J. Membrane Biol. 5, 297–318 (1971)

The Magnitude of Nonelectrolyte Selectivity in the Gallbladder Epithelium

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Received 13 January 1971

Summary. The permeability of the rabbit gallbladder epithelium to nonelectrolytes was determined by radioactive tracer techniques and by a rapid osmotic procedure. As expected from empirical and theoretical considerations, there was a good agreement between the selectivity sequences obtained by the two methods for the sixteen compounds used in this study. Although the permeability coefficients are directly related to their bulk-phase partition coefficients, the gallbladder behaves as if the membranes controlling selectivity are more hydrophilic than isobutanol. The relation between permeability coefficients and molecular weight also show that these membranes are less viscous than other single cell membranes. Small polar solutes exhibit lower apparent activation energies for permeation than larger solutes, and this is taken as support for the view that small polar molecules permeate across this tissue via a polar pathway. Inulin and sucrose permeability coefficients are in the ratio of their free-solution diffusion coefficients, and the apparent sucrose activation energy is indistinguishable from that reported for diffusion in aqueous solution. These latter observations may be explained by the presence of a few large "pores" in the epithelium.

In a previous study of nonelectrolyte permeation across the gallbladder epithelium, an osmotic method was used to measure relative rates of permeation. On the basis of these experimental results, it was possible to describe the selectivity of nonelectrolyte permeation and to analyze the nature of the molecular forces controlling permeation (Wright & Diamond, 1969*a*, *b*; Diamond & Wright, 1969*a*). Although the selectivity pattern can be obtained from osmotic procedures, the magnitude of the selectivity spread, the ratio of the least to the most permeable solutes, cannot be readily obtained from such measurements. Since the range of the selectivity sequence provides an important clue about the composition of the membranes controlling permeation (Diamond & Wright, 1969*b*), we have set out to measure permeability coefficients by radioactive tracer techniques. The low spread of the permeability coefficients obtained suggests that the

lipid of the membranes controlling permeation across the epithelium contain a high ratio of hydrogen bonding sites to methylene groups compared with most single cell membranes. This is taken to mean that permeation of nonelectrolytes across the gallbladder is via a highly hydrated membrane or membranes.

The Principle of the Experiments

In the previous study, the parameter used to measure the permeability of the rabbit gallbladder was the reflection coefficient (σ). Reflection coefficients are defined as the ratio of the osmotic flow produced by a concentration gradient of the test molecule to the flow produced by the same concentration gradient of an impermeable solute (Staverman, 1951). The value of σ depends on the nature of the membrane and the molecule: for an impermeant molecule, $\sigma=1$; for increasingly permeant solutes, σ decreases progressively below 1. Reflection coefficients have been related quantitatively to permeability coefficients by the following expression:

$$\sigma_i = 1 - \frac{\omega_i \bar{V}_i}{L_p} - \frac{\omega_i f_{im} d}{\phi_w} \tag{1}$$

where ω_i is the permeability coefficient ($P_i = \omega_i RT$) of molecule i, $\bar{V}_i$ the partial molar volume of i, L_p the hydraulic conductivity of the membrane, d the thickness of the membrane, f_{im} the frictional coefficient between i and the membrane, and ϕ_w the volume fraction of water in the membrane (Katchalsky & Kedem, 1962). The term $\frac{\omega_i \bar{V}_i}{L_p}$ represents the volume flow of i across the membrane, and $\frac{\omega_i f_{im} d}{\phi_w}$ is the frictional interaction between i and water in the membrane. This frictional term appears in the equation if, and only if, water and the solute interact while crossing the membrane. Although the precise form of the frictional term depends to some extent on the model used to describe frictional interaction, this interaction is probably negligible in most biological membranes except for small polar solutes such as urea.

Reflection coefficients in this study are determined by the procedure described previously (Smyth & Wright, 1966; Wright & Diamond, 1969*a*). This method takes advantage of the fact that osmotic flow across the gallbladder generates so-called streaming potentials. As the magnitude of the streaming potentials is directly proportional to the rate of flow, the ratio of the streaming potentials produced by a test solute and by an equal concentration of an impermeant solute yields σ. Permeability coefficients are determined from measurements of nonelectrolyte tracer fluxes, and it is assumed that the rates of permeation of the labelled and unlabelled species are identical.

Unstirred Layer Effects

Owing to the fact that it is impossible to achieve perfect mixing of the solutions adjacent to biological membranes, some consideration must be given to unstirred layer effects (*see* Dainty, 1963). In the solutions on each side of membranes there is a region adjacent to the membrane in which diffusion is the only mechanism of transport. A direct consequence of the presence of these unstirred layers is that the flux of a molecule across the membrane tends to dissipate the concentration gradient of that molecule (or isotope) across the membrane; the concentration gradient is reduced by an amount

$\phi\left(\frac{\delta_1}{D_1}+\frac{\delta_2}{D_2}\right)$ where ϕ is the steady state flux of the molecule across the membrane, D_1 and D_2 the diffusion coefficients in the unstirred layers δ_1 and δ_2 on side 1 and side 2 of the membrane. Thus the permeability coefficient (P) determined experimentally is related to the true membrane permeability coefficient (P_m) by

$$\frac{1}{P}=\frac{1}{P_m}+\frac{\delta_1}{D_1}+\frac{\delta_2}{D_2}. \tag{2}$$

Another factor which affects the magnitude of the effective concentration gradient is the flow of water across the membrane. A water flow tends to reduce the solute concentration gradient by convectively enhancing the solute concentration in the unstirred layer on one side of the membrane and by depleting the solute concentration on the other side. The solute concentration adjacent to the membrane (C_m) is related to the velocity of the water flow (V) by

$$C_m=C_b e^{\pm\frac{V\delta}{D}} \tag{3}$$

where C_b is the bulk phase concentration, δ the unstirred layer thickness, and D the free solution diffusion coefficient. In the present study, permeability coefficients are determined under conditions of zero water flow, i.e., in the absence of concentration gradients and in the absence of solute-linked water flow, and so only the former unstirred layer effect need be considered. In the case of σ determinations, both unstirred layer effects have to be evaluated; the sweeping-away effect is more important for the least permeable solutes, whereas the flux effect becomes more important the more permeable the solute. Two lines of evidence suggest that water flows have negligible effects on σ measurements in the gallbladder: (1) σ's determined by the described method here and by a zero flow method give identical results (Wright & Diamond, 1969*a*); and (2) the solute concentration adjacent to the mucosal surface of the gallbladder is reduced by less than 5% even at the maximum flow rates encountered (Smulders, Tormey & Wright, 1971). As in previous papers, we have not attempted to correct σ's for the unstirred layer effects as we will limit the discussion to those conclusions that can be drawn in the absence of such corrections. However, permeability coefficients are corrected for the unstirred layers by the use of Eq. (2).

Materials and Methods

In these experiments, the rabbit gallbladder was mounted as a flat sheet between two lucite chambers similar in design to those first described by Ussing and Zerahn (1951) for the frog skin. A schematic representation of the apparatus is shown in Fig. 1 (*see* Wright & Diamond, 1968, and Wright, Barry & Diamond, 1971, for procedural details).

Reflection coefficients were determined by the procedure described in detail by Wright and Diamond (1969*a*). Briefly, σ's were obtained by measuring the streaming potentials produced by test solutes (0.05 mole) and by an impermeant solute (sucrose, 0.05 mole) which were added to the mucosal solutions.

The use of sucrose as the reference solute is justified by two lines of reasoning: (1) 38 fully hydroxylated compounds ranging from 4-carbon to 18-carbon compounds gave σ's indistinguishable from 1 (Wright & Diamond, 1969*b*); and (2) sucrose flux and L_p measurements show that $\omega\bar{V}_s>1\%$ L_p [$\omega\bar{V}_i=3.6\times10^{-8}$ cm/sec/atm (Table 2), and $L_p=4.5\times10^{-4}$ cm/sec/atm (Smulders *et al.*, 1971)].

Permeability coefficients were determined through the use of radioactive tracer techniques. The nonelectrolytes were added to both the mucosal and serosal solutions to

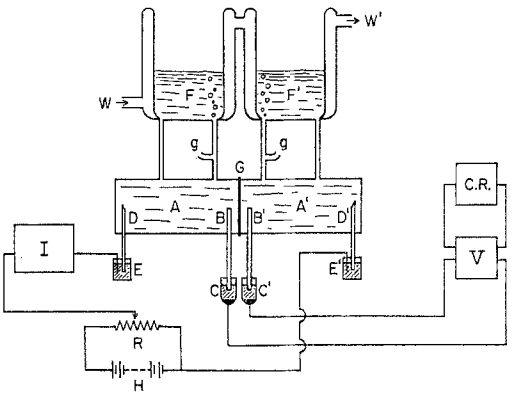

Fig. 1. A schematic drawing of the apparatus employed in this study. The gallbladder (*G*) is clamped between two lucite half chambers (*A* & *A'*) which are connected to two reservoirs (*F* & *F'*). Saline was recirculated through the chambers and reservoirs by means of gas lifts (*g*), and the temperature of the saline was controlled by perfusing water through the water jackets (*W* & *W'*) of the glass reservoirs. The potential difference across the tissue was measured on an electrometer (*V*) and displayed on a chart recorder (*C.R.*); the electrometer was connected to each chamber by means of salt bridges (*B* & *B'*) and calomel half cells (*C* & *C'*). The resistance of the tissue was monitored by passing a direct current across the gallbladder from a battery (*H*) using a potentiometer (*R*), silver/silver chloride electrodes (*E* & *E'*), and salt bridges (*D* & *D'*). The current passed was recorded on an electrometer (*I*)

give a final concentration of 0.05 mole to avoid the "sweeping-away" effects and the asymmetrical osmotic behavior of the epithelium (*see* Smulders *et al.*, 1971). A tracer quantity of the ^{14}C radioactive isotope was added to the mucosal solution, and the rate of appearance of the tracer in the serosal solution was determined by withdrawing aliquots of the serosal solution at 15-min intervals. After each aliquot was withdrawn, the volume of the serosal fluid was maintained constant by the addition of "cold" saline. Samples were withdrawn for at least 1 hr, and at the end of each flux determination a sample was withdrawn from the mucosal solution to determine the activity of the isotope in the "hot" solution. All radioactive samples were assayed, after dilution if necessary, by conventional liquid scintillation counting techniques where each sample was counted to at least 1 % counting efficiency. The radioactive tracer flux is expressed in μmoles/cm^2/hr, and the permeability coefficient in cm/sec.

The physiological saline used to perfuse the mucosal and serosal surfaces of the gallbladder contained NaCl (0.148 mole), KCl (0.006 mole) and $CaCl_2$ (0.0025 mole) buffered at pH 7.4 with Na_2HPO_4/NaH_2PO_4 (0.0025 mole). The saline was recirculated and oxygenated by means of an oxygen gas lift. Nonelectrolytes were obtained in the purest form commercially available, generally from Eastman Kodak Organic Chemicals or from Aldrich Chemical Company, and these were added to the saline to give a final concentration of 0.05 mole. In this study we were limited to 16 ^{14}C labelled radioactive isotopes which are commercially available; these were obtained from International Chemical and Nuclear Company, Irvine, Calif. (sucrose, glycerol, urea, 1,6-hexanediol and 1,2-propanediol), New England Nuclear, Boston, Mass. (inulin, sucrose, nicotinamide, acetamide, antipyrine, caffeine), and Tracerlab, Waltham, Mass. (n-butanol, n-propanol, 1,4-butanediol).

Although most experiments were carried out at room temperature (22–24 °C), in a few experiments the temperature was varied between 5 and 35 °C by varying the tem-

perature of the water circulated through the water-jacketed reservoirs (*see* Fig. 1). By using two water baths, it was possible to bring about 90% of the desired temperature change within 1 min.

NaCl diffusion potentials were obtained by partially replacing the NaCl in either the mucosal or serosal solutions with iso-osmotic mannitol. These dilution potentials were corrected for junction potentials as described by Barry and Diamond (1970). The half-times for the buildup of both these diffusion potentials and streaming potentials were used to estimate the thickness of the unstirred layers at the mucosal and serosal surfaces of the gallbladder by the following expression:

$$t_{\frac{1}{2}} = \frac{0.38\,\delta^2}{D} \tag{4}$$

where $t_{\frac{1}{2}}$ is the half-time, δ the unstirred layer thickness, and D the free-solution diffusion coefficients (Diamond, 1966; Dainty & House, 1966).

All calculations were performed on a programmed Hewlett Packard Calculator (Model, 9100 A), and all errors are expressed as standard errors with the number of estimates in parentheses.

Results

Measurements of Unstirred Layers

The thicknesses of the unstirred layers adjacent to the mucosal and serosal surfaces of the gallbladder epithelium were estimated from NaCl diffusion and sucrose streaming potential measurements. In three gallbladders, the mucosal and serosal unstirred layers were estimated from both potentials, and there was no significant difference in the results which suggests that neither the mucosal nor the serosal unstirred layers behave as selective diffusion barriers. The half-times for the mucosal streaming potentials and the serosal diffusion potentials are given in Table 1 together with the estimated thickness of the unstirred layers. The results–

Table 1. *Unstirred layers in the gall bladder*

Surface	$t_{1/2}$ (sec)	δ (microns)
Mucosal	6.4 ± 0.4 (147)	95
Serosal	158 ± 17 (16)	780

Streaming potentials were used to evaluate the mucosal-surface unstirred layers; NaCl diffusion potentials were used to evaluate the serosal unstirred layers. Streaming potentials were produced by the addition of sucrose (0.05 mole) to the mucosal fluid, and diffusion potentials were produced by replacing half the NaCl concentration with iso-osmotic mannitol. The diffusion potentials were corrected for junction potentials as described by Barry and Diamond (1970). Unstirred layers were calculated from the relation $t_{1/2}=0.38\,\delta^2/D$ assuming free-solution diffusion coefficients in both the mucosal and serosal unstirred layers; i.e., $D_{\text{sucrose}}=5.2\times10^{-6}$ cm^2/sec, and $D_{\text{NaCl}}=1.48\times10^{-5}$ cm^2/sec.

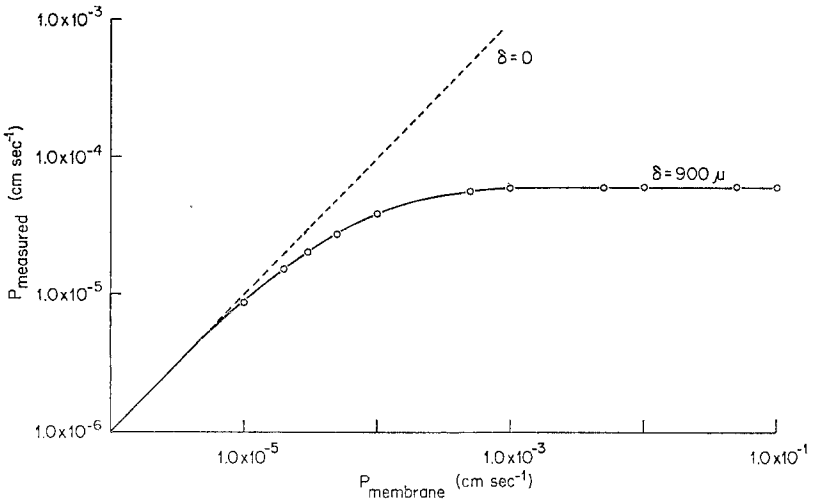

Fig. 2. A theoretical curve calculated from Eq. (2) relating the measured permeability coefficients (ordinate) to the membrane permeability coefficients (abscissa). The solute diffusion coefficient is assumed to be the same in both unstirred layers (i.e., $D_1 = D_2$), and the thickness of the unstirred layers ($\delta_1 + \delta_2$) was taken as 900 μ. A solute diffusion coefficient of 5×10^{-6} cm²/sec was used in the construction of the curve. The dotted line represents the relation between the two permeability coefficients when the unstirred-layer thickness is negligible

95μ in the mucosal and 780μ in the serosal solution – are essentially the same as those obtained by Diamond (1966) for sac preparations of the isolated gallbladder. These unstirred layers are calculated assuming free-solution diffusion coefficients in both the mucosal and serosal unstirred layers; this is probably correct for diffusion in the mucosal unstirred layer but not for the serosal unstirred layer where diffusion through the muscle and connective tissue may be lower than that in free solution. Consequently these calculations only give effective widths of the unstirred layers.

Using these estimates of the thickness of the unstirred layers, we calculated the theoretical relationship between the apparent permeability coefficients (P) and the true membrane permeability coefficients (P_m) using expression (2). The results are illustrated in Fig. 2 where it is shown that the presence of unstirred layers becomes apparent at P_m values greater than 10^{-5} cm/sec and that permeability measurements become unstirred-layer limited when P_m is greater than 5×10^{-4} cm/sec.

Flux Measurements

Preliminary flux experiments showed that steady state unidirectional fluxes were obtained within 15 min after the addition of the isotope to either the mucosal or serosal solutions and that the fluxes of at least five

compounds (sucrose, antipyrine, 1,4-butanediol, urea and acetamide) were constant for up to 8 hr. This contrasts with the conductance of the gallbladder which increased by about 20% over the first 60 to 90 min of the experiment (see Wright *et al.*, 1971, Fig. 3; and Barry, Diamond & Wright, 1971, Fig. 6). For two compounds, n-butanol and n-propanol, the unidirectional fluxes decreased exponentially with time. As judged by subsequent measurements of diffusion potentials and solute fluxes, this decrease was not due to irreversible effects of these compounds on the epithelium. Calculations show that the permeability of these two compounds is limited by the unstirred layers. Consequently the decrease in the flux is ascribed to the dissipation of the isotope gradients across the tissue. Thus the permeability coefficients of both n-butanol and n-propanol are calculated from the extrapolated zero time fluxes. These preliminary experiments also showed that there was no difference in the two unidirectional fluxes, mucosa to serosa and serosa to mucosa, for the six nonelectrolytes tested (sucrose, urea, acetamide, 1,4-butanediol, 1,7-heptanediol and nicotinamide).

In each gallbladder, it is possible to measure up to six permeability coefficients by measuring the flux of each compound for 1 hr and then washing both the mucosal and serosal compartments free of the compound before exposing the tissue to another compound. Initially sucrose fluxes were determined before and after every other compound to ensure that the tissue was not adversely affected by long exposures to the compounds used in this study. Since it soon became apparent that the compounds produced no irreversible effects[1], that permeability coefficients were constant over the concentration range 0.01 to 0.1 mole, and that sucrose fluxes were constant over the course of the experiments, we decided to measure sucrose fluxes only once in each gallbladder.

The Correlation between P's and σ's

In each gallbladder we measured permeability coefficients for at least four compounds (including sucrose) in a random fashion such that each of the sixteen compounds was compared directly with each of the other fifteen compounds in at least one gallbladder. Also in each gallbladder we determined the nonelectrolyte σ's before and after the flux determinations. No effort was made to determine the inulin σ owing to the fact that it is only slightly soluble in water. All the permeability coefficients and reflection coefficients measured in this study are listed in Table 2 in order of decreasing

1 However, ethyl acetoacetate reduced P_{Cl}/P_{Na} toward free solution even though subsequent sucrose fluxes were unaffected by this compound.

Table 2. *Gallbladder P's and σ's*

Compound	σ	P (cm/sec × 10^5)	P_m (cm/sec × 10^5)
Inulin	–	0.06 ± 0.01 (50)	0.06
Sucrose	1.00	0.38 ± 0.01 (285)	0.4
Erythritol	0.95 ± 0.01 (12)	1.2 ± 0 (28)	1.4 ± 0.1
Glycerol	0.92 ± 0.02 (12)	1.5 ± 0 (30)	1.8 ± 0.1
1,4-Butanediol	0.74 ± 0.01 (16)	2.6 ± 0 (58)	3.4 ± 0.1
Nicotinamide	0.65 ± 0.02 (13)	2.7 ± 0 (39)	3.8 ± 0.1
1,2-Propanediol	0.63 ± 0.03 (8)	2.9 ± 0.1 (25)	3.7
Acetamide	0.50 ± 0.03 (17)	4.7 ± 0.1 (63)	7.0
Urea	0.48 ± 0.03 (15)	5.5 ± 0.1 (59)	8.9
1,6-Hexanediol	0.33 ± 0.01 (16)	3.9 ± 0.1 (42)	6.4
Antipyrine	0.28 ± 0.02 (14)	3.9 ± 0.1 (58)	7.6
1,7-Heptanediol	0.11 ± 0.02 (12)	4.0 ± 0.1 (36)	6.8
Caffeine	0.10 ± 0.02 (12)	3.9 ± 0.1 (36)	7.6
Ethyl acetoacetate	0.06 ± 0.02 (11)	8.6 ± 0.2 (30)	≫10
n-Propanol	0.01 ± 0.01 (7)	14.2 ± 0.5 (4)[a]	≫10
n-Butanol	0.00 ± 0.01 (15)	16.6 ± 1.5 (8)[a]	≫10

[a] Calculated from zero time fluxes as indicated in the text.

The nonelectrolytes are listed in column 1 in order of decreasing σ. The second column gives the average value, the standard error of the mean (SEM) and the total number of estimates of σ. The third column gives the average value, SEM and total number of estimates of P. The fourth column lists the membrane permeability coefficients (P_m) obtained from the measured permeability coefficients in column 3 after correction for the presence of unstirred layers using Eq. (2); unstirred layers were obtained from Table 1 and diffusion coefficients were calculated from the relation $DM^{1/2}$=constant using sucrose ($D = 5.2 \times 10^{-6}$ cm^2/sec) as the reference solute. Both P's and σ's were determined in the same gallbladders – a minimum number of four gallbladders for each compound. σ's were uncorrected for the presence of unstirred layers for the reason outlined in the text. P's were calculated assuming that the area of the gallbladder was identical to the area of the window between the half chambers, i.e., 1.13 cm^2. All P's were determined from fluxes measured at concentrations of 0.05 mole in both the mucosal and serosal solutions, except for inulin which was determined at 0.001 mole owing to the low solubility of this compound. For the same reason, the inulin σ was not determined.

σ. Comparison of these σ results with those obtained previously (Wright & Diamond, 1969*b*) shows that there is good qualitative and quantitative agreement between the two studies. Permeability coefficients were corrected for the presence of unstirred layers by the use of Eq. (2). As predicted (*see* Table 2 and Fig. 2), the correction is insignificant for inulin and sucrose, but for the other compounds the correction increases in magnitude with increasing P until the unstirred layers become rate limiting for butanol and propanol. In other words, the resistance of the unstirred layers becomes

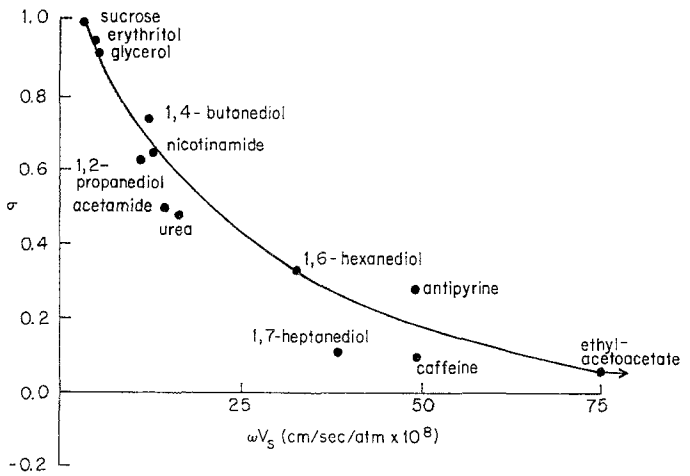

Fig. 3. A plot of $\sigma/\omega V_s$. The σ values are those obtained from Table 2 and the ωV_s values are those calculated from the permeability coefficients listed in Table 2 after correction for unstirred layers according to Eq. (2). As an approximation, molar volumes are equated with partial molar volumes. The curve drawn in the figure is the theoretical curve predicted for the relation between σ and ωV_s on the basis of Eq. (10) where σ is uncorrected for unstirred layers and ωV_s is corrected. The parameters used in Eq. (10) are $P_{H_2O}=7.5\times10^{-4}$ cm/sec, $D_{H_2O}=2.3\times10^{-5}$ cm^2/sec, $\delta_1+\delta_2=9\times10^{-2}$ cm, and $V_s/V_{H_2O}=7$. (Also *see* Appendix)

greater than the resistance of the epithelium for compounds with P's greater than 10^{-4} cm/sec.

The corrected permeability coefficients, expressed as ωV_s, are plotted against σ in Fig. 3; the curve fitted to the points is the theoretical expectation for the relation between the observed σ and membrane permeability coefficients (for further details *see* the figure legend and the Appendix). This figure shows that σ's decrease below 1 as the permeability coefficients increase in a fashion expected from theoretical considerations.

The Relation between P's and K's

In view of the empirical and theoretical relation between nonelectrolyte permeability coefficients and bulk-phase partition coefficients (*see* Diamond & Wright, 1969*b*), we have plotted the gallbladder permeability coefficients against olive oil, ether and isobutanol partition coefficients. In all cases there is a good straight-line relationship between P and K, the correlation coefficient in all cases being greater than 0.95. The plot of $P/K_{\text{isobutanol}}$ is shown in Fig. 4 where it can be seen that P's increase linearly with increasing K, the slope of the line being 0.45.

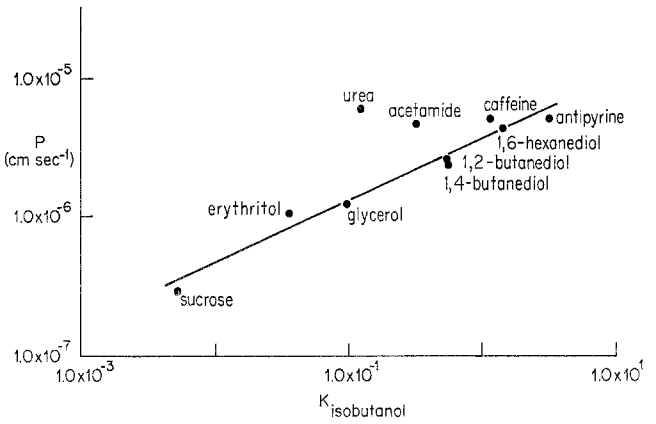

Fig. 4. The correlation between permeability coefficients and isobutanol partition coefficients. The isobutanol partition coefficients, plotted on the abscissa, were obtained from the sources indicated in Table 3. The gallbladder permeability coefficients, on the ordinate in cm/sec, were taken from Table 2 (corrected for unstirred layers) and expressed per unit area of the mucosal surface. Although the area of the window between the lucite half chambers was 1.13 cm^2, the actual surface area of the epithelium is greater owing to the presence of folds and the microvilli. It is estimated that the mucosal surface area is about 14 times greater than the serosal area in this tissue. However it should be noted that the slope of the $P/K_{\text{isobutanol}}$ plot is unaffected by this correction

Recently Lieb and Stein (1969) have published an approach to this problem which enables us to extract from such a plot of P/K the relationship between P and K on one hand and the relationship between P and molecular weight on the other. They derive the expression

$$\log P = \log P_0 + s_k \log K - s_m \log M \tag{5}$$

where P is the permeability coefficient, P_0 a constant, K the partition coefficient of the model system under consideration (e.g., olive oil or ether), M the molecular weight of the solute under consideration, s_k the correlation between P and K (if $s_k = 1$, then the model system mimics the biological membrane under consideration, and if $s_k = 0$, there is no correlation between P and K), and s_m the correlation coefficient between P and M. Double linear regression analysis of the gallbladder results has yielded values of s_k and s_m for plots of P against K_{ether}, $K_{\text{olive oil}}$ and $K_{\text{isobutanol}}$. These are listed in Table 3 where it is seen that $s_m = -0.7$ for all three plots and that the isobutanol K's provide the best model for the gallbladder permeability barrier. Even though $K_{\text{isobutanol}}$ provides the best fit for the gallbladder, the membranes are even more hydrophilic than isobutanol since $s_k = 0.36$ and not 1.

Table 3. *Values of s_m and s_k. For each model system obtained by application of multiple linear regression analysis to the gallbladder permeability coefficients and isobutanol, olive oil and ether partition coefficients according to Eq.* (5)

System	s_m	s_k
Olive oil	−0.74	0.22
Ether	−0.73	0.18
Isobutanol	−0.73	0.36

Partition coefficients were obtained from Collander (1949, 1950*b*, 1954). For 1,4-butanediol, 1,2-propanediol and 1,6-hexanediol, $K_{\text{isobutanol}}$ was obtained from the following expression: $\log K_{\text{isobutanol}} = 0.5 \log K_{\text{ether}} + 0.8$ (Collander, 1947).

Effects of Temperature on P

It has been established previously that σ's for small polar solutes, such as urea and acetamide, are less sensitive to change in temperature than other solutes which permeate solely by virtue of their "lipid solubility" (Wright & Diamond, 1969*b*). This observation is in accord with other lines of evidence which suggest that these small polar solutes permeate via polar pathways. We have investigated temperature effects further by measuring the apparent activation energy of solute permeation across the gallbladder. According to the classical theory of Arrhenius, the relation between permeation and temperature takes the form

$$P = P_0 e^{-A/RT}$$

where P is the permeability, P_0 a constant, and A the apparent activation energy for permeation (*see* also Stein, 1967). The fluxes of five compounds are plotted against temperature in Fig. 5 where it can be seen that there is a linear relationship between the log of the flux and $1/T$ as expected from the Arrhenius theory. The apparent activation energies are obtained from the slopes of these curves. The apparent activation energies of sucrose, urea and acetamide are between 5 and 7.5 kcal/mole which is comparable to that expected for diffusion in aqueous solution; e.g., the activation energy for urea and mannitol diffusion in aqueous solution is 4.5 kcal/mole (Taylor, 1938; Longsworth, 1954). In contrast, the apparent activation energies for 1,4-butanediol and antipyrine – between 12 and 15 kcal/mole – are approximately twice that expected for diffusion in aqueous solutions. Although the temperature effects are undoubtedly complex (*see* Diamond & Wright, 1969*a*, *b*), the apparent activation energies would seem to suggest

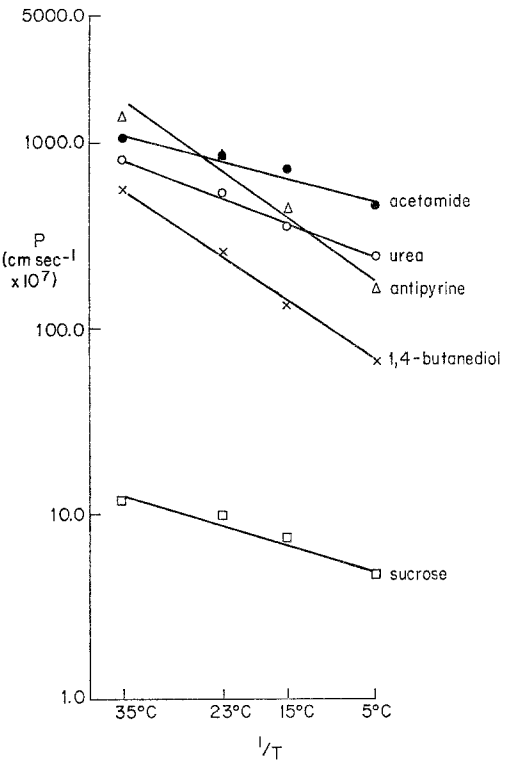

Fig. 5. The effect of temperature on gallbladder permeability coefficients. Nonelectrolyte permeability coefficients, on the ordinate, are plotted against the reciprocal of temperature, on the abscissa. The graph shows the results of one experiment each on five compounds (sucrose, urea, acetamide, 1,4-butanediol, and antipyrine). In each experiment the nonelectrolyte flux at each temperature was bracketed with measurements at room temperature. The membrane permeability coefficients were calculated from the apparent permeability coefficients from Eq. (2) using the unstirred layers and solute diffusion coefficients at the appropriate temperature

that the small polar solutes and sucrose permeate via a more polar route than the solutes which are expected to permeate by virtue of their lipid solubility alone. This view is substantiated by the fact that there is no obvious correlation between the apparent activation energies and molecular weight; e.g., the molecular weight of antipyrine is about twice that of 1,4-butanediol although there is little difference between their activation energies.

Discussion

In general this study confirms the validity of using the osmotic method described previously (Wright & Diamond, 1969*a*) to determine relative rates of nonelectrolyte permeation across biological membranes as expected

from empirical and theoretical considerations (Collander, 1950*a*, 1954; Kedem & Katchalsky, 1958). In particular we have established that there is a close correlation between the permeabilities recorded by this osmotic method and by tracer techniques (*see* Fig. 3). Further support for the method comes from preliminary experiments which show that there is good agreement between these σ's and those determined by (1) direct measurement of osmotic flows, and (2) osmotically induced resistances changes (*see* Smulders *et al.*, 1971).

Although there is good agreement between the nonelectrolyte selectivity sequences as determined by the osmotic procedure and by direct permeability measurements, the presence of the rather thick unstirred layers adjacent to the epithelium precludes a rigorous quantitative correlation between σ and P. However, the empirical nonlinear relationship between σ and ωV_s is that expected from theoretical considerations (*see* Fig. 3 and Appendix). Further analysis is complicated by the fact that σ's are neither measured at zero water flows nor can they be corrected for the unstirred-layer effects. Flux measurements show that the unstirred layers contribute significantly to the resistance of permeation when P_m is greater than 10^{-5} cm/sec; when P_m is greater than 5×10^{-4} cm/sec, permeation is unstirred-layer limited. Furthermore L_p measurements are particularly sensitive to the presence of unstirred layers and are further complicated by osmotically induced structural changes in the epithelium (Smulders *et al.*, 1971).

Regression analysis of plots of P/K show that $P \propto M^{-0.7}$ (Table 3) which is greater than that expected for diffusion in aqueous solution ($D \propto M^{-0.5}$) but is less than that obtained for *Nitella* membranes ($P \propto M^{-1.5}$, Collander, 1954). If the Stokes-Einstein relation[2] holds for the diffusion of nonelectrolytes across biological membranes, these results suggest that the membrane or membranes controlling permeation across the gallbladder have a low viscosity compared with those in *Nitella* membranes.

Although permeability coefficients are directly related to bulk-phase partition coefficients (Fig. 4), it appears that even isobutanol is more hydrophobic than the barrier or barriers controlling nonelectrolyte permeation across the gallbladder epithelium. This view stems from the observation that the slope of the plot of $P/K_{\text{isobutanol}}$ is less than 1 and that multiple regression analysis of the results in terms of Eq. (5) reveals that $P \propto (K_{\text{isobutanol}})^{0.36}$. Yet another expression of the same information is the relatively small effect of hydroxyl groups on permeation; e.g., addition

2 $D = \dfrac{kT}{6\pi r\eta}$ where k is the Boltzmann constant, T the absolute temperature, r the radius of the molecule, and η the viscosity of the medium.

of one –OH group to 1,2-propanediol to form glycerol only reduces permeation by a factor of two whereas isobutanol partition coefficients are reduced on the average by a factor of 5.3 (Collander, 1950*b*). These observations suggest that the barriers or barrier controlling permeation through the gallbladder are more hydrated than single cell membranes. Consistent with this view is the observation that the apparent activation energies for 1,4-butanediol and antipyrine are somewhat lower than that generally reported for the permeation of similar compounds across single cell membranes [*see*, e.g., Stein, 1967 (Tables 3, 4), and Wartiovaara, 1956].

Previously, on the basis of σ measurements alone, it was tentatively concluded that the gallbladder membranes must be virtually free of hydrogen bonding sites (Diamond & Wright, 1969*a*). This conclusion was based on a comparison of the relative effects of –OH and $-CH_2-$ groups on gallbladder σ's and on partition coefficients. For instance, removal of one hydroxyl group from 1,2-propanediol to give propanol has a slightly greater effect than the addition of five methylene groups to give 1,8-octanediol which suggests that one –OH group is offset by slightly more than five $-CH_2-$ groups. Similar conclusions can be made from the σ's and P's obtained in the present study. If these results are compared with the average effects of –OH and $-CH_2-$ groups on partition coefficients, it is concluded that the gallbladder membranes are more hydrophobic than olive oil. It is now apparent that this line of reasoning is invalid owing to the fact that for the diols the number of $-CH_2-$ groups required to balance the effect of an –OH group is independent of the lipid solvent (*see* Table 4).

The membranes controlling ion permeation across the gallbladder and other epithelial tissue also behave as if they are more hydrated than single cell membranes (Wright *et al.*, 1971; Barry *et al.*, 1971). This conclusion is based mainly on the relative rates of alkali metal ion permeation. There is a considerable array of evidence which suggests that ions permeate via the so-called tight junctions which join adjacent epithelial cells at the apical surface to separate the mucosal fluid from the lateral intercellular spaces. Although this is a possible route for the permeation of the nonelectrolytes, there is not sufficient evidence at this time to distinguish between this and the transcellular pathway. This question may be resolved by the use of autoradiographic techniques.

On the basis of σ measurements alone, it has been concluded that small polar molecules permeate across the gallbladder via a polar pathway that bypasses the membrane lipids (Wright & Diamond, 1969*b*). In the present series of experiments, there are two lines of evidence which support

Table 4. *The effect of –OH and –CH_2– on alcohol partition coefficients*

System	Increase in K or P			n	
	1	2	3	4	5
$K_{\text{isobutanol}}$	10	14	1.4±0.3	7.0	7.8
$K_{\text{olive oil}}$	76	252	1.8±1.0	7.7	9.9
K_{ether}	106	405	2.0±0.9	6.5	8.4
P_{Nitella}	424	664	2.5±2.4	6.7	7.2

The effect of removing an hydroxyl group or adding a methylene group on solute partition coefficients in various solvent systems. Columns 1 and 2 list the effect of removing an –OH group; 1 from 1,2-propanediol to form n-propanol and 2 from 1,4-butanediol to form n-butanol. Column 3 lists the average effect of adding a –CH_2– group in the dihydroxy alcohol series (1,2-propanediol, 1,4-butanediol, 1,5-pentanediol, 1,6-hexanediol, 1,7-heptanediol). Columns 4 and 5 give the number of –CH_2– groups (n) needed to offset the affect of one –OH group in columns 1 and 2, respectively, according to the equation:

(effect of removing one –OH) = (effect of adding one –CH_2–)^n. Also included in the table is the effect of these groups on *Nitella* permeability coefficients (taken from Collander, 1954). The changes in K and P listed in columns 1, 2 and 3 are all factors.

this hypothesis. (1) The position of urea and acetamide in the plot of P/K (Fig. 4) would indicate that these two solutes permeate faster than predicted from their partition coefficients alone. However, this is not fully conclusive owing to the limited number of points on the graph. (2) The effect of temperature shows that the apparent activation energies for urea and acetamide permeation (Fig. 5) are indistinguishable from the activation energies for diffusion in aqueous solution but are significantly less than the apparent activation energies of 1,4-butanediol and antipyrine permeation. The difference between urea and acetamide on one hand and 1,4-butanediol and antipyrine on the other is not due simply to a molecular weight dependence of diffusion on temperature since there is no obvious correlation between molecular weight and the apparent activation energies; e.g., there is no significant difference between the apparent activation energies for 1,4-butanediol and antipyrine although their molecular weights differ by a factor of two. The interpretation of the temperature experiments is complex (for discussion, *see* Wartiovaara & Collander, 1960, and Diamond & Wright, 1969*b*), but the results suggest that the two small polar solutes permeate via "pores". This view is further strengthened by experiments on the goldfish gallbladder which show that the apparent activation energies of acetamide and 1,4-butanediol are not significantly different (Wright, *unpublished observation*). In this gallbladder, D. J. Hingson and J. M.

Diamond (*personal communication*) found no separate pathway for the permeation of the small polar solutes.

In recent reviews Lieb and Stein (1969, 1971) have questioned the evidence for "pores" in the gallbladder and in *Nitella*. First, in the case of *Nitella*, they analyzed the permeability coefficients obtained by Collander (1954) by the use of Eq. (5) to obtain the relationship between P and M. They found that $P \propto M^{-3.7}$ and suggested that *Nitella* membranes behaved as a homogeneous membrane of hydrophobic polymers where the high rates of permeation of the small polar solutes were no longer anomalous. Since their conclusions are markedly different from those reached by Collander himself, their analysis deserves close scrutiny. Of the 70 permeability coefficients obtained by Collander, Lieb and Stein selected 13 P's and the molecular weight of 9 of these compounds was less than 90 g/mole, average molecular weight 95 ± 15 (13). On repeating their analysis on 53 of Collander's P's (neglecting compounds 1, 2, 3, 8, and 57–70 because in Collander's view these were the least accurate), we find that $P \propto M^{-1.8}$. Furthermore, if we (1) neglect compounds with $M < 70$, then we find that $P \propto M^{-1.2}$, and (2) if we only consider compounds with $M < 70$, we find that $P \propto M^{-3.7}$; i.e., we reach the same conclusions reached by Collander himself, namely, that the low molecular weight compounds have a steeper relationship to M than the higher molecular weight compounds. Thus Lieb and Stein's conclusions for the *Nitella* data are only correct insofar as they apply to compounds of low molecular weight.

In their review of the gallbladder studies, Lieb and Stein (1971) suggest that there is no need to postulate the existence of pores to explain the deviation of the small polar solutes from the main pattern in plots of σ/K. Their line of reasoning is as follows: since most of the small molecules deviating from the σ/K plot are amides, they suggest that the membranes controlling permeation are amidophilic. To test this suggestion, they extract relative P's from σ values using $\sigma = 1 - \frac{\omega V_s}{L_p}$, calculate relative membrane diffusion coefficients using the relation $P \propto K_{(\text{olive oil} + \text{oleic acid})} D_m$, and finally plot $D_{m\,(\text{relative})}/M$. The plot indicated that a single permeation mechanism was operative for solutes of all sizes and that $D_{m\,(\text{relative})} \propto M^{-2.8}$. These conclusions are erroneous for the following four reasons. (1) The gallbladder membranes are not "amidophilic". Although some of the small polar compounds happen to be amides, others are not amides (the remainder are ureas and polyhydroxy alcohols). The significant effect is that all small polar solutes, whether amides or not, are deviant. (2) The position of larger amides (e.g., butyramide, valeramide and hexanamide) on the σ/K plots do not deviate from the main pattern. (3) Of the 21 compounds selected

for their analysis, 13 have molecular weights less than 90, and of the remaining 8 compounds 2 are branched compounds which are known to be less permeant than predicted from bulk phase K's. Neglecting the compounds with molecular weights less than 65 $D_{m\,(\text{relative})}$ is proportional to $M^{-1.2}$. Thus the steep molecular weight dependence of permeation implied by Lieb and Stein applies mainly to low molecular weight compounds, i.e., compounds that would be expected to permeate via the polar pathway. (4) Unstirred layer effects were ignored. It is obvious from Fig. 2 that the unstirred layers have profound effects on solute permeation and it is for this reason that we have urged caution in this kind of analysis of gallbladder σ's (*see* also Wright & Diamond, 1969*a*). Consequently we see no need to revise our earlier conclusion that small polar solutes permeate across the rabbit gallbladder via a polar pathway which bypasses the membrane lipid. Furthermore, the recent experiments on artificial membranes doped with Nystatin or with Amphotericin B (Andreoli, Dennis & Weigl, 1969; Holz & Finkelstein, 1970) show that the antibiotics introduced into artificial bilayer membranes the same type of permeation properties associated with "pores" in biological membranes and suggest a possible molecular basis for this pathway.

The final point for discussion concerns the mechanism of sucrose permeation across the gallbladder. In Table 2 it is apparent that the ratio of the sucrose and inulin permeability coefficients ($P_{\text{sucrose}}/P_{\text{inulin}}=6$) is close to the ratio of the free-solution diffusion coefficients ($D_{\text{sucrose}}/D_{\text{inulin}}=4$); in three experiments where the fluxes of these two compounds were directly compared, the permeability ratio was even closer, i.e. ($P_{\text{sucrose}}/P_{\text{inulin}}=3$). Another point to note is that the apparent activation energy for the permeation of sucrose (Fig. 5) is indistinguishable from that expected for sucrose diffusion in aqueous solution. These observations suggest that inulin and sucrose permeate across the tissue via a free-solution shunt. The question that now arises concerns the nature of the shunt: is it due to edge-damage effects brought about by clamping the tissue between the lucite half chamber [compare the effect in frog skin (Dobson & Kidder, 1968)], or is permeation via "pores" evenly distributed over the surface of the epithelium? If sucrose permeation were confined to damaged regions, this would amount to about 0.2% of the total area of the mucosal surface. Edge damage, however, is ruled out by experiments where the edge/surface area ratio was varied by changing the area of the window between the lucite chambers. There was no change in membrane resistance or in NaCl diffusion potentials (Wright *et al.*, 1971) or in P_{sucrose} (J. W. Prather & E. M. Wright, *unpublished observations*) when the area of the tissue was reduced from 1.13 cm^2 to 2.6 mm^2.

Furthermore, microscopic examination showed no evidence of damage (J. McD. Tormey, *personal communication*). Finally there is a substantial body of evidence which suggests that the lateral intercellular spaces provide a common pathway for the permeation of water, ions and nonelectrolytes (including sucrose) across the gallbladder epithelium (Smulders *et al.*, 1971).

Thus sucrose and inulin permeation across the gallbladder is probably due to the presence of a few large "pores" (>12 Å radius) distributed over the surface of the epithelium. These pores may be located at the tight junctions of the epithelial cells (see Wright, Smulders & Tormey, 1971, for a further discussion of this problem). A similar explanation would also account for the permeation of large molecules across a number of other epithelial tissues, e.g., in the choroid plexus (Welch, 1967), small intestine (Loehry, Axon, Hilton, Hoder & Creamer, 1970) and stomach (Altamirano & Martinoya, 1966). Direct evidence for the route of permeation of these solutes across epithelia may be obtained by the use of autoradiographic techniques.

These few large pores are not the pathway used by the small polar solutes to traverse the gallbladder epithelium since (1) the urea and acetamide fluxes are about five times greater than would be predicted from the sucrose flux and the ratio of the free-solution diffusion coefficients, and (2) the sucrose σ is indistinguishable from 1 as is the case for 37 other fully hydroxylated compounds. This means that all 38 compounds fail to get through the urea pathway and that in the few large pores all solutes have σ's close to zero. Finally it should be pointed out that correction of the permeability coefficients in Table 2 for permeation via the sucrose shunt does not alter the slope of the P/K plots; i.e., the relation between P and M, and between P and K are unaffected by the presence of the sucrose shunt.

We are grateful to Drs. Peter H. Barry and Jared M. Diamond for valuable discussion throughout this study and to Dr. Diamond for his valuable comments on the manuscript.

This project was supported by grants from the Los Angeles Country Heart Association and the Life Insurance Medical Research Fund. A.P.S. was a Predoctoral Fellow under U.S. Public Health Service training grant 5 T01 GM 00448-10 during this study.

Appendix

The Effect of Unstirred Layers on Reflection Coefficient Measurements

In the absence of frictional interaction between solutes and water in a membrane, Eq. (1) reduces to:

$$\sigma = 1 - \frac{\omega \bar{V}_s}{L_p} \tag{6}$$

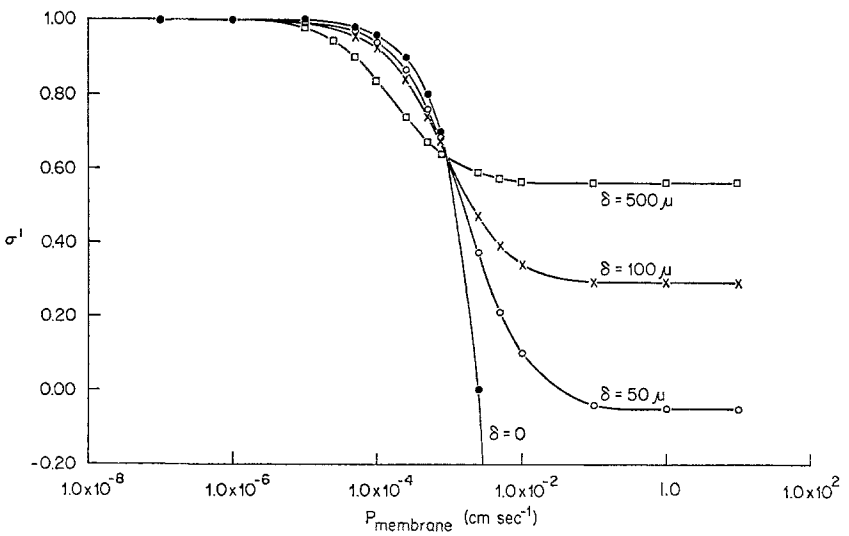

Fig. 6. The theoretical relationship between σ' and P_m; the effects of varying the unstirred-layer thickness. The reflection coefficient, on the ordinate, is uncorrected for unstirred layers whereas the permeability coefficient, on the abscissa, is the true membrane permeability coefficient. The affect of increasing the unstirred layer thickness from 0 to 500 μ is shown on the graph; in the presence of unstirred layers at the higher permeability coefficients, σ' becomes independent of P_m and the thicker the unstirred layer the curves level out at higher values of σ'. In the construction of the graph, it was assumed that $D_{H_2O}=2.3\times10^{-5}$ cm²/sec, $P_{H_2O}=2.5\times10^{-3}$ cm/sec, and $D_s=8.5\times10^{-6}$ cm²/sec

where ω is related to P by the expression

$$\omega = P/RT. \tag{7}$$

If we assume that water crosses the membrane by diffusion, the water permeability coefficient P_{H_2O} is related to L_p by the expression:

$$P_{H_2O} = L_p RT/\overline{V}_{H_2O} \tag{8}$$

where $\overline{V}_{H_2O}$ is the partial molar volume of water (Dainty & House, 1966). Eq. (6) can then be rewritten in terms of P_{H_2O} and P_s,

$$\sigma = 1 - \frac{P_s \overline{V}_s}{P_{H_2O}\,\overline{V}_{H_2O}}. \tag{9}$$

When sweeping-away effects are negligible, reflection coefficients measured in the presence of unstirred layers (σ') may be related to membrane permeability coefficients by substitution of Eq. (2) into Eq. (9)

$$\sigma' = 1 - \frac{\overline{V}_s \dfrac{1}{P_{H_2O}} + \dfrac{\delta_1}{D_{(H_2O)_1}} + \dfrac{\delta_2}{D_{(H_2O)_2}}}{\overline{V}_{H_2O} \dfrac{1}{P_s} + \dfrac{\delta_1}{D_{(s)_1}} + \dfrac{\delta_2}{D_{(s)_2}}}. \tag{10}$$

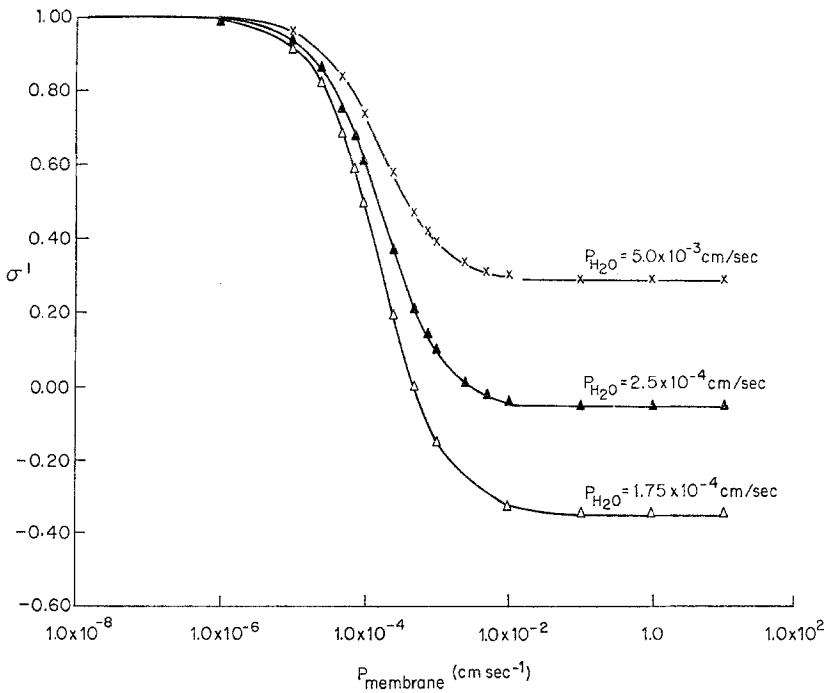

Fig. 7. The theoretical relationship between σ' and P_m; the effect of varying water permeability. The plots are similar to those shown in Fig. 6 except that the unstirred-layer thickness was held constant at 500 μ and P_{H_2O} was varied between 1.75×10^{-4} and 5×10^{-3} cm/sec. This figure shows that the precise shape of the plot of σ' against P_m depends on the permeability of the membrane to water; at low water permeabilities, negative reflection coefficients are obtained, whereas at higher values only positive σ's are obtained. The curve obtained for $P_{H_2O} = 1.75 \times 10^{-4}$ cm/sec corresponds to the behavior expected for the choroid plexus (Wright & Prather, 1970) whereas the curve for $P_{H_2O} = 2.5 \times 10^{-4}$ cm/sec would correspond to that for the rabbit gallbladder

This equation defines reflection coefficients (uncorrected for unstirred layers) in term of membrane permeability coefficients (P_{H_2O} and P_s), the thickness of the unstirred layers, and the diffusion of the solutes and water through the unstirred layers.

The influence of unstirred layers and P_{H_2O} on the relationship between σ' and P_s (membrane) are shown in Figs. 6 and 7 where we have assumed for simplicity that $D_1 = D_2$ and $\bar{V}_s = \bar{V}_{H_2O}$. In Fig. 6 all parameters except the thickness of the unstirred layers were kept constant, and it can be seen that the most striking deviation between the presence and absence of unstirred layers is that σ' becomes independent of P when the permeability coefficients are high; σ' becomes independent of P at lower values of P the thicker the unstirred layer. In Fig. 7 all parameters except P_{H_2O} were kept constant at an unstirred layer thickness of 500 μ. This figure shows that σ' levels out at different values depending on the permeability of the membrane to water. Thus the precise form of the relation between σ' and P depends critically on both the thickness of the unstirred layers and the water permeability. Consequently, extreme caution is required in the interpretation of reflection coefficients obtained from different membranes in the presence of unstirred layers.

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