of the means, except for those three cases marked by asterisks where the errors were less than 20% of the means. All permeability coefficients were corrected for the presence of unstirred layers (*see* Smulders & Wright, 1971). The thickness of the unstirred layers was estimated from diffusion and streaming potential transients, and the permeability coefficients were corrected accordingly (*see* Smulders & Wright, 1971). Under the experimental conditions used here the thicknesses of the unstirred layers were: choroid plexus 900 μ, toad urinary bladder 200 μ, and rabbit gallbladder 365 μ. The permeability coefficients were arranged according to the solute olive oil partition coefficients. These were taken from Collander (1954) except for nicotinamide, *n*-butyramide, iso-butyramide, and 1,7-heptanediol which we measured ourselves.

Permeation of Branched-Chain Compounds

Many biological membranes discriminate between branched and straight-chain isomers such that the branched compounds are generally less permeable than the straight-chain isomers (*see* Diamond & Wright, 1969*b*). In this investigation we have been able to compare the permeability of the two isomers, *n*- and iso-butyramide. The ratio of $P_{\text{iso-butyramide}}$ to $P_{n\text{-butyramide}}$ was 0.30 for the urinary bladder, 0.63 for the gallbladder, and 0.82 for the choroid plexus (Table 1). On the other hand, the ratio of the olive oil partition coefficients for these two isomers is 0.82 (0.014/0.017, Table 1).

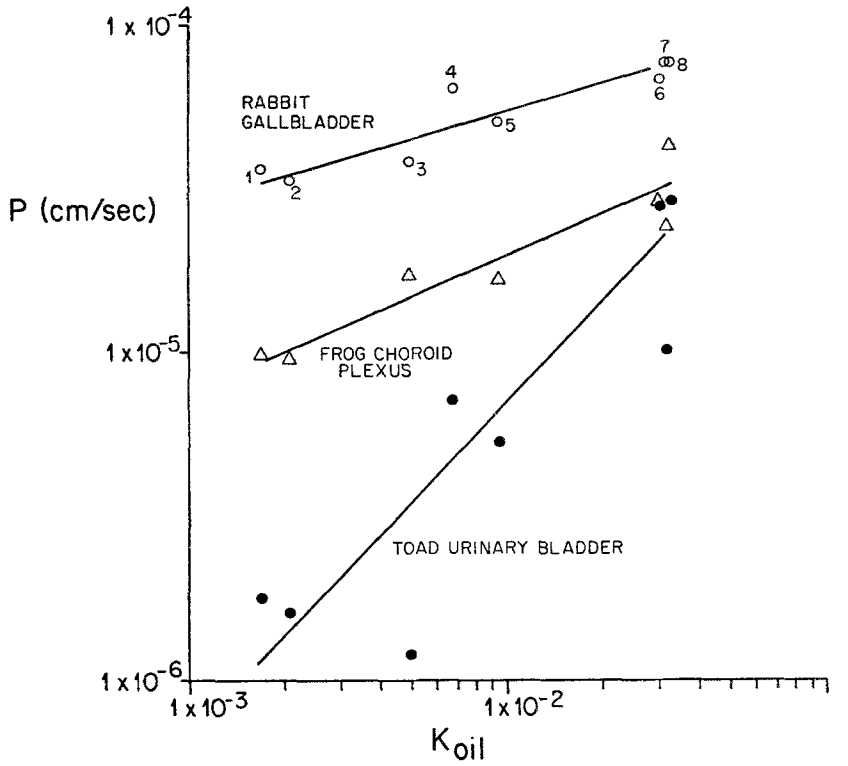

Fig. 1. The relation between P and K_{oil} for the toad urinary bladder (•), frog choroid plexus (△) and the rabbit gallbladder (○). Both the P's and the K's were taken from Table 1; (1) 1,2-propanediol, (2) 1,4-butanediol, (3) nicotinamide, (4) 1,6-hexanediol, (5) *n*-butyramide, (6) 1,7-heptanediol, (7) antipyrine, and (8) caffeine. The regression lines were drawn through the data points for each tissue. The regression coefficients were 0.86 for the urinary bladder, 0.88 for the choroid plexus and 0.89 for the gallbladder

Thus, in gallbladder and urinary bladder, the branched isomer is less permeable than expected from the ratio of the bulk phase partition coefficients. The gallbladder results are consistent with earlier observations that the reflection coefficients of branched compounds are generally greater than the straight-chain isomers (Wright & Diamond, 1969*b*). The results for the choroid plexus are also consistent with previous reflection coefficient measurements (Wright & Prather, 1970) which failed to show significant differences between straight and branched molecules.

Permeation of Hydrophilic Molecules

Earlier studies on the gallbladder suggested that hydrophilic solutes traversed the gallbladder epithelium by two separate polar pathways; one for small molecules such as urea and acetamide – probably through the

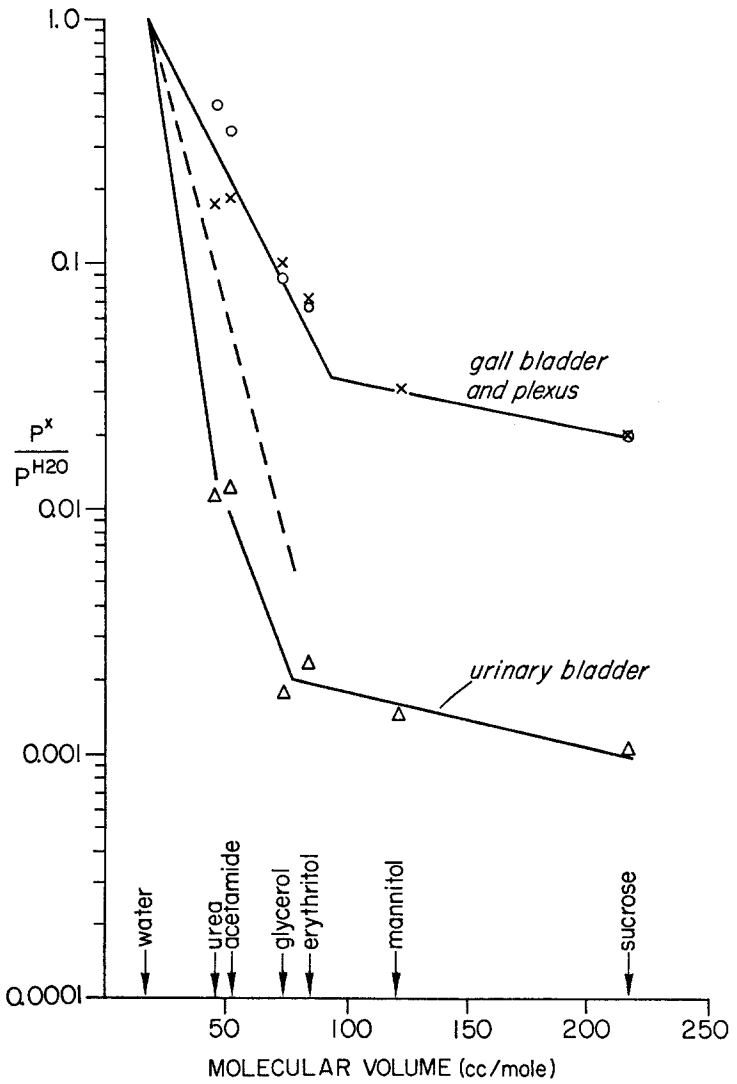

Fig. 2. The relative permeability of three epithelial membranes to hydrophilic solutes. Permeability coefficients, relative to water, are plotted as a function of molecular volume (MV) for the toad urinary bladder (△), rabbit gallbladder (○), and frog choroid plexus (×). The dotted line is the relationship between P and MV for human red blood cells and bilayer membranes doped with either amphotericin B or nystatin (Solomon & Gary-Bobo, 1972)

cell membranes, and the other for larger molecules such as sucrose and inulin – probably through the tight junctions (Smulders & Wright, 1971; Smulders, Tormey & Wright, 1972; Wright, Smulders & Tormey, 1972). To test the possibility that such polar pathways exist in other epithelial mem-

branes we have measured the permeability of six hydrophilic solutes in the choroid plexus and urinary bladder.

In order to compare P's in these three epithelia we have expressed P's relative to P_{water}, and plotted the log of the ratio against molecular volume (Fig. 2). [Horowitz and Fenichel (1964) have pointed out that the diffusion coefficients are a smooth function of the molecular volume, and Sha'afi, Gary-Bobo and Solomon (1971) found in red cells an empirical correlation between the P's of hydrophilic solutes and molecular volume.] The figure shows that: (*i*) There are two components in the relationship between P and MV for these epithelial membranes – one component for MV less than 80 cc/mole and another for MV greater than 80 cc/mole. (*ii*) The plots for the P ratios in the gallbladder and choroid plexus are, apart from urea and acetamide, almost identical. In the gallbladder the ratios for urea and acetamide are about a factor or two higher than in the choroid plexus, but this difference is eliminated by the action of phloretin *(see below)*. (*iii*) For small molecules the slope of the relationship between P and MV in the urinary bladder is similar to that found for the red cell membrane and lipid bilayer membranes doped with the channel-forming antibiotics, nystatin and amphotericin B (Solomon & Gary-Bobo, 1972). This suggests that the steric hindrance offered to the small molecules in these diverse systems is rather similar. The slope for the gallbladder and choroid plexus is less than that for urinary bladder. (*iv*) In the case of the larger molecules (i.e. MV > 80 cc/mole) the slope of the relationship between P and MV is similar for all three epithelia, and substantially less than the slopes obtained for the smaller molecules. However, with respect to water permeability, the relative importance of the pathway for these large molecules in the urinary bladder is about an order of magnitude less than that in the plexus and gallbladder. Nevertheless, it should be pointed out that the permeability of the urinary bladder to sucrose is about three orders of magnitude greater than that expected from the sucrose partition coefficient and the relationship between P and K for the lipophilic solutes shown in Fig. 1.

Effect of Phloretin

Macy, Karen and Farmer (1972) reported that phloretin selectively reduced the permeability of the red blood cell to urea, methyl urea and glycerol. This observation was confirmed and extended by Owen and Solomon (1972), and, in addition, they observed that phloretin increased the permeability of lipophilic solutes such as antipyrine. These reports led us to test the effect of phloretin on the permeation of selected molecules

Table 2. Effect of phloretin on nonelectrolyte permeability

Nonelectrolyte	Toad urinary bladder	Frog choroid plexus	Rabbit gallbladder
	$P_{phloretin}/P_{control}$		
urea	0.52±0.08 (6)	0.93±0.05 (2)	0.55±0.12 (2)
acetamide	0.56±0.17 (2)	1.24±0.17 (5)	0.51±0.25 (2)
water	0.94±0.01 (6)	1.04±0.23 (2)	0.93±0.03 (3)
1,7-heptanediol	1.16±0.05 (4)	1.07±0.20 (2)	0.51±0.07 (4)

The ratio of the permeabilities were determined in individual experiments by first measuring the flux in the absence of phloretin, and measuring the flux after the addition of phloretin (final concentration 0.5 mM). (Ethanol, at the concentrations used to carry the phloretin into solution, had no significant effect on the unidirectional fluxes.)

across the three epithelia used in this study. The results, summarized in Table 2, show: (*i*) phloretin caused a substantial reduction in the permeability of urea and acetamide in the urinary bladder and gallbladder, but not in the choroid plexus. In fact there was a slight increase in acetamide permeability in the choroid plexus; (*ii*) phloretin had little or no effect on water permeability in all three epithelia; and (*iii*) phloretin caused a significant increase in the heptanediol permeability in the urinary bladder. In contrast there was a significant reduction in the permeability of the gallbladder to heptanediol. We also observed that phloretin has no effect on the permeability of the gallbladder to sucrose (two experiments), but that there was a significant increase in the permeability of 1,4-butanediol (two experiments). In the urinary bladder there was no significant effect on the permeability of sucrose and *n*-butyramide (one experiment each), nor on the conductance of the tissue (18 experiments). As reported by Owen and Solomon (1972) for the red cell, there appears to be a relationship between the effect of phloretin on solute permeation across the toad bladder and the partition coefficient of the solute; phloretin inhibits those solutes with low partition coefficients but enhances those solutes with high partition coefficients.

Discussion

The patterns of nonelectrolyte permeation across the toad urinary bladder, frog choroid plexus, and rabbit gallbladder are very similar. This is quite understandable in view of the fact that permeation of nonelectrolytes across natural membranes is controlled largely by solute membrane partition coefficients and the molecular size of the solutes. In the case of the larger

lipophilic solutes, the predominant factor appears to be the partition coefficients, whereas for the small polar solutes (e.g., urea and acetamide), molecular size plays a prominent role.

Lipophilic Solutes

The correlation between permeability coefficients and bulk phase partition coefficients suggests that permeation is largely controlled by the balance of solute/water and solute/lipid intermolecular forces (*see* Diamond & Wright, 1969*a*, *b*). Solute/water intermolecular attractive forces are generally much stronger than the solute/lipid attractive forces, and so the selectivity for nonelectrolyte permeation is largely controlled by differences in solute/water intermolecular forces. The chief intermolecular forces in the aqueous phase have been identified as hydrogen bonds and, therefore, the greater the number and strength of the hydrogen bonds that the solute can make with water the lower the permeability. In these series of experiments evidence in support of this interpretation is provided by the observations that (*i*) the polyhydroxyl alcohols were less permeable than the diols, e.g. glycerol (6 H^+ bonds) *vs.* 1,2-propanediol (4 H^+ bonds) and mannitol (2 H^+ bonds) *vs.* 1,6-hexanediol (4 H^+ bonds), and (*ii*) diols were less permeable than amides, e.g. 1,4-butanediol *vs.* *n*-butyramide – the diol makes stronger H^+ bonds with water than the amide.

Another important class of solute/water interactions underlies the effect of methylene groups on solute permeation. The permeability of a solute increases with the successive addition of methylene groups to a molecule; e.g., in the case of the diols there is an increase in P on going from 1,4-butanediol to 1,7-heptanediol in all three epithelia. This effect is largely due to an entropy, or "iceberg", effect in the aqueous phase, and an increase in van der Waal's interactions between the solute and the membrane hydrocarbons. The entropy effect is due to the fact that nonpolar molecules in aqueous solution are surrounded by a zone of water with a more highly organized structure than bulk water. Thus, on the addition of each methylene group the change in entropy in the aqueous phase "pushes" the solute out of the aqueous phase into the membrane interior (*see* Diamond & Wright, 1969*a*, *b*).

Thermodynamic analysis has also shown that quantitative differences between selectivity patterns, the differences in the spread of the P's and K's are largely due to differences in solute/lipid intermolecular forces; the weaker these forces the greater the spread in the selectivity. Although the weak van der Waal's forces are probably the principal solute/lipid inter-

molecular forces, it has long been recognized that the number of hydrogen bonding sites within the membrane lipids are also important. Collander (1954) was the first to suggest parameters for gauging the "hydrophobic" character of cell membranes, namely: (*i*) *the slope of the relation between P and* K_{oil}. As shown in Fig. 1 the slope of the regression line for the urinary bladder is greater than either the gallbladder or choroid plexus. Thus, the membrane, or membranes, controlling permeation across the urinary bladder are, by this criterion, more hydrophobic than those in gallbladder and choroid plexus. *P*'s are proportional to K_{oil}^{1} in *Chara*, $K_{oil}^{1.15}$ in *Nitellopsis*, $K_{oil}^{1.5}$ in *Nitella* (Collander, 1954) and $K_{oil}^{1.4}$ in bovine red blood cells (Lieb & Stein, 1969); (*ii*) *the effect of an* $-OH$ *group.* Addition of a hydroxyl group to a molecule reduces its permeability to an amount dependent on the magnitude of the solute/lipid interactions. For example, the addition of $-OH$ to 1,2-propanediol to form glycerol reduces *P* by a factor of 2.1 in the gallbladder, 0.7 in choroid plexus, 7.8 in urinary bladder, 24 in olive oil (Table 1) and 530 in *Nitella* (Collander, 1954); (*iii*) *the increase in P upon increasing the chain length.* As an example we can use the diols 1,4-butanediol and 1,6-hexanediol. *P* increases by a factor of 1.9 in the gallbladder, *K* by 3.2 in olive oil, *P* by 4.5 in urinary bladder and by 12.6 in *Nitella.* On the basis of these three criteria it may be concluded that the "hydrophobic" character of these cell membranes is in the sequence: *Nitella*, bovine red blood cells > *Nitellopsis* > *Chara*, toad urinary bladder > rabbit gallbladder, frog choroid plexus.

In other words, in the low resistance epithelia (choroid plexus and gallbladder), the membrane, or membranes, controlling the permeation of lipophilic solutes behave as if the solute/lipid interactions are substantially stronger than in the high resistance epithelium. These conclusions are independent of the presence or absence of additional pathways for the permeation of polar solutes across these epithelia, since the diffusion coefficients of the lipophilic solutes vary by less than a factor of 1.5; i.e., the slopes of P/K in Fig. 1 are unaffected by the presence or absence of special permeation mechanisms for polar solutes.

Branched Compounds

The toad urinary bladder and the rabbit gallbladder, like many other biological membranes (*see* Diamond & Wright, 1969*b* and Sha'afi *et al.*, 1971), discriminate between branched and straight-chain isomers. This is interpreted to mean that both the partition of branched compounds into membranes and the mobility of branched compounds in the membrane are

preferentially reduced by the close packing of the hydrocarbon tails of the membrane lipids. Evidence for the role of membrane fluidity in the control of nonelectrolyte permeation comes from observations that, (*i*) increasing the fluidity of membranes increases nonelectrolyte permeability (Wilson, Rose & Fox, 1970; Kroes & Ostwald, 1971; Demel, Bruckdorfer & Van Deenen, 1972) and (*ii*) the partition of solutes into membranes decreased when the hydrocarbon tails of membrane lipids changed from a fluid into a crystalline state on passing the temperature through the phase transition point (Hubbell & McConnell, 1971; Diamond & Katz, 1974; Dix, Diamond & Kivelson, 1974).

The membranes of the choroid plexus and the mesophyll cells of the plant *Vallisneria* (Collander, 1959) do not discriminate between the branched and straight-chain isomers. This suggests that the membrane lipids of these two cell types are in a less ordered configuration than in most other biological membranes.

Antidiuretic hormone reduces the discrimination between *n*- and isobutyramide in the toad urinary bladder, and this has been used as evidence that the hormone increases nonelectrolyte permeability by increasing the fluidity of the apical plasma membrane (Pietras & Wright, 1974).

Small Polar Solutes

Permeation of small polar solutes across the three epithelial membranes resembles that in single cell membranes, namely: (*i*) permeation is greater than predicted on the basis of their partition coefficients and the relationship between P and K obtained for the lipophilic solutes, e.g. the permeability coefficient for urea was greater than predicted by a factor of 10 in the toad urinary bladder, and by a factor of 5 in the gallbladder and choroid plexus; (*ii*) there is an empirical relationship between P and molecular volume (Fig. 2). If these solutes actually cross the membranes via "pores", and if these "pores" are otherwise similar, the relationship between P and MV suggests that the steric hindrance offered to the passage of small molecules is very similar in doped bilayer membranes, red blood cells and epithelial membranes; (*iii*) the apparent activation energies for the permeation of small polar solutes across the toad urinary bladder (Bindslev & Wright, 1974)[3], gallbladder (Smulders & Wright, 1971) and red blood cell (Galey, Owen & Solomon, 1973) are substantially less than those for the permeation

3 Bindslev, N., Wright, E. M. 1974. Temperature dependence of nonelectrolyte permeation across the toad urinary bladder. *(In preparation)*

of lipophilic solutes; and (*iv*) in the red blood cell, toad urinary bladder, and gallbladder urea and acetamide permeability is reduced by phloretin *(see below)*. Consequently, we conclude that the pathway for the permeation of small polar solutes across these epithelial membranes is through the cell membranes.

The three alternative explanations for the anomalous permeation of small polar solutes across biological membranes include (1) permeation occurs through small "pores", (2) permeation mediated by membrane carriers, and (3) high permeability of small polar solutes as a direct consequence of the highly ordered configuration of the plasma membrane lipids. Although we have no information to allow us to distinguish between these explanations, we do note that in the most "fluid" membrane, the choroid plexus, the phloretin-sensitive permeation of urea and acetamide is absent, and that in the least fluid membrane, the urinary bladder, the anomalous permeation of water is most apparent. Furthermore, it has been observed that the lower members of the monohydroxyl alcohol series, methanol and ethanol, violate Overton's rules in both the toad urinary bladder (Leaf & Hays, 1962) and in the red blood cell (Naccache & Sha'afi, 1973). These observations suggest that the anomalous behavior of small solutes may be, at least in part, due to the highly ordered structure of the membrane lipids.

Large Polar Solutes

In the gallbladder, permeation of large polar solutes is thought to occur via a few "large" pores in the tight junctions (Smulders & Wright, 1971). This conclusion was based on the observations that (*i*) the apparent activation energy for sucrose permeation was indistinguishable from the activation energy for sucrose diffusion in aqueous solution, and (*ii*) the sucrose to inulin P ratio was approximately equal to the ratio of the free solution diffusion coefficients. Edge damage was ruled out since, (1) there was no change in sucrose permeability with variations in the edge-to-surface ratio, (2) sucrose permeability varied with the width of the lateral intercellular spaces (Smulders *et al.*, 1972), and (3) in *Necturus* gallbladder, Frömter (1972) found no leakage of current around the edge of the tissue. Frömter also failed to find current leaks that might be equated with desquamating cells.

The plots of P *vs.* MV in Fig. 2 suggest that a similar pathway exists for the large polar solutes in the choroid plexus and toad urinary bladder. In the urinary bladder, additional evidence for the presence of this pathway comes from the observations that the apparent activation energy for sucrose

permeation (~10 kcal/mole) is substantially less than that for the permeation of lipophilic solutes[3], and ADH does not increase the permeability of the large polar solutes even though the hormone significantly increases the permeability of all other nonelectrolytes[1]. In the rabbit choroid plexus (in vivo) Welch and Sadler (1966) found a relationship between the permeability of large polar solutes and their diffusion coefficients.

The plot of *P vs.* MV, in Fig. 2, also indicates the extent to which small polar solutes use the pathway for the larger polar solutes; e.g., less than 25% of the urea and acetamide fluxes occur through the larger pathway in the gallbladder, choroid plexus, and toad urinary bladder.

So far there is no direct evidence that the large polar pathway is located in the tight junctions, but observation that the sucrose permeability in the gallbladder varied with the width of the lateral intracellular spaces (Smulders *et al.*, 1972) provides indirect evidence that this large polar pathway resides in the junctions of the gallbladder.

Considerable insight into the nature of the permeation pathway across the gallbladder tight junctions has been obtained by Moreno & Diamond (1974*a*, *b*) in their studies of organic cations. They find that the permeation of amines, in the charged form, *increases* with the ability of the amines to form H^+ bonds, and conclude that the selectivity of the tight junction pathway depends upon (*i*) the charge on the solute, (*ii*) the membrane charge in the tight junctions, (*iii*) the ability of the solute to form H^+ bonds with proton acceptor groups in the junctions, and (*iv*) steric factors. It is probable that the same factors, apart from the lack of a net charge on the molecules, are involved in the permeation of neutral solutes across the tight junctions. Osmotic and electrical gradients have been shown to increase the permeability of the toad urinary bladder to mannitol (Bindslev, Tormey, Pietras & Wright, 1974), but we were unable to find a consistent change in the ultrastructure of the tight junctions. The positive identification of the pathway of sucrose or mannitol permeation across these three epithelia would provide important clues about the differences between epithelial membranes.

Water Permeability

Some comments about the magnitude of the water permeability in these three epithelial membranes are necessary. First, in both the gallbladder[4]

4 The permeability of the gallbladder to water reported here and by Moreno[5] is about 5 times less than that obtained previously by Van Os and Slegers (1973). Our unstirred layer correction only increases P_{H_2O} by a factor of 1.3 whereas that used by Van Os and Slegers increases P_{H_2O} by a factor of 10.

5 Moreno, J. H. 1974. The pathway of nonelectrolyte permeability in gallbladder epithelium. *(In preparation)*

and choroid plexus, P_{water} (diffusional permeability) is about an order of magnitude greater than expected from the relationship between P and K for the lipophilic solutes, and in the toad urinary bladder P_{water} is about three orders of magnitude greater than expected. A common explanation for such observations is that water permeates across cell membranes via small aqueous "pores". Evidence taken in support of this view is: (*i*) the ratio of the hydraulic (or osmotic) water permeability, L_p, to the diffusional permeability, P_{water}, is usually found to be greater than 1. This is the case for the urinary bladder [$L_p/P_{water} \sim 6$ (Hays, 1972)] and gallbladder [$L_p/P_{water} \sim 15$; $L_p = 3 \times 10^{-3}$ cm/sec (Wright *et al.*, 1972), and $P_{water} = 2 \times 10^{-4}$ cm/sec (Table 1)]. Moreover, recent experiments with red blood cells showed that when 90% of the water permeation was eliminated by PCMBS the ratio of L_p/P_{water} fell from 2.5 towards unity, and that the apparent activation energy for water permeation increased from 4.8 to 11.5 kcal/mole (Macy *et al.*, 1972). We cannot rule out the alternative possibility that most of the water permeates across the native lipid component of the epithelial cell membranes and that the unexpectedly high P_{water} is due to the structural organization of the membrane lipids (*see* pp. 303–304) and the extremely low molecular volume of water. In this respect it should be noted that the permeability of bilayer membranes to water is similar to that for biological membranes, and that the apparent activation energies for water permeation across bilayer membranes and the toad urinary bladder are about 12 to 15 kcal/mole (Dick, 1970; Hays, 1972; Bindslev & Wright, 1974[3]). Finally, as in the case of osmotic water flow (Wright *et al.*, 1972), it is unlikely that the tight junctions are an important pathway for the diffusional flux of water across these epithelia.

Phloretin

In general, our results with phloretin are essentially the same as those found by Levine, Franki and Hays (1973*a*) for the toad urinary bladder and by Moreno (1974)[5] for the frog gallbladder. Furthermore, the results in both the toad urinary bladder and the gallbladder (except for the effect on heptanediol, for which we have no explanation at present) are essentially identical to those reported for the effect of phloretin on permeation across the red blood cell (Macy *et al.*, 1972; Owen & Solomon, 1972).

It may be concluded that (*i*) the same phloretin-sensitive mechanism for the permeation of urea and acetamide occurs in the red blood cell, toad urinary bladder and gallbladder. One interpretation of this finding is that phloretin inhibits carrier-mediated urea and acetamide transport across

these membranes. Support for this view comes from observations that show (a) saturation of the acetamide flux across the urinary bladder, and (b) inhibition of the urea flux across the urinary bladder by acetamide (Levine, Franki & Hays, 1973*b*). Inconsistent with this interpretation is the finding that the apparent activation energies for urea and acetamide permeation across the red cell (Galey *et al.*, 1973) and toad urinary bladder (Bindslev & Wright, 1974)[3] are less than 14 kcal/mole, whereas carrier-mediated sugar transport processes have activation energies greater than 20 kcal/mole (e.g. *see* LaCelle & Passow, 1971). However, regardless of the actual mode of urea and acetamide permeation across the bladders, it would appear that this phloretin-sensitive mechanism is absent from the choroid plexus. (*ii*) The action of phloretin on the permeation of lipophilic solutes across the urinary bladder appears to be identical to that observed in the red blood cell. The increase in the permeability of lipophilic solutes may be related to the phloretin-induced increase in permeability of native lipid bilayer membranes (Cass, Andersen, Katz & Finkelstein, 1973), which is thought to be due to a decrease in membrane viscosity, i.e. phloretin may increase nonelectrolyte permeability by increasing the partition of the solutes into the membrane and the mobility of the solute within the membrane[6].

A general conclusion about the action of phloretin on nonelectrolyte permeation across biological membranes is that the effects are nonspecific, i.e. there are at least two effects of phloretin and there are variations in each effect from one membrane to another.

Conclusions

Although the patterns of nonelectrolyte permeation across the three epithelia are rather similar, there are striking differences in detail. First, the membrane or membranes, controlling permeation of lipophilic solutes behave as if they are more hydrophobic in the urinary bladder than in either the gallbladder or choroid plexus, i.e. the solute/lipid intermolecular attractive forces are stronger in the choroid plexus and gallbladder than in the urinary bladder. This observation raises the possibility that in epithelia there is a correlation between the permeability of lipophilic solutes and the electrical conductance of the epithelium; i.e., the high conductance of some epithelial membranes may be in part related to the high permeability of the plasma membranes. However, a more definite conclusion must await further

6 Although phloretin does not appear to affect nonelectrolyte permeation across the choroid plexus, it should be noted that phloretin does inhibit active anion transport by this tissue (Wright, 1974).

studies on the conductance and permeability of a larger sample of cells and epithelial tissues.

The lipids of the tight junction complexes may also represent a pathway for the permeation of lipophilic solutes across epithelia, but, as yet, there is no information available about the significance of such a route.

Second, the membranes of the urinary bladder and the gallbladder discriminate between branched and straight-chain isomers, whereas the membranes of the choroid plexus do not. These results suggest that the membranes of the choroid plexus are in a less ordered configuration than the lipids in most other membranes. There does not appear to be any correlation between the fluidity of the epithelial membranes and the conductance of the tissue.

Third, hydrophilic solutes appear to cross all three epithelial membranes by two pathways, one for small polar solutes and the other for the larger polar solutes. There is no obvious correlation between the conductance of the three epithelia and the size of the pathway for the small polar solutes. Such a correlation might be expected if "pores" were the major route of ion permeation and if the properties of the "pores" varied from one epithelium to another. The pathway for the larger polar nonelectrolytes, which is probably extracellular (in the tight junction?), is less important in the toad urinary bladder than in the choroid plexus or gallbladder. Consequently, we expect that the magnitude of the large polar pathway is a significant factor in the determination of the transcellular resistance in epithelial membranes.

Finally, this study emphasizes that the following factors have to be taken into consideration when comparing permeation across epithelial membranes: (*i*) the composition and configuration of plasma membrane lipids; (*ii*) the presence or absence of special transport mechanisms for the permeation of small polar solutes, water, ions, and metabolites; (*iii*) the magnitude of the permeability of the tight junctions – to ions, water and nonelectrolytes; (*iv*) the presence or absence of "shunts" for the permeation of large polar molecules; these may reside in the tight junctions, desquamated cells, or damaged cells; (*v*) the thickness of the unstirred layers; these provide significant diffusion barriers to the highly permeable molecules; and (*vi*) the dimensions of the lateral intercellular spaces – in low resistance epithelia diffusion along these spaces may be rate-limiting transepithelial transport. Detailed differences between passive permeation of molecules across epithelia may be due to variations in one or more of these factors.

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