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Concentration-Dependence of Nonelectrolyte Permeability of Toad Bladder

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Summary. A theoretical formulation was derived for the dependence of bulk solute permeability, P, defined as net flux ÷ concentration gradient, Δc, across any membrane in which solute concentration is controlling for net flux, $\overset{\Delta}{J}$. According to this formulation, $\overset{\Delta}{J}$ is stimulated by increments in trans concentration, c_2, in the range $c_2/c_1 = 0.0 - 0.1$. Net flux of urea across toad bladder down concentration gradients was shown to be stimulated threefold by small increments in trans urea concentration. The theory also predicts that, in the absence of concentration gradients, tracer permeability, P^*, defined as tracer flux ÷ tracer concentration, will be independent of c provided that $P = P^*$, but will diminish with increasing c if $P/P^* < 1$. P/P^* was not significantly different from unity for urea, and both P and P^* were independent of c in the absence of concentration gradients. However, P/P^* was significantly less than unity (0.90 and 0.85) for thiourea and mannitol, respectively. In conformity with theory, P^* (and also P) of these two solutes, measured as c was increased by 3–4 orders of magnitude, diminished progressively. These effects are more consistent with this formulation than with transport via a saturable carrier.

Membrane permeability to uncharged solutes, defined as net flux/concentration gradient, is often presumed to be independent of solute concentration, in the absence of specific carrier mechanisms or interaction between the solute and other solutes (or solvent) crossing the membrane. Diminution in permeability with increasing concentration is usually taken as evidence for a saturable carrier mechanism. However, physicochemical studies employing wide ranges of solute concentrations have often shown some diminution of diffusion coefficients with increas-

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ing concentration, whether measurements are based on transmembrane flux [8, 24] or "intra-diffusion" between labeled and unlabeled molecules in solution [1].

A related question concerns the validity of permeability measurements based on tracers, which may yield results differing from true or bulk permeability, as defined above, under a variety of conditions [7, 22, 23]. Yet the most readily performed measurement of membrane permeability consists of the observation of unidirectional tracer flux between two media containing equal concentrations of unlabeled solute.

In recent studies of electrolyte permeation of toad bladder, we have observed that the effects of gradients of electrical potential [4], concentration [5], or both [6] on unidirectional and net fluxes of ions conform closely to the predictions of a model based on a constant ratio of bulk permeability coefficient to tracer permeability coefficient. This ratio is the same as the ratio of bulk diffusion coefficient to "intradiffusion coefficient", as defined by Albright and Mills [1].

In the present work, this approach has been extended to the permeation of nonelectrolytes across toad bladder.

Materials and Methods

Bladder sacs of Dominican toads (*Bufo marinus*) were prepared and mounted as previously described [4–6].

Unless otherwise specified, hemibladders were first bathed on both sides with amphibian Ringer's solution (in mM: NaCl, 114; $NaHCO_3$, 3; $CaCl_2$, 2.7; $MgCl_2$, 2; KCl, 3.4) containing an appropriate quantity of a single nonelectrolyte to be tested. The initial mucosal bath volume was 20 ml and the initial serosal bath volume was 50 ml. After 60–120 min equilibration, bladders with spontaneous potentials of less than 60 mV were rejected.

Preliminary experiments showed that unidirectional fluxes of urea, mannitol, or thiourea were unaffected by hyperpolarizing potentials or by ouabain (1.83×10^{-3} M), as others have observed in frog skin [19]. The nonelectrolyte concentrations used were found to have little or no effect on short-circuit current. In all experiments, fluxes were measured at room temperature (22–25 °C) with potential clamped to 0 mV.

The experimental protocol consisted of an initial equilibration period of 1–2 hr, after which a nonelectrolyte (urea, thiourea, or mannitol) was added to one or both bathing media. Two types of experiments were performed. In the first type, net flux of solute was measured directly by maintaining constant specific activity on both sides of the membrane. ^{14}C-labeled solute was mixed with unlabeled solute in a concentrated solution, and a sufficient quantity of this mixture was added to one of the two media to achieve the desired concentration. A small portion of the same mixture was subsequently added to the opposite bath. Under these circumstances, radioactivity determinations can be equated with chemical determinations and isotope flux divided by specific activity is equivalent to net flux (neglecting chemical isotope effects) [22]. However, high precision in radioactivity determination is required to achieve useful data by this method. This was done by transferring four samples of 1 ml, obtained at 12–18 min intervals, into weighed,

capped scintillation vials, reweighing them after recapping, and then adding 15 ml PCS solution (Amersham Corp., Arlington Heights, Ill.). At least 10^6 total counts were accumulated for each vial, and three cycles of counting were performed for each experiment. When mucosal-to-serosal flux was measured in this way, it was sometimes necessary to correct for the concentrative effect of evaporation of the serosal bath, which amounted to about 0.6% per hr. In most cases, this correction was negligible. Evaporation of the mucosal bath could safely be neglected, since the surface exposed to the atmosphere was far smaller.

In the second type of experiment, unlabeled solute was added to both sides of the hemibladders (at equal or unequal concentrations), and ^{14}C-labeled solute was then added to one side only. Sampling from the opposite medium then yields unidirectional flux. In some cases, label was added to the mucosal bath of one paired hemibladder and to the serosal bath of the other paired hemibladder. After 10 min of equilibration, a single sample of the bath to which isotope was added and three or four (usually four) successive samples at 10–15 min intervals of the opposite bath were obtained. Additional nonelectrolyte was then added to one or both media, and after re-equilibration, sampling of both baths was again carried out. In some cases, a third addition of unlabeled nonelectrolyte was made and sampling repeated.

At the end of each experiment, the bladders were blotted to remove surface fluid and weighed. Mean weight was 185 mg. Since sac area was approximately 36 cm^2, 1 mg of wet wt corresponds to about 0.19 cm^2 of surface area. Nonelectrolyte flux and its standard error were calculated from the linear regression of bath radioactivity against time, using a computer program which takes into account the changes in bath volume with time. In the unidirectional flux experiments, no correction was necessary for backflux of isotope from the bath opposite to that to which it was added. Flux periods in which the standard error of the estimated flux was more than 20% of the flux were rejected.

Symbols

In addition to those defined below, other symbols used in the theoretical derivation are defined in the text or the *Appendix*.

c_m	Concentration of the test species in the mucosal bathing medium (M)
c_s	Concentration of the test species in the serosal bathing medium (M)
$\bar{c}$	Logarithmic mean of the concentrations in the two bathing media (M), defined as $(c_m - c_s)/\ln(c_m/c_s)$
J_{sm}	Serosal-to-mucosal unidirectional flux (mole/mg hr)
J_{ms}	Mucosal-to-serosal unidirectional flux (mole/mg hr)
$J_{\leftrightarrows}$	Unidirectional flux (mole/mg-hr) when $c_m = c_s$ and $\Delta p = 0$; $J_{\leftrightarrows} = J_{ms} = J_{sm}$
$\overset{\Delta}{J}$	Net flux (mole/mg hr) defined as $J_{sm} - J_{ms}$
P	Bulk permeability coefficient (mole/mg hr M)
P^*	Tracer permeability coefficient (mole/mg hr M)
Q	Ratio of P/P^*
Δp	External hydrostatic pressure gradient
Δp_{eq}	Hydrostatic pressure gradient at which $\overset{\Delta}{J} = 0$ when $c_m \neq c_s$
$J_{\leftrightarrows}^{eq}$	Unidirectional flux at $\Delta p = \Delta p_{eq}$ (mole/mg hr) when $c_m \neq c_s$
$\overset{\Delta}{J}_w$	Net flow of water (mole/mg hr)
L_p	Filtration coefficient or hydraulic conductivity of the membrane
σ	Reflection coefficient of the membrane for the solute
$\bar{v}$	Partial molar volume of the solute.

Theoretical

First, we consider a membrane separating two solutions containing different concentrations of a single nonelectrolyte. The set of equations so obtained is then used to derive an expression for analyzing tracer fluxes of a nonelectrolyte in the presence of a concentration gradient. We assume no solvent drag, no coupling between nonelectrolyte flux and flux of any other species, absence of unstirred layers adjacent to the membrane surface, and membrane symmetry. The validity of these assumptions is considered in the *Discussion.*

The flux of passively transported ions in the presence of gradients of ionic concentration, hydrostatic pressure, electrical potential, and electro-osmotic solvent drag can be described [20] by

$$\overset{\Delta}{J}_i^{\psi} = -U_i c_i [d(RT \ln c_i + \bar{v}_i p + z_i F \psi)/dx] + c_i u_o \tag{1}$$

where R, T, c_i, ψ, x, and $\overset{\Delta}{J}_i^{\psi}$ are as previously defined [4–6], U_i is ionic mobility, and u_o is the velocity of the solvent in a given frame of reference. Therefore, the net flux of a nonelectrolyte, $\overset{\Delta}{J}$, may be written in general as

$$\overset{\Delta}{J} = -U c' [d(RT \ln c' + \bar{v} p)/dx] + c' u_o \tag{2}$$

where U is its mobility, and the superscript prime is used to denote solute concentration within the membrane. Note that this equation is applicable only when solute concentration within the membrane is controlling for transmembrane flux.

In the absence of solvent flow, i.e., $u_o = 0$, Eq. (2) reduces to

$$\overset{\Delta}{J} = U c' [d(RT \ln c' + \bar{v} p)/dx]. \tag{3}$$

If we assume that dc'/dx is independent of x, then in the steady state, U must also be independent of x in order for $\overset{\Delta}{J}$ to have the same value along the gradient. Integrating over the thickness of the membrane, δ,

$$\overset{\Delta}{J} = P[\Delta c' + (\bar{c}' \bar{v}/RT) \Delta p], \tag{4}$$

where P is defined as

$$P = URT/\delta. \tag{5}$$

We postulate, as before [5, 6], that this integrated equation is valid even if the concentration gradient is not linear, and that concentrations in the bulk media bathing the membrane can be substituted for concentrations within the membrane in the integrated equation.

The defined volume flow, $\overset{\Delta}{J}_v$, for passive transport consisting of the flow of a single permeant nonelectrolyte, $\overset{\Delta}{J}$, and of water, $\overset{\Delta}{J}_w$, is described by the equation [10]

$$\overset{\Delta}{J}_v = \bar{v}\overset{\Delta}{J} + \bar{v}_w \overset{\Delta}{J}_w. \tag{6}$$

At equilibrium, $\overset{\Delta}{J} = 0$. Under this condition, from the equations given,

$$\Delta p_{eq} = -(RT/\bar{v}) \ln (c_s/c_m). \tag{7}$$

In the absence of Δp, the only driving force for the transport of the nonelectrolyte is its transepithelial concentration gradient. Therefore, its net flux becomes, according to Eq. (4),

$$\overset{\Delta}{J} = P\Delta c \tag{8}$$

which defines P.

The physical meaning of P in terms of phenomenological coefficients of interaction between nontracers, between tracers, and/or between tracers and nontracers has been described by Meares and Sutton [21].

Let $J_{\leftrightarrows}^{eq}$ be the unidirectional flux of the nonelectrolyte when $\overset{\Delta}{J} = 0$, according to Eq. (4). We then define P^* as

$$P^* = J_{\leftrightarrows}^{eq}/\bar{c}. \tag{9}$$

P^* is again referred to as the tracer permeability coefficient under these conditions, although the exact definition of P^* is still obscure. In what follows, we assume that $P/P^*(=Q)$ is independent of c.

By extension from the theoretical formulation of Meares and Sutton [21], we have shown [5, 6] mathematically and experimentally that in the presence of an electrochemical potential gradient of a solute, in general,

$$J_{\leftrightarrows}^{eq} = \overset{\Delta}{J}/\ln (J_{sm}/J_{ms}). \tag{10}$$

By mathematical techniques similar to those employed previously, Eq. (10) can also be derived for transport systems subjected to gradients

of solute concentration and pressure. From Eqs. (8), (9), and (10), it can be shown [5] that

$$\ln (J_{ms}/J_{sm}) = Q \ln (c_m/c_s). \tag{11}$$

It should be noted here that Eqs. (9)–(11) are not postulates, but are mathematical and physically defined expressions based on the stated assumptions. Moreover, for nonelectrolytes or ionic species, when c_s is fixed and c_m is varied, if Q is invariant, we can also derive the equation for $J_{\leftrightarrows}^{eq}$ without recourse to the assumptions made previously [5] (*see* Appendix *A*). The equilibrium nonelectrolyte flux, $J_{\leftrightarrows}^{eq}$, in terms of $J_{\leftrightarrows}$ observed when bath nonelectrolyte concentrations are identical and equal to c_s, is

$$J_{\leftrightarrows}^{eq} = J_{\leftrightarrows} (c_m/c_s)^{Q/2}. \tag{12}$$

The unidirectional nonelectrolyte fluxes, J_{sm} and J_{ms}, are given by combining Eqs. (10), (11), and (12)

$$J_{sm} = J_{\leftrightarrows} [n/(e^{n/2} - e^{-n/2})] \tag{13}$$

and

$$J_{ms} = J_{\leftrightarrows} [n e^{n}/(e^{n/2} - e^{-n/2})] \tag{14}$$

in which $n = Q \ln c_m/c_s$.

Similarly, when c_m is fixed and c_s varied,

$$J_{\leftrightarrows}^{eq} = J_{\leftrightarrows} (c_m/c_s)^{-Q/2}. \tag{15}$$

The unidirectional nonelectrolyte fluxes can be obtained by combining Eqs. (10), (11) and (15)

$$J_{sm} = J_{\leftrightarrows} [n e^{-n}/(e^{n/2} - e^{-n/2})] \tag{16}$$

and

$$J_{ms} = J_{\leftrightarrows} [n/(e^{n/2} - e^{-n/2})]. \tag{17}$$

In practice, it is important to derive a general expression that can be used to predict how $J_{\leftrightarrows}$ varies with changes in bath nonelectrolyte concentration when $c_m \neq c_s$. To this end, we start from the general equation (12) for $J_{\leftrightarrows}^{eq}$ when $c_s \neq c_m$ and write, by fixing $c_s = c_1$ and varying c_m,

$$J_{\leftrightarrows}^{eq} = J_{\leftrightarrows} (c_m/c_s)^{Q/2} = J_{\leftrightarrows} (c_m/c_1)^{Q/2} \tag{18}$$

in which $J_{\leftrightarrows}$ is the unidirectional flux of the nonelectrolyte when $c_m = c_s = c_1$. If $c_m = c_2$ when $c_2 \neq c_1$, Eq. (18) becomes, then,

$$ {}_1J_{\leftrightarrows}^{eq} = {}_1J_{\leftrightarrows}\,(c_2/c_1)^{Q/2}. \tag{19} $$

Similarly, we can also write Eq. (12) by fixing $c_s = c_2$ and varying c_m, as

$$ {}_2J_{\leftrightarrows}^{eq} = {}_2J_{\leftrightarrows}\,(c_m/c_2)^{Q/2} \tag{20} $$

in which ${}_2J_{\leftrightarrows}$ is the unidirectional flux of the nonelectrolyte when $c_m = c_s = c_2$. Now, replacing c_m by c_1 with $c_1 \neq c_2$, Eq. (20) becomes

$$ {}_2J_{\leftrightarrows}^{eq} = {}_2J_{\leftrightarrows}\,(c_1/c_2)^{Q/2}. \tag{21} $$

Clearly, under the conditions as described above, ${}_1J_{\leftrightarrows}^{eq} = {}_2J_{\leftrightarrows}^{eq}$, and ${}_1J_{\leftrightarrows} \neq {}_2J_{\leftrightarrows}$. Hence, from Eqs. (19) and (21), we obtain, by appropriate arranging,

$$ {}_2J_{\leftrightarrows} / {}_1J_{\leftrightarrows} = (c_2/c_1)^Q \tag{22} $$

in the absence of a solute concentration gradient. Thus, one of the goals of the present study is to determine whether Q is invariant with changes in bath solute concentrations.

Equation (22), if proved valid, can be used to estimate Q. If Q and unidirectional flux at any value of c can be measured, then unidirectional flux at any other value of c can be calculated. Furthermore, by combining Eqs. (10) and (22), we obtain an expression for the concentration dependence of P and P^*,

$$ \log(P_2/P_1) = \log(P_2^*/P_1^*) = (Q-1)\log(c_2/c_1) \tag{23} $$

when $c_m = c_s$. Also, P^* or P as a function of c_m, when c_s is fixed, can be derived by combining Eqs. (8), (10), (11), and (12),

$$ P = J_{\leftrightarrows}\,(c_s - c_m)^{-1}\,Q(c_m/c_s)^{Q/2}\ln(c_s/c_m) \tag{24} $$

and when c_m is fixed,

$$ P = J_{\leftrightarrows}\,(c_m - c_s)^{-1}\,Q(c_s/c_m)^{Q/2}\ln(c_m/c_s). \tag{25} $$

The limit of applicability of these assumptions as $|\ln(c_s/c_m)|$ increases is unknown. Clearly, the assumption that Eq. (4) is valid when concentrations in the bathing media are substituted for concentrations within the membrane becomes more questionable as either concentration approaches zero.

An interpretation of the parameter Q in terms of irreversible thermodynamic theory is given in Appendix *B*.

Results

Absence of Net Flux of Nonelectrolytes in the Absence of Concentration Gradients

As shown in Group I, Period II in Table 2 and in Groups I and II, Period I in Table 4, observed mucosal-to-serosal fluxes of urea and mannitol were not significantly different from the observed serosal-to-mucosal fluxes of these solutes in the absence of a concentration gradient. This suggests that the transport of these solutes was passive. Tables 2–4 also show that both thiourea and mannitol are less permeant than urea across toad bladder, as reported by Bindslev and Wright [2] and Lichtenstein and Leaf [17].

Concentration-Dependence of Solute Permeability in the Presence of Concentration Gradients

Solutions to Eq. (24), normalized by letting $c_s = 1$ and varying c_m from 1 to 0, are shown in Figs. 1 and 2. When $Q = 1$, predicted permeability

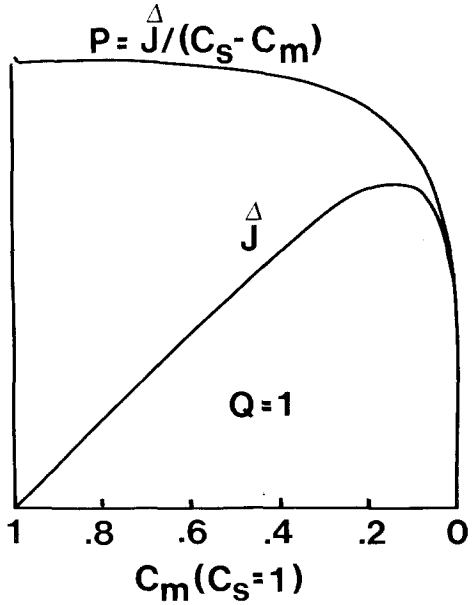

Fig. 1. Predicted variations [from Eq. (24)] in permeability, P, and net flux, J, as a function of concentration gradient. Serosal concentration, c_s, has been fixed at unity. The horizontal axis shows mucosal concentration, c_m. The vertical scales are in arbitrary units. The ratio, Q, of bulk permeability to tracer permeability has been taken as unity

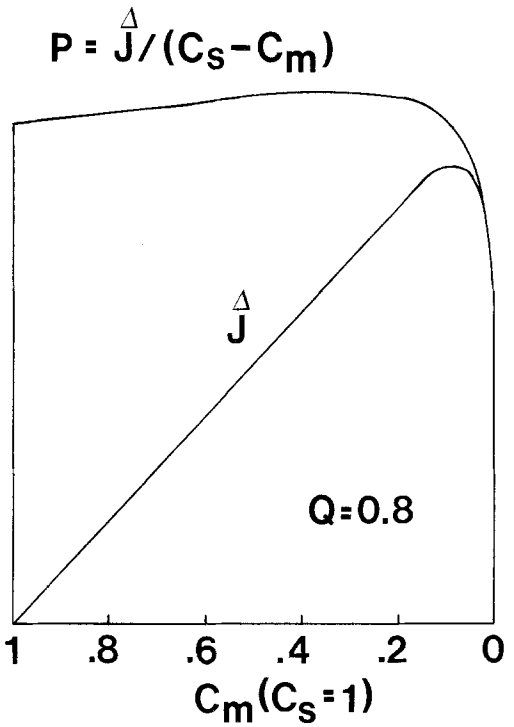

Fig. 2. The same relationships as shown in Fig. 1, recalculated for $Q=0.8$

falls only slightly as c_m approaches zero, until it becomes about 0.2. Thereafter, permeability falls progressively more rapidly, becoming zero when c_m is identically zero. Although this result at first seems absurd, it is obviously true that permeability must indeed be zero if there is no solute on the trans side of the membrane. As soon as any solute crosses, c_m is of course no longer zero. The steepness of the curve near $c_m=0$ means that even slight changes in solute concentration beyond the limiting barrier, such as would be expected from unstirred layers, could have profound effects on Q, according to this formulation.

When $Q<1$, the curve has a somewhat different shape (Fig. 2). Permeability at first rises somewhat as c_m is lowered. It passes through a maximum, and then falls, more steeply than when $Q=1$.

Using uniform specific activity urea or mannitol, net transepithelial flux was measured at two or three different concentrations in the range of c_m/c_s or $c_s/c_m \leqq 0.1$. The results are summarized in Table 1, and a representative experiment is shown in Fig. 3.

Table 1. Effect of increments in trans nonelectrolyte concentration on net cis-trans nonelectrolyte flux

Expt. and (n)	Period	c_s (mM)	c_m (mM)	$\overset{\Delta}{J}$ (nmol/mg · hr)	P (μmol/mg · hr · M)
Urea Flux					
I	1	4[a]	0.04[a]	0.70 ±0.12	0.177 ±0.024
(11)	2	4[a]	0.38[a]	1.13 ±0.11	0.312 ±0.032
II	1	0.001[b]	4[a]	0.36 ±0.06	0.090 ±0.017
(7)	2	0.04[a]	4[a]	0.58 ±0.07	0.147 ±0.019
	3	0.21[a]	4[a]	0.77 ±0.11	0.206 ±0.030
Mannitol Flux					
III	1	5[a]	0.05[a]	0.121 ±0.010	0.024 ±0.002
	2	5[a]	0.47[a]	0.160 ±0.015	0.035 ±0.002

[a] Quantity added.

[b] Determined from mean measured radioactivity of serosal bath and specific activity of added solute.

Increments in trans concentration were invariably associated with increments in net flux, $\overset{\Delta}{J}$, and calculated permeability. The design of these experiments made impossible the determination of P^* with $c_m = c_s$ in each experiment. Furthermore, the degree of uncertainty as to the true value of trans concentration at the limiting barrier becomes greater as trans concentration approaches zero. Hence it is difficult to compare these results quantitatively with the predictions of Eq. (24). Nevertheless, it is clear that a substantial increase in permeability, at least for urea, is induced by small increments in c_m/c_s or c_s/c_m.

Concentration-Dependence of Nonelectrolyte Permeability in the Absence of Concentration Gradients

Urea permeability was the same, within experimental error, between 0.2 and 20 mM (Table 2). Q for urea was found to be insignificantly different from unity. Thus, no change in permeability with concentration would be predicted [Eq. (23)], within this range of concentration. In contrast, thiourea permeability (Table 3) and mannitol permeability (Table 4) fell progressively as concentration increased from 0.005 to 5 mM (Fig. 4). This change was qualitatively distinct from the fall in permeability to be expected on the basis of saturation kinetics. As shown in

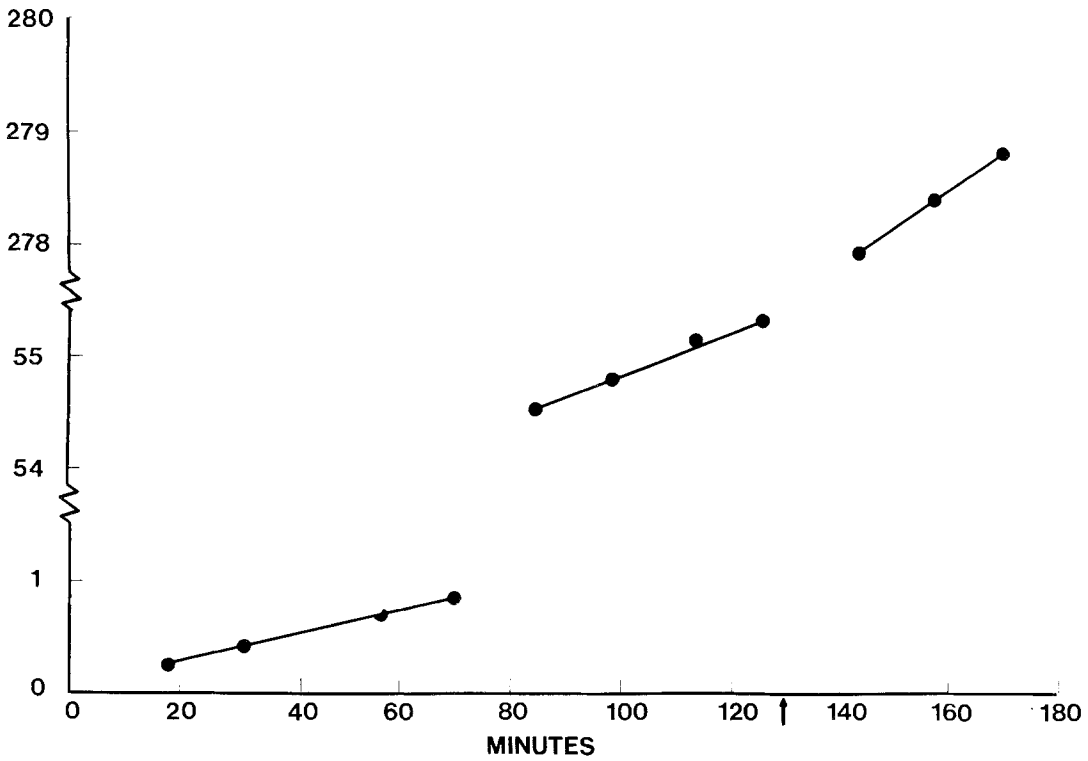

Fig. 3. Results of a representative experiment in which uniform specific activity urea was added first to the mucosal bath at 4 mM, and then, at the times indicated by the arrows, to the serosal bath in small amounts. The points represent measured radioactivity of the serosal bath, divided by specific activity and hence expressed in units of concentration. The broken vertical scale is uniform. Note that the slope of increases in serosal urea concentration, which is a direct measure of net urea flux under these conditions, increases with each successive addition of urea to the serosal bath

the figure, nonelectrolyte penetration following Michaelis-Menten kinetics should yield a curve relating permeability to log concentration with a sigmoidal shape, quite distinct from the shape of a curve drawn through the experimentally derived points. The figure shows theoretical curves for both thiourea and mannitol permeability as functions of log concentration obtained from Eq. (23), using the average estimates of Q for these two solutes (*see* below) and the observed permeability at the lowest concentration studied. The correspondence between the observed fall in permeability and the change predicted from this equation is quite close.

This correspondence can also be expressed by comparing the two values for Q obtained from two successive tenfold increases in thiourea

Table 2. Unidirectional fluxes of labeled urea across toad bladder in the presence and absence of urea concentration gradients

Group	Period	C_m	C_s	J_{sm}	J_{ms}	Q	P^*	P
		(mM)		(nmol/mg · hr)			(μmol/mg · hr · M)	
I	1	8	20	6.65	2.63	1.00[a]	0.36[f]	0.38[e]
($n=7$)				±3.44	±1.18	±0.06	±0.18	±0.21
	2	20	20	7.23	7.98	1.09[b]	0.35[f]	0.38[g]
				±3.79	±3.96	±0.08	±0.16	±0.17
II	1	4	20		1.33		0.35[f]	0.36[g]
($n=6$)					±0.68		±0.17	±0.17
	2	20	20		7.92	1.00[b]	0.39[h]	0.39[i]
					±3.82	±0.07	±0.17	±0.17
III	1	4	4		1.45		0.36[i]	0.36[i]
($n=7$)					±0.15		±0.04	±0.04
	2	8	8		2.92	1.02[c]	0.37[h]	0.37[i]
					±0.29	±0.04	±0.04	±0.04
IV	1	0.2	0.2		0.073		0.36[h]	0.35[i]
($n=3$)					±0.016		±0.08	±0.07
	2	2.0	2.0		0.626	0.93[c]	0.31[h]	0.31[i]
					±0.143	±0.01	±0.07	±0.07
	3	20	20		6.31	1.01[d]	0.32[h]	0.31[i]
					±1.23	±0.02	±0.06	±0.06

[a] Calculated from Eq. (8) using data from 6 paired hemibladders.
[b] Calculated from Eq. (14) in individual hemibladders.
[c] Calculated from Eq. (19) in individual hemibladders using data from periods *1* and *2*.
[d] Calculated from Eq. (19) in individual hemibladders using data from periods *2* and *3*.
[e] Calculated from Eq. (8) using 6 paired hemibladders.
[f] Calculated as P/Q.
[g] Calculated from Eq. (24) in individual hemibladders using data from periods *1* and *2*.
[h] Calculated as $J_{\rightleftarrows}/C$.
[i] Calculated as P^*/Q.

concentration (Experiment I, Table 3). The mean ratio of the two values of Q so obtained was 1.02 ± 0.01 (SEM, $n=6$).

Comparison of Tracer and Bulk Permeability Coefficients

As noted above, P_{urea} was not significantly different from P^*_{urea}. However, the ratio P/P^* for both thiourea (0.90, *see* Table 3) and mannitol (0.85; Table 4) was significantly less than unity ($p<0.001$).

Table 3. Unidirectional flux of thiourea across toad bladder at varying concentrations

Group	Period	C_m	C_s	J_{ms}	Q	P^*	P
		(mM)		(pmol/mg · hr)		(μmol/mg · hr · M)	
I	1	0.005	0.005	0.45 ±0.2		0.090[c] ±0.005	0.081[d] ±0.003
	2	0.05	0.05	3.60 ±0.12	0.90[a] ±0.02	0.072[c] ±0.002	0.064[d] ±0.003
	3	0.5	0.5	27.6 ±1.8	0.88[b] ±0.02	0.055[c] ±0.004	0.049[d] ±0.004
II	1	0.5	0.5	25.8 ±1.7			
	2	5.0	5.0	208 ±17	0.90[a] ±0.02	0.042[c] ±0.003	0.038[d] ±0.004

[a] Calculated from Eq. (19) using periods *1* and *2*.
[b] Calculated from Eq. (19) using periods *2* and *3*.
[c] Calculated as $J_{\rightleftarrows}/C$.
[d] Calculated as P^*Q.

Table 4. Unidirectional fluxes of labeled mannitol across toad bladder in the presence and absence of mannitol concentration gradients

Group	Period	C_m	C_s	J_{ms}	J_{sm}	Q[a]	P^*	P
		(mM)		(pmol/mg · hr)			(μmol/mg · hr · M)	
I ($n=5$)	1	0.05	0.05	3.12 ± 0.32			0.062[b] ±0.007	0.053[d] ±0.005
	2	5.0	0.05	97.7 ± 10.5			0.021[c] ±0.002	0.018[c] ±0.002
	3	5.0	5.0	162.2 ± 15.2		0.86 ±0.02	0.033[b] ±0.003	0.028[d] ±0.003
II ($n=5$)	1	0.05	0.05		3.4 ± 0.5		0.067[b] ±0.010	0.055[d] ±0.007
	2	0.05	5.0		101.7 ± 14.6		0.022[c] ±0.003	0.018[c] ±0.002
	3	5.0	5.0		164.1 ± 24.0	0.84 ±0.01	0.033[b] ±0.005	0.028[d] ±0.004
III ($n=6$)	1	0.05	0.05	2.85 ± 0.29			0.057[b] ±0.006	0.048[d] ±0.005
	2	0.50	0.50	19.57 ± 2.26		0.84 ±0.03	0.039[b] ±0.004	0.033[d] ±0.004

[a] Calculated from Eq. (19).
[b] Calculated from Eq. (9).
[c] Calculated from $J_{\rightleftarrows}$ observed at $C_m = C_s$ and Q estimated in the same experiment, using Eq. (6) and $P^* = P/Q$.
[d] Calculated as QP^*.

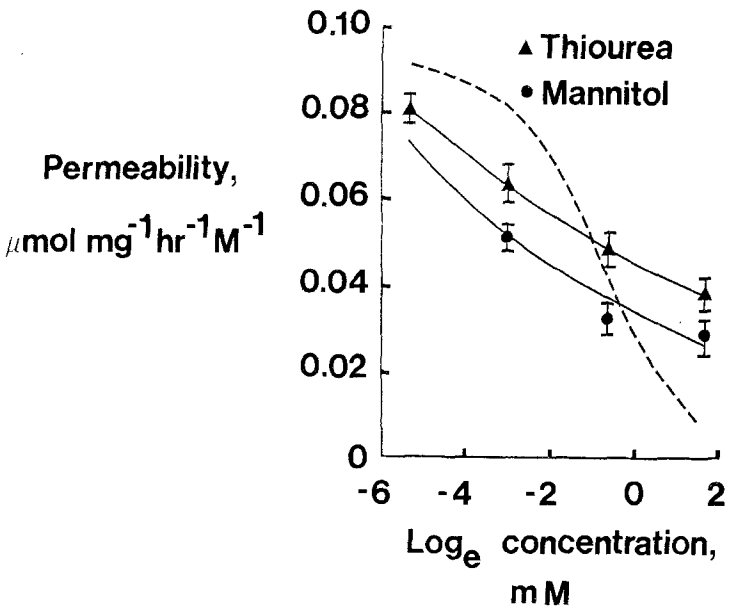

Fig. 4. Permeability of toad bladder to mannitol and thiourea as a function of ln concentration, in the absence of concentration gradients. The solid curves are calculated from Eq. (23), using average estimates of Q for these solutes and observed permeability at the lowest concentration studied. The points are mean values observed ± 1 SEM. The dotted line shows the shape of a representative curve relating permeability to concentration predicted by saturation kinetics, using in this case $k_m = 0.45$ mM and $J_{max} = 408$ pmol/mg · hr

Unidirectional and Net Solute Fluxes Across Concentration Gradients

Bidirectional urea fluxes were measured with $c_s = 20$ mM and $c_m = 8$ mM (Table 2). In six paired hemibladders, net flux, $\overset{\Delta}{J}$, could therefore be estimated, and permeability, P, estimated as $\overset{\Delta}{J}/(c_s - c_m)$. The resulting value was not significantly different from P estimated from unidirectional flux and estimated values of Q.

Unidirectional fluxes of urea and mannitol across concentration gradients corresponded closely to those predicted by the above equations. For example, mannitol flux in Group I, Period 2 (Table 4) predicted from the data obtained in Periods 1 and 3, using Eq. (14), was 93.3 ± 10.3 pmol/mg · hr, compared with an observed value of 97.7 ± 10.5 pmol/mg · hr. Similarly, in Group II, Period 2, predicted flux, using Eq. (16), was 93.4 ± 13.2 pmol/mg · hr, compared with an observed value of 101.7 ± 14.6 pmol/mg · hr.

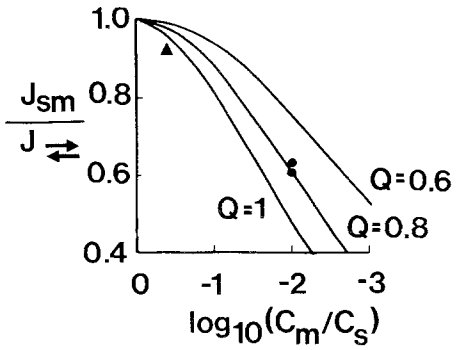

Fig. 5. Trans-stimulation of serosal-to-mucosal nonelectrolyte flux, J_{sm}, as a function of mucosal concentration with serosal concentration fixed. The curves shown are Eq. (13) with three different values of Q, as shown. The triangle is urea flux from Group I, Table 2, and the dots are mannitol fluxes from Groups I and II, Table 4. J_{sm} is shown as a fraction of $J_{\leftrightarrows}$, the bidirectional flux observed in the absence of a concentration gradient

Stimulation of unidirectional flux by increasing trans concentration is predicted by these equations, except for very small values of Q, and is demonstrated by Table 4, Experiments I and II. The magnitude of the changes observed corresponded closely to predictions from Eqs. (13) and (14) or (16) and (17) (Fig. 5).

These equations also predict that, starting with equal bath concentrations, a substantial increase in concentration of one side will lower permeability, but a subsequent equal increase on the other side, thus eliminating the concentration gradient, will raise permeability again. This is shown to be the case in Table 4, Groups I and II, in which mannitol concentration was increased 100-fold first on one side and then the other, the sequence being reversed in Group II as compared to Group I. The permeability changes were almost identical in the two groups: 0.053, 0.018 and 0.028 μmol/mg · hr · M in Group I and 0.055, 0.018 and 0.028 μmol/mg · hr · M in Group II.

Discussion

The experimental data obtained on nonelectrolyte permeation of toad bladder in this study correspond closely to the predictions of the theoretical formulation. The major postulates of this formulation are (i) that the ratio of bulk permeability to tracer permeability is concentration-independent for each solute and (ii) that the Nernst-Planck

equation, integrated on the assumption of a linear concentration gradient within the tissue, using concentrations in bathing media instead of concentrations within the membrane, is applicable. The results support the first postulate, in that calculated values of Q are nearly constant for each of the solutes studied. The second postulate has not been rigorously tested, but the data are consistent with this formulation within the range of concentrations examined.

The other three assumptions made in the derivation are supported by direct experimental evidence. Thus, solvent drag can be neglected because water flux across toad bladder in the absence of antidiuretic hormone is very low [9] and only small osmotic gradients were imposed in these experiments. The absence of change in nonelectrolyte fluxes with short-circuiting, hyperpolarization, or ouabain is strong evidence against coupling between ion fluxes and the fluxes of these nonelectrolytes. The data presented in the tables give no evidence of membrane asymmetry.

Several of the equations yield relationships which are not intuitively expected. The most important is Eq. (23), which predicts a continuous variation of permeability with concentration whenever $Q<1$. Clearly, the form of this variation is very different from saturation kinetics, and its explanation cannot lie in the existence of a mobile carrier. On the other hand, exchange diffusion proportional to concentration could produce values of $Q<1$. In our first study [4], exchange diffusion was shown to be an unlikely explanation for the low values of Q seen for ion permeation through the passive path, on the basis of fluxes measured at various clamping potentials. For nonelectrolytes, the force analogous to potential is hydrostatic pressure, which cannot be readily varied experimentally. Hence, we cannot exclude exchange diffusion as an explanation for Q values less than unity, provided it is proportional to concentration. Other conceivable explanations for the low value of Q observed here include heterogeneous parallel pathways [16], series barriers, and unstirred layers [8].

Bresler, Mason and Wendt [3] have criticized the use of logarithmic mean concentration in kinetic equations for nonelectrolyte flux. However, the use of arithmetic mean concentration in their derivation is based in part on their assumption that the diffusion coefficient is independent of concentration, which is clearly not the case in toad bladder, as shown here. The use of an arithmetic mean in the present data leads to high (>1) and variable values for Q.

A second major implication of this formulation concerns the relationship between net flux and trans concentration when cis con-

centration is fixed. Intuitively, one would expect an inverse linear relationship, since, at constant P, net flux is proportional to Δc. But the change in P predicted by Eqs. (24) and (25) as concentration is reduced on one side of the membrane leads to the prediction that net flux would reach a maximum at a concentration ratio of 10 to 50 (depending on Q) and then fall as trans concentration is further reduced. This has been partially verified in the present work.

A third point of interest is the trans stimulation predicted by the unidirectional flux equations (14) and (16). This is illustrated in Fig. 2, in which J_{sm}, expressed as a fraction of the value observed when $c_s = c_m$, is plotted as a function of c_m/c_s for diminishing values of c_m. Only three groups of experiments have been performed to validate this relationship, but the correspondence between theory and data is close. Again, the limit of c_m beyond which this relationship breaks down is not known. The important point is that trans stimulation need not imply a carrier mechanism, as we have previously noted for ion permeation [5, 6].

The most important practical implication of this work is that P and Q can be evaluated by measuring tracer flux in one direction in the absence of a chemical concentration gradient, at two different concentrations. With these values it should be possible to predict P at any pair of bath concentrations as well as net flux and both unidirectional fluxes, within the limit of applicability of these equations. Clearly, these equations do not apply (i) in the presence of solvent drag or (ii) whenever solute concentration within the membrane is not controlling for transmembrane flux, as, for example, in a porous barrier.

Wright and Pietras [25] have concluded, from a study of nonelectrolyte permeation of toad urinary bladder, frog choroid plexus, and rabbit gallbladder, that small polar molecules such as urea cross the epithelia by passing through the cell membrane rather than through pores or via membrane carriers. While we have not established that permeation through pores would exhibit a different form of concentration-dependence than that found in our experiments, the fundamental assumption underlying the theoretical derivation would be inconsistent with passage through aqueous channels. In addition, indirect evidence has been presented here against the saturable carrier mechanism. Hence, our results support the inference drawn by Wright and Pietras [25].

Levine and Worthington [15] have observed a reduction in labeled urea flux following the addition of 150 mM urea to both sides of the bladder, and Levine, Franki and Hays [14] have made analogous observations using acetamide. Their findings with regard to acetamide

are consistent with our formulation if Q for acetamide is approximately 0.89. Their findings with respect to urea differ from ours, but could be explained by the use of a far higher urea concentration than we have employed, or by a value of Q for urea slightly less than 1. On the other hand, Lief and Essig [18] have found no effect of 100 mM urea on isotopic urea permeability of toad bladder; 300 mM urea induced coupling of tracer and abundant urea fluxes which was attributed to the associated hypertonicity.

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Appendix A

The mathematical derivation of Eq. (12) can be carried out as follows:

To begin with, we express, mathematically, the equilibrium unidirectional flux of the solute, $J_{\leftrightarrows}^{eq}$, when $c_m \neq c_s$, as a function of mucosal solute concentration, c_m, in terms of the equilibrium unidirectional flux of the solute, $J_{\leftrightarrows}$, when $c_m = c_s$, and the fixed serosal solute concentration, by the method of Taylor's expansion, i.e.,

$$J_{\leftrightarrows}^{eq}/J_{\leftrightarrows} = 1 + \sum_{k=1}^{j} a_k (c_m - c_s)^k \tag{A1}$$

where $a_k's$ are the coefficients to be determined. Note that according to Eq. (A1), when $c_m = c_s$, $J_{\leftrightarrows}^{eq} = J_{\leftrightarrows}$, *a priori* requisite. We can now write Eq. (A1), for fixed c_s, as

$$J_{\leftrightarrows}^{eq}/J_{\leftrightarrows} = 1 + \sum_{k=1}^{j} \xi_k [(c_m - c_s)/c_s]^k \tag{A2}$$

in which $\xi_k = a_k c_s^k (k = 1, 2, \ldots, j)$.

In practice, however, Eq. (A2) can be further transformed into a binomial series as shown below,

$$J_{\leftrightarrows}^{eq}/J_{\leftrightarrows} = 1 + \underset{1}{\xi}_1 [(c_m - c_s)/c_s] + \underset{2}{\xi}_1 [(c_m - c_s)/c_s]^2 + \underset{3}{\xi}_1 [(c_m - c_s)/c_s]^3 + \ldots \tag{A3}$$

where $\underset{k}{\xi}_1 s$ are the binomial coefficients and where we have expressed the other coefficients $\xi_k (k = 2, 3, \ldots 1)$ in terms of ξ_1, i.e., $\xi_k = \underset{k}{\xi}_1 = a_k c_s^k$.

Hence, Eq. (A3) can be simply written as

$$J_{\leftrightarrows}^{eq} = [1 + (c_m - c_s)/c_s]^{\xi_1} \tag{A4}$$

from which we immediately obtain

$$J_{\leftrightarrows}^{eq}/J_{\leftrightarrows} = (c_m/c_s)^{\xi_1}. \tag{A5}$$

Hence, our next goal is to evaluate the parameter, ξ_1.

Returning to the general equations, (10) and (11), there follows by appropriate rearranging

$$J_{\leftrightarrows}^{eq} = J_{sm}(e^n - 1)/n \tag{A6}$$

where $n = Q \ln (c_m/c_s)$, as previously defined. By comparing Eq. (A5) with Eq. (A6), we have

$$J_{\leftrightarrows} (c_m/c_s)^{\xi_1} = J_{sm}(e^n - 1)/n \tag{A7}$$

from which

$$\xi_1/Q = \ln [J_{sm}(e^n - 1)/nJ_{\leftrightarrows}]/n. \tag{A8}$$

Since ξ_1 is a constant, it can be evaluated at $c_m = c_s$. However, when $c_m = c_s$, the right-hand side of Eq. (A8) is indeterminate. Thus, we use L'Hospital's rule and differentiate both numerator and denominator with respect to n. Since $J_{sm} = J_{ms}$ when $c_m = c_s$, there follows, from Eq. (A8),

$$\xi_1/Q = \lim_{n \to 0} [\ln \{e^n - 1)/n\}/n = \lim_{n \to 0} (ne^n - e^n + 1)/(e^n - 1)n]. \tag{A9}$$

Since the right-hand side of Eq. (A9) is still indeterminate, we repeat the same procedure as described above. There follows

$$\xi_1/Q = \lim_{n \to 0} [ne^n/(ne^n + e^n - 1)] \tag{A10}$$

which, by repeating the same procedure, finally leads to

$$\xi_1/Q = \lim_{n \to 0} [(ne^n + e^n)/(ne^n + e^n + e^n)] = \lim_{n \to 0} [e^n/2e^n] = 1/2 \tag{A11}$$

from which ξ_1 is shown to depend on Q, $\xi_1 = Q/2$. (A12)

Substitution of Eq. (A12) into Eq. (A5) yields

$$J_{\leftrightarrows}^{eq} = J_{\leftrightarrows} (c_m/c_s)^{Q/2} = J_{\leftrightarrows} e^{n/2}. \tag{A13}$$

As we can see in the derivation of Eq. (12), no other assumptions were made except that Q is invariant with changes in c_m.

Appendix B

According to Kedem and Katchalsky [11, 12], the phenomenological relations for the total volume flow and for the exchange flow of a permeant nonelectrolyte are derived by

$$\overset{\Delta}{J}_v = L_p \Delta p + L_{pD} RT \Delta c = L_p(\Delta p - \sigma RT \Delta c) \tag{B1}$$

and

$$\overset{\Delta}{J}_D = L_D RT \Delta c + L_{pD} \Delta p \tag{B2}$$

where σ is defined as $\sigma = -L_{pD}/L_p$ and where the exchange flow, $\overset{\Delta}{J}_D$, also corresponds to the velocity of the solute relative to water in the membrane [13],

$$\overset{\Delta}{J}_D = \overset{\Delta}{J}/\bar{c} - \overset{\Delta}{J}_w/\bar{c}_w \tag{B3}$$

where $\bar{c}_w$ is the average concentration of water in the membrane. By rearranging, Eq. (B3) becomes

$$\overset{\Delta}{J} = \bar{c}\overset{\Delta}{J}_D + (\bar{c}/\bar{c}_w)\overset{\Delta}{J}_w. \tag{B4}$$

In the absence of solvent drag, $\overset{\Delta}{J}_w = 0$, and Eq. (B4) becomes, by the use of Eq. (B2),

$$\overset{\Delta}{J} = \bar{c}\overset{\Delta}{J}_D = \bar{c} L_{pD} \Delta p + \bar{c} L_D RT \Delta c \tag{B5}$$

which can be further rearranged to give

$$\overset{\Delta}{J} = P[\Delta c + (L_{pD}/RTL_D)\Delta P] \tag{B6}$$

in which L_D is related to the bulk permeability coefficient by

$$L_D = P/\bar{c}RT. \tag{B7}$$

By comparing Eqs. (B6) and Eq. (4), we immediately obtain, by the use of Eq. (B7),

$$L_{pD} = P\bar{v}/RT. \tag{B8}$$

Introducing $P^* = P/Q$ into Eq. (9) and eliminating P with the aid of Eq. (B8), there follows

$$Q = RTL_{pD}^{-}/J_{\leftrightarrows}^{eq}\bar{v} = -RT\sigma L_p\bar{c}/J_{\leftrightarrows}^{eq}\bar{v} \tag{B9}$$

where we have used $L_{pD} = -\sigma L_p$.

As we see from Eq. (B9), both σ and L_p are membrane properties which are functions of bath nonelectrolyte concentrations [8]. We have previously shown [5] that for ionic species, Q_i is given by

$$Q_i = RTg_i/z^2F^2J_{\leftrightarrows}^{eq} \tag{B10}$$

where g_i is the partial ionic conductance. Clearly, by comparison of Eq. (B9) with Eq. (B10), the membrane properties σ and L_p would give rise to the phenomena of nonelectrolyte flows different from those of ionic flows induced by g_i.

It should be remarked here that in the present theoretical treatment, we deal only with tracer diffusion of an uncharged solute under the influence of the concentration gradient of a single nonelectrolyte of interest. For better understanding of the detailed mechanistic interpretation of this process concerning interactions between abundant species and between tracer and nontracer species, please refer to work by Meares and Sutton [21].

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