

ORIGINAL ARTICLE

# The Reliability of Relative Anion–Cation Permeabilities Deduced From Reversal (Dilution) Potential Measurements in Ion Channel Studies

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## Abstract

Measurements of anion–cation permeability ratios (e.g.,  $P_{Cl}/P_{Na}$ ) are most readily made by measuring changes in zero-current reversal potential when the salt concentration on one side of the membrane (e.g., external NaCl) is decreased. This is particularly useful for measuring changes in ion selectivity in wild-type and mutant channels, such as those of the ligand-gated ion channel superfamily, and has shown that many of these channels have a significant permeability to counter-ions. One Brownian dynamics study of ion permeation through such narrow ion channels failed to observe such counter-ion movement, although later, another Brownian dynamics study did observe counter-ion movement through simulations of the same channels. The question has been raised as to the reliability of such reversal potential measurements for determining permeability ratios, particularly given the use of an equation such as the Goldman–Hodgkin–Katz (GHK) equation, which is often used to calculate such ratios. A new derivation of the GHK equation in terms of activity coefficients is also included. The application of irreversible thermodynamics will be shown to qualitatively support the reliability of such experimental anion–cation permeability values derived from reversal potential measurements. It will then be shown that for such zero-current situations, different electrodiffusion models, with very different underlying assumptions, produce almost identical relative permeabilities (and reversal potentials). Finally, the results of the two Brownian dynamics simulation studies and the relationship between reversal potentials and relative permeability will be discussed.

**Index Entries:** Reversal potentials; anion–cation permeability ratios; counter-ion permeation; Goldman–Hodgkin–Katz equation; Planck equation; irreversible thermodynamics; Brownian dynamics; ligand-gated ion channels.

## INTRODUCTION

The ion selectivity of ion channels, and particularly their relative anion–cation permeability, plays a critical role in their physiological function. This can be clearly demonstrated in the case of ligand-gated ion channels (LGICs), where cation-selective channels like the acetylcholine receptor channel and the 5-hydroxytryptamine type 3 receptor (5-HT<sub>3</sub>R) channels are normally excitatory, and anion-selective channels such as the  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub>R) and the glycine receptor (GlyR) channels are normally inhibitory.

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Structure–function experiments, using site-directed mutagenesis, have sought to investigate the molecular and biophysical mechanisms underlying ion selectivity and permeation in these and other channels by investigating the effects of mutations on anion–cation permeability ratios (typically  $P_{Cl}/P_{Na}$ ). The most unequivocal and sensitive way of determining such permeability ratios is to measure changes in the zero-current reversal potential as the solution on one side of the membrane (e.g., for a whole cell configuration or an outside-out membrane patch, the external solution) is diluted. The Goldman equation (1), expressed in the form of the Goldman–Hodgkin–Katz equation (GHK equation) to give the zero current potential across a membrane by Hodgkin and Katz (2), is typically used to determine

the relative permeability ratios from the shifts in the reversal potentials in the diluted solutions. This approach has been used in a number of situations to determine relative anion–cation permeabilities of various channels. For example, Blatz and Magleby (3) showed from dilution potential measurements that Cl<sup>-</sup>-selective channels in cultured rat skeletal muscle had a relative permeability to K<sup>+</sup>,  $P_K/P_{Cl}$ , of 0.15–0.2; Franciolini and Nonner (4) showed that a rat neuronal hippocampal Cl<sup>-</sup>-selective channel had a relative permeability to Na<sup>+</sup>,  $P_{Na}/P_{Cl}$ , of about 0.2. In recent site-directed mutagenesis work on the selectivity switching of LGICs, the native channels are generally found to be very selective between anions and cations (e.g., GABA<sub>A</sub> and glycine receptor channels have  $P_K/P_{Cl}$  values < 0.05; Bormann et al. [5]), whereas the mutant channels are often much less selective. For example, dilution potential measurements indicated that the wild-type (WT)  $\alpha 1$  GlyR had a  $P_{Cl}/P_{Na}$  value of about 25, whereas the cation-selective triple mutant value was 0.27 (6) and the single cation-selective A-1'E mutant GlyR resulted in a  $P_{Cl}/P_{Na}$  value of 0.34 (7). Similarly, the WT cation-selective 5-HT<sub>3A</sub> receptor had a  $P_{Cl}/P_{Na}$  value of about 25 and for its anion-selective triple mutant 5-HT<sub>3AR</sub> a value of 0.27 (8) with an E-1'A mutation producing a nonselective  $P_{Cl}/P_{Na}$  value of 1.12 (9). Carland et al. (10) also showed that the  $\rho 1$  GABA<sub>C</sub>R channel permeability ratios went from an anion-selective WT  $P_{Cl}/P_{Na}$  value of about 7 to a value of 0.08 for its P2'E cation-selective mutant channel.

Recently, ion permeation through WT and cation-selective mutant  $\alpha 1$  GlyR channels has been simulated using Brownian dynamics (BD) to endeavor to investigate the physical mechanisms underlying ion permeation through these channels. The first of these studies was by O'Mara et al. (11), who showed that BD simulations with models of both GlyR channels could give reasonably good fits to the experimental current-voltage curves obtained by Keramidas et al. (6,7). From these simulations, relative anion–cation permeability ratios were calculated with the GHK equation (see Analytical Techniques section), from reversal potential values determined for the channels under conditions of salt dilution. For the simulated WT GlyR channel,  $P_{Cl}/P_{Na}$  was  $34 \pm 1$  and for the (A-1'R) cation-selective mutant GlyR channel  $P_{Cl}/P_{Na}$  was  $0.17 \pm 0.02$  (11), which appeared to compare reasonably favorably with the actual experimental values of  $25 \pm 1$  for the anion-selective WT channel and  $0.34 \pm 0.04$  for the cation-selective (A-1'R) mutant GlyR channel (6,7). It should be noted that for the BD study (11), the permeability ratios were calculated from reversal potentials and direct measurements of counter-ion fluxes were not made. However, when O'Mara subsequently ran the same BD simulations again, but this time specifically tried to directly

measure counter-ion fluxes, she found that no counter-ions were actually observed to permeate through the channel (O'Mara, personal communication; see also Barry and O'Mara [12], in which it was stated that "Brownian dynamics calculations on such channels [e.g., refs. 7 and 11] have seemed to indicate no counterion permeation, but have not given Nernstian potentials"). The question was then raised as to whether reversal potentials were a reliable measure of relative permeabilities and, in particular, whether using the GHK equation to evaluate relative permeabilities from reversal potentials had any validity. In the light of the importance and growing use of BD simulations to investigate the physical principles underlying ion selectivity and permeation through ion channels, it is important to address this question and justify the widespread use of reversal potentials for determining relative ion permeabilities and stress some basic fundamental principles relating to reversal potentials and ion permeation.

However, a subsequent study by Cheng et al. (13) also using BD simulations of the same channels, but with slightly different channel assumptions, again produced reversal potentials for their simulations similar to what had been observed in the original LGIC experiments by Keramidas et al. (6,7). In this case, though, Cheng et al. (13) did observe counter-ion fluxes of about the right magnitude to account for their reversal potentials.

The aim of this article is therefore to reevaluate the reliability of anion–cation permeability ratios determined from reversal potential measurements under dilution conditions and the validity of using the GHK equation for such calculations. It will briefly mention a critical aspect of BD simulations, namely the solution boundary conditions at each end of the channel and their implementation, which could affect the link between reversal potentials and counter-ion fluxes.

## MATERIALS AND METHODS

### Recording Techniques

Ideally, to simplify subsequent analysis, the pipette solution would be maintained in a simplified salt solution essentially similar to the composition of a standard saline solution for those cells (e.g., mainly about 150 mM NaCl), provided the simplified nature of the solution does not affect the viability of the cell membrane. Initially, the composition of the external solution would also be made as close as possible to that of the pipette solution. Current-voltage curves would be generated for the appropriate channels in the presence of the relevant agonist that activates them, and their zero-current reversal potential,  $V_{rev}$ , determined. Normally,  $V_{rev}$  should be very close to zero in such symmetrical solutions, provided the symmetry is close to perfect, regardless of the

relative permeability properties of the channels. Typically for whole-cell experiments and externally activated ligand-gated channels, the external concentration of NaCl would be approximately halved (referred to as the “0.5 NaCl solution”) and the shift in the reversal potential measured (with reference to the control value in symmetrical solutions). In the same way, the external concentration of NaCl would be approximately halved again (referred to as the “0.25 NaCl solution”) and again the shift in the reversal potential measured (with reference to the control value). In both diluted solutions, the decrease in NaCl concentration is normally osmotically balanced with sucrose (in many cases D-mannitol has also been used, though being smaller than sucrose it is more likely to permeate through some large ion channels). Generally, the actual concentrations of Na<sup>+</sup> and Cl<sup>−</sup> are not exactly equal because of the presence of an appropriate buffer (e.g., 10 mM HEPES, with about 5 mM free Na<sup>+</sup> and 5 mM HEPES<sup>−</sup> at pH 7.4 when buffered with NaOH), and in some cases the solutions may also contain small concentrations of such salts as KCl and CaCl<sub>2</sub>. Experimentally, it is also very important to correct all measurements of membrane potentials for liquid junction potentials, which can be particularly significant for selectivity studies in patch-clamp experiments (14). For example, for a 1:0.25 activity gradient of NaCl, the liquid junction potential would be −7.3 mV; for the same gradient of LiCl, it would be −11.5 mV; and for NMDG-Cl, it would be −18.1 mV (using the Windows version of the program *JPCalc* [15] and mobilities of  $u_{\text{Na}^+}$ ,  $u_{\text{Li}^+}$ ,  $u_{\text{NMDG}^+}$ , and  $u_{\text{Cl}^-}$  of 0.682, 0.525, 0.33, and 1.088 each relative to  $u_{\text{K}^+}$  (see [http://web.med.unsw.edu.au/phbsoft/mobility\\_listings.htm](http://web.med.unsw.edu.au/phbsoft/mobility_listings.htm)). Hence, the corrections are almost invariably very significant.

### Analytical Techniques

After the reversal potential shifts have been determined, then they can be plotted as a function of either  $a_{\text{Na}^+}^o$ , the activity of Na<sup>+</sup> in the external solution, for a predominantly cation-selective channel, or against  $a_{\text{Cl}^-}^o$ , the activity of Cl<sup>−</sup> in the external solution, for a predominantly anion-selective channel (see Fig. 1 for the principles involved). Because the ionic strength of the diluted solutions differs from that in the control solution, it is also important that activities rather than concentrations are used. The data can then be fitted to the GHK equation (2), which is a special zero-current potential case of the more general Goldman equation or Constant field equation (1). The Goldman equation was derived assuming that the ionic fluxes are independent of each other and that the electric field within the membrane or channel is a constant (1). For a shift in the (zero-current) reversal potential,  $\Delta V_{\text{rev}}$ , from a symmetrical to a dilution situation, with the assumption that only Na<sup>+</sup> and

Cl<sup>−</sup> ions are permeant, the GHK equation can be shown (Appendix A) to be given by:

$$\Delta V_{\text{rev}} = \frac{RT}{F} \ln \left( \frac{a_{\text{Na}^+}^o + (P_{\text{Cl}^-} / P_{\text{Na}^+}) a_{\text{Cl}^-}^o}{a_{\text{Na}^+}^i + (P_{\text{Cl}^-} / P_{\text{Na}^+}) a_{\text{Cl}^-}^i} \right) \quad (1)$$

in which  $R$ ,  $T$ , and  $F$  are the gas constant, temperature in K and Faraday constant respectively;  $a_{\text{Na}^+}$  and  $a_{\text{Cl}^-}$  are the activities of Na<sup>+</sup> and Cl<sup>−</sup> ions, respectively;  $o$  and  $i$  refer to external and internal solutions, respectively; and  $P_{\text{Na}^+}$  and  $P_{\text{Cl}^-}$  refer to the relative permeability of the Na<sup>+</sup> and Cl<sup>−</sup> ions, respectively. A question that is often raised is: “Should the GHK equation be expressed and used with activities rather than concentrations?” The GHK equation was originally derived in terms of concentrations, though clearly it should reduce to the Nernst equation when only one of the ions is permeant. Hence it should correctly be written in terms of activities especially under conditions of salt dilution, in which the ionic strength on both sides of the membrane can be very different. A new derivation of the GHK equation in terms of activities is given in Appendix A. The equation can be fit to the data using a numerical fitting procedure to determine  $P_{\text{Cl}^-}/P_{\text{Na}^+}$ . Our research group does this for each experiment and then averages the values of  $P_{\text{Cl}^-}/P_{\text{Na}^+}$ , rather than averaging the data over all the experiments and then trying to fit the averaged data and correct for the error bars in the fitting.

In this article, the permeability ratio obtained with the GHK equation will be compared with the value obtained with the Planck equation. This latter equation has been derived in terms of ionic activities by solving the Nernst–Planck flux equations, with the only assumption being that of electroneutrality over short distances within the membrane channel (16). The Planck equation is given by:

$$\Delta V_{\text{rev}} = \frac{RT}{F} \frac{[1 - (P_{\text{Cl}^-} / P_{\text{Na}^+})]}{[1 + (P_{\text{Cl}^-} / P_{\text{Na}^+})]} \ln \left[ \frac{a_{\text{NaCl}}^o}{a_{\text{NaCl}}^i} \right] \quad (2)$$

in which the parameters are the same as for Eq. 1.

## RESULTS

The question was raised previously as to whether relative anion–cation permeabilities could be reliably determined from measurements of zero-current reversal potentials in dilution potential measurements. To investigate this question, this section will initially go back to first principles and consider such a relationship in a relatively model-independent analysis using the princi-

ples of nonequilibrium irreversible thermodynamics. Irreversible thermodynamics does assume that the fluxes are linearly related to driving forces, which is a reasonable assumption provided the driving forces are not too large. However, it makes no assumptions about the underlying physicochemical principles involved in the permeation process. This article will then compare the predictions of the GHK equation with those of the Planck equation. It will finally discuss the results of the second BD simulation article previously mentioned (13) and will comment on the relationship between reversal potentials and counter-ion fluxes in the first BD simulation article (11) and their subsequent unpublished measurements on the lack of observed counter-ion fluxes.

### ***The Relationship Between Reversal Potentials and Relative Permeabilities as Predicted by Irreversible Thermodynamics***

This analysis is shown in Appendix B, in which three main cases are considered for a NaCl dilution situation. The situation to be considered is that of a concentration gradient across a membrane (NaCl [C<sub>1</sub>]:NaCl [C<sub>2</sub>]). The irreversible flux:energy equations for the movement of Na<sup>+</sup> and Cl<sup>-</sup> can then be set up following the Onsager approach; for example, as given in Katchalsky and Curran (17):

$$J_{\text{Na}} = L_{\text{Na}} \cdot X_{\text{Na}} + L_{\text{Na-Cl}} \cdot X_{\text{Cl}} \quad (3)$$

$$J_{\text{Cl}} = L_{\text{Cl-Na}} \cdot X_{\text{Na}} + L_{\text{Cl}} \cdot X_{\text{Cl}} \quad (4)$$

in which  $L_{\text{Na}}$  is the coupling coefficient linking the flux,  $J$ , of Na<sup>+</sup> with the energy difference,  $X_{\text{Na}}$ , across the membrane for Na<sup>+</sup>, and  $L_{\text{Cl}}$  is the coupling coefficient linking the flux of Cl<sup>-</sup> with the energy difference,  $X_{\text{Cl}}$ , across the membrane for Cl<sup>-</sup>.  $L_{\text{Na-Cl}}$  and  $L_{\text{Cl-Na}}$  are cross-coupling coefficients.  $X_{\text{Na}}$  and  $X_{\text{Cl}}$  represent the electrochemical potential energy differences for Na<sup>+</sup> and Cl<sup>-</sup>, respectively, across a membrane and will be given by:

$$X_{\text{Na}} = F(V_{\text{m}} - V_{\text{Na}}) \quad (5)$$

$$X_{\text{Cl}} = -F(V_{\text{m}} - V_{\text{Cl}}) \quad (6)$$

With

$$V_{\text{Na}} = (RT/F) \ln (a_{\text{Na}}^{\circ} / a_{\text{Na}}^{\text{i}}) \quad (7)$$

$$V_{\text{Cl}} = (-RT/F) \ln (a_{\text{Cl}}^{\circ} / a_{\text{Cl}}^{\text{i}}) \quad (8)$$

in which  $V_{\text{Na}}$  and  $V_{\text{Cl}}$  represent the equilibrium potentials for Na<sup>+</sup> and Cl<sup>-</sup>, respectively, and the other parameters are as previously defined.

As shown in Appendix A, the results for the three cases considered are as follows.

#### **CASE 1**

The anion is assumed to be impermeant ( $P_{\text{Cl}} = 0$ ). Under these conditions, the coefficient for chloride transport,  $L_{\text{Cl}}$ , should = 0. Equation B15 in Appendix B indicates that, under such conditions,  $V_{\text{rev}} = V_{\text{Na}}$ , the Na<sup>+</sup> equilibrium (or Nernst) potential.

#### **CASE 2**

The cation is assumed to be impermeant ( $P_{\text{Na}} = 0$ ). Under these conditions, the coefficient for sodium transport,  $L_{\text{Na}}$ , should = 0. Equation B16 indicates that under such conditions  $V_{\text{rev}} = V_{\text{Cl}}$ , the Cl<sup>-</sup> equilibrium (or Nernst) potential.

#### **CASE 3**

Both ions are considered to be equally permeant ( $P_{\text{Na}} = P_{\text{Cl}}$ ). Under these conditions, the transport coefficients for both Na<sup>+</sup> and Cl<sup>-</sup> should be equal ( $L_{\text{Na}} = L_{\text{Cl}}$ ). Equation B18 indicates that under such conditions,  $V_{\text{rev}} = (V_{\text{Na}} + V_{\text{Cl}})/2$ . For a typical symmetrical dilution situation (e.g., a simple gradient of NaCl), where  $V_{\text{Na}} = -V_{\text{Cl}}$ , then  $V_{\text{rev}} = 0$ .

Logically, cases 1 and 2 are also what one would expect from equilibrium thermodynamics. If only one ion is able to permeate across a membrane, then that ion would keep moving down its electrochemical gradient and those ions would move across the membrane until the membrane potential, created by the resultant separation of charge across the membrane, equalled the equilibrium potential for that ion, irrespective of the mechanism of ion permeation. Conversely, if the reversal potential is not equal to the equilibrium potential of one of the two permeant ions, then each ion must be permeant to at least some degree.

Similarly, one would expect that if the activity gradients of two ions were the same across a membrane, then a reversal potential of 0 mV would imply that both ions were moving at the same rate across the membrane for the same electrochemical energy gradient, so that there would be no buildup of charge across the membrane, and hence the two ions must be equally permeant. If the salt concentrations in each solution remained constant (a reasonable approximation, if the two bathing solutions were large and well stirred), then this would represent a steady-state situation. However, even if the concentrations of the two salt solutions bathing the membrane interfaces did change, and the two ions were equally permeant, one would not expect  $V_{\text{rev}}$  to change because there would still be no buildup or decrease in charge across the membrane.

It should be stressed that the analysis is also completely model independent and at three pivotal points, the relationship between reversal potentials and relative permeabilities through a channel, is identical to that predicted by the GHK equation.

Table 1  
A Comparison of the Shifts in Reversal Potentials Under External Salt (NaCl) Dilution Conditions as Predicted by the GHK and Planck Equations for a Range of Different Permeability Ratios ( $P_{\text{Cl}}/P_{\text{Na}}$ )

$a_{\text{NaCl}}^{\circ}$ in mM $P_{\text{Cl}}/P_{\text{Na}}$	GHK equation Calculated $V_{\text{r}}$ s in mV			Planck equation Calculated $V_{\text{r}}$ s in mV		
	100	50	25	100	50	25
0	0	-17.5	-35.0	0	-17.5	-35.0
0.001	0	-17.5	-34.9	0	-17.5	-35.0
0.01	0	-17.1	-34.1	0	-17.2	-34.3
0.1	0	-14.1	-27.1	0	-14.3	-28.7
0.2	0	-11.4	-21.4	0	-11.7	-23.4
0.4	0	-7.3	-13.3	0	-7.5	-15.0
0.6	0	-4.2	-7.6	0	-4.4	-8.8
0.8	0	-1.9	-3.4	0	-2.0	-3.9
1	0	0.0	0.0	0	0.0	0.0
1.25	0	1.9	3.4	0	2.0	3.9
1.67	0	4.2	7.7	0	4.4	8.8
2.5	0	7.3	13.3	0	7.5	15.0
5	0	11.4	21.4	0	11.7	23.4
10	0	14.1	27.1	0	14.3	28.7
100	0	17.1	34.1	0	17.2	34.3
1000	0	17.5	34.9	0	17.5	35.0
$\infty$	0	17.5	35.0	0	17.5	35.0

In each case, the same  $P_{\text{Cl}}/P_{\text{Na}}$  value was used for both equations to calculate the shift in reversal potential from the symmetrical situation (100 mM: 100 mM NaCl) to one in which the external solution *activity* was dropped to 50 and 25 mM. The values of the  $\text{Na}^+$  equilibrium potential,  $V_{\text{Na}}$ , for the two solution dilutions were -17.5 and -35.0 mV, respectively, and of the  $\text{Cl}^-$  equilibrium potential,  $V_{\text{Cl}}$ , were 17.5 and 35.0 mV, respectively.

### Comparison of Reversal Potentials and Permeability Ratios Determined From the GHK and Planck Equations

As previously mentioned, the Goldman equation was derived on the basis of independence of the different ion fluxes and a constant electric field across the membrane. In contrast, the derivation of the Planck equation assumes electroneutrality over small elemental distances across the membrane, the opposite of ionic independence, and results in a nonconstant electric field and a linear concentration gradient across the membrane (16). Keramidas et al. (18) have already shown by comparing relative permeability data for the WT and six mutant GlyRs, with  $P_{\text{Cl}}/P_{\text{Na}}$  values ranging from 0.12 to 28.5, that there was very good agreement between the relative permeabilities obtained by fitting the GHK equation and those obtained by fitting the Planck equation to reversal potential measurements (e.g., 0.12 [0.14], 0.37 [0.38], 3.8 [3.3], and 28.5 [26.2]; the values being GHK [Planck], respectively, and for the following mutant and WT

GlyRs, respectively: SDM (P-2' $\Delta$ , A-1'E), A-1'E, SDM+R19'A, SDM+R19'E, SDM+T13'V, P-2' $\Delta$ , and WT (from Table 2 of ref. 18). These values were obtained from averaged  $V_{\text{rev}}$  data and some of the GHK:Planck variability between the relative permeabilities calculated from the two equations probably reflects the cell-to-cell variability in the experimental data.

To more systematically compare the predictions of the two equations over a range of permeability values, the predicted shifts in reversal potential have been calculated for a control symmetrical 100:100 mM activity solution, a 50% (100:50), and a 25% (100:25) NaCl solution dilution with both the GHK and Planck equations (see Table 1). It may immediately be seen that for  $P_{\text{Cl}}/P_{\text{Na}}$  values of 0, 1.0, and  $\infty$ , both sets of  $\Delta V_{\text{rev}}$  values are absolutely identical. For the 25% dilution case, the difference is less than 1 mV for the intermediate values, except in the range of 0.2–0.6 (or its reciprocals) where it is less than about 2 mV. In each case, the magnitude of the Planck value is slightly greater than the value predicted by the GHK equation. Of

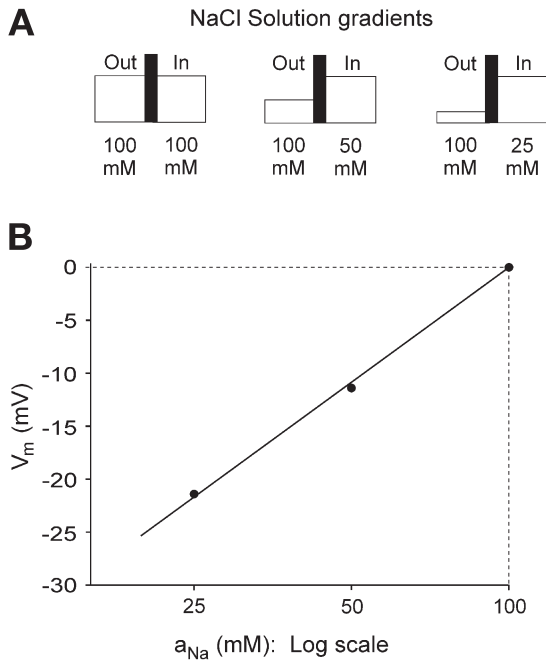


Fig. 1. A dilution potential experiment used to determine relative anion–cation permeability ratios from reversal potential data, in this case to compare the predictions of both the GHK and Planck equations. The principle is similar to what normally would be used for the analysis of experimental data. (A) The NaCl activity gradients for a 50% and 25% dilution of the external solution. However, in this case, the reversal potential “data” ( $V_m$  in mV) for  $\text{Na}^+$  activities ( $a_{\text{Na}}$ ) of 100, 50, and 25 mM were generated as GHK predictions for a  $P_{\text{Cl}}/P_{\text{Na}}$  value of 0.2. (B) The fitting of these data. Regular experimental data would normally be fitted to the GHK equation. However, for this article, the “GHK data,” displayed as filled circles, is fitted by the Planck equation, shown as a solid line, for a  $P_{\text{Cl}}/P_{\text{Na}}$  value of 0.235 (numerically fitted by Sigma Plot). The same procedure was used to compare the predictions of both equations for the range of  $P_{\text{Cl}}/P_{\text{Na}}$  values, which are listed in Table 2 and displayed in Fig. 2.

course, experimentally it is very difficult to distinguish relative permeabilities of 0.001 or less (or 1000 or greater), because the magnitude of the difference in reversal potential is  $\leq 0.1$  mV in a 100:25 saline gradient. In addition, even the difference for a  $P_{\text{Cl}}/P_{\text{Na}}$  ratio of 0.2 between the two calculations is only 2 mV in a 100:25 saline gradient and this is relatively minor compared with other experimental errors that need to be correctly accounted for, such as liquid junction potentials or accuracy of solution composition. In the second comparison between the predictions of the two equations, a range of different permeabilities was chosen and used to calculate the  $\Delta V_{\text{rev}}$  values using the GHK equation, as in the first four columns of Table 1. Then these values were numeri-

cally fitted to the Planck equation using SigmaPlot (V.5, Jandell Scientific, San Rafael, CA), to obtain the values of  $P_{\text{Cl}}/P_{\text{Na}}$  calculated from that equation, as illustrated in Fig. 1. These Planck equation  $P_{\text{Cl}}/P_{\text{Na}}$  values are given together with the original GHK  $P_{\text{Cl}}/P_{\text{Na}}$  values in Table 2, and the results are displayed graphically in Fig. 2. It is seen that there is perfect agreement between the predictions of the two equations for a  $P_{\text{Cl}}/P_{\text{Na}}$  value of 1.0, agreement to within about 10% between a value of 0.4 to 2.5 and always within 25% for  $P_{\text{Cl}}/P_{\text{Na}}$  values below 0.4. Given the very different assumptions underlying the two different equations, this is actually very reassuring. In addition, the sequence of relative permeabilities for different ions or different channels is absolutely maintained between the two equations and the values of relative permeabilities calculated by each equation will also generally be closer than may be apparent from a comparison of the absolute values listed in Table 1. For example, for two channels with permeability ratios of 0.001 and 0.2 (GHK), with a ratio of 1:200, the corresponding ratio for the Planck equation from Table 2 would be about 1:185.

It should be noted that the predictions of both the GHK and Planck equations also agree perfectly for the three pivotal cases derived using the principles of irreversible thermodynamics and supported by considerations of equilibrium thermodynamics.

### Reversal Potential and Flux Data From Numerical BD Simulations of WT and Mutant GlyR Channel Experiments

Although the earlier study by O’Mara et al. (11) had been able to simulate conductances for both the WT and cation-selective mutant GlyR channels and to observe reversal potentials of reasonably similar magnitudes ( $34 \pm 1$  and  $0.17 \pm 0.02$ , respectively) compared with those observed experimentally ( $25 \pm 1$  and  $0.34 \pm 0.04$ ), when they had subsequently looked for counter-ion permeation under the same conditions, they had not been able to observe any permeation of counter-ions through the channel. However, the more recent study of Cheng et al. (13), also using BD to simulate ion permeation through both WT and mutant cation-selective channels, did observe counter-ion permeation; some of the results of their study will now be discussed. For their permeation of the anion-selective WT  $\alpha 1$  GlyR channel, they obtained a reversal potential of  $15.5 \pm 0.8$  mV (13) for conditions similar to the experimental ones of Keramidis et al. (6,7). From this value, they calculated a  $P_{\text{Cl}}/P_{\text{Na}}$  permeability ratio of  $24 \pm 11$  (using the GHK equation for their calculations), which was within the experimental range of approx 24–28 for real GlyR channels (6,7). Significantly, Cheng et al. (13) did observe spontaneous transport of the counter-ion  $\text{Na}^+$  through

Table 2  
A Comparison of the Permeability Ratio ( $P_{Cl}/P_{Na}$ ) Predicted by the Planck Equation for Reversal Potential Data Calculated for Different  $P_{Cl}/P_{Na}$  Values Using the GHK Equation

Eqn.	$P_{Cl}/P_{Na}$														
GHK	0.0010	0.010	0.10	0.20	0.40	0.60	0.80	1.0	1.25	1.67	2.5	5.0	10	100	1000
Planck	0.0013	0.013	0.12	0.24	0.44	0.64	0.82	1.0	1.22	1.57	2.3	4.3	8.2	77	750

For each  $P_{Cl}/P_{Na}$  value, the shifts in reversal potential were calculated using the GHK equation for dilutions to activities of 50 mM and 25 mM NaCl. These reversal potential values were then numerically fitted to the Planck equation using Sigma Plot to determine the best value of  $P_{Cl}/P_{Na}$  predicted by that equation for those values. These values are also graphically displayed in Fig. 2.

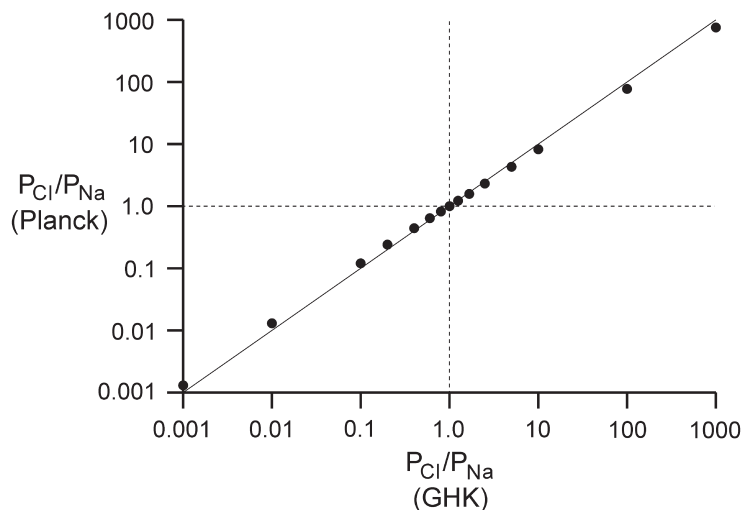


Fig. 2. A comparison of the permeability ratio ( $P_{Cl}/P_{Na}$ ) predicted by the Planck equation for reversal potential data calculated for different  $P_{Cl}/P_{Na}$  values using the GHK equation. The procedure for the calculation of the Planck equation  $P_{Cl}/P_{Na}$  values is outlined in Fig. 1. In each case, the reversal potential values were generated for a particular  $P_{Cl}/P_{Na}$  values using the GHK equation, and the generated data numerically fitted to the Planck equation to determine the best value of  $P_{Cl}/P_{Na}$  that would be predicted by that equation for those values (see Table 2). The solid line indicates the linear relationship between the two sets of permeabilities that would have resulted if both equations gave identical predictions and the intersection of the dashed lines indicates that both equations give identical predictions when  $P_{Cl}/P_{Na} = 1$ .

such WT GlyR channels, although it was noted that such events were rare. They also investigated permeation through the A251E (A-1'E) cation-selective mutant GlyR channel, which Keramidas et al. (7) had shown had an experimental  $P_{Cl}/P_{Na}$  value of  $0.34 \pm 0.04$ . Cheng et al. (13) obtained a reversal potential from their BD simulation studies of  $-14.0 \pm 1.5$  mV from which they calculated a  $P_{Cl}/P_{Na}$  value of  $0.11 \pm 0.05$  (again using the GHK equation). In addition, they also observed clear counterion fluxes of  $Cl^-$  through these cation-selective channels. For example, at a membrane potential of +60 mV, they observed a current ratio for  $I_{Na^+}/I_{Cl^-} = -16 \pm 7$  (the minus sign has been added to indicate that the  $Na^+$  and  $Cl^-$  ions were moving in opposite directions) (13). However, there would be different driving forces acting on each

ion species and it would be expected that for an ion,  $j$ , the ionic conductance,  $g_j$ , should be proportional to  $z_j(V_m - V_j)$ , where  $V_m$  is the membrane potential and  $z_j$  and  $V_j$  are the valency and equilibrium (Nernst) potential for ion  $j$ , as in Eqs. 7 and 8 for  $Na^+$  and  $Cl^-$ . For  $Na^+$ ,  $(V_m - V_{Na}) = +60 - (-17.1)$  mV = +77 mV, correcting for activity coefficients for their two averaged NaCl concentrations of 148 mM and 71 mM (with activities,  $\gamma$ , of 111.6 and 56.8 mM, and assuming that  $\gamma_{Na} = \gamma_{Cl}$ , the Guggenheim assumption; MacInnes [19], p. 242). Similarly, for  $Cl^-$ ,  $-(V_m - V_{Cl}) = +60 - (17.1)$  mV = -43 mV. From the ratio of  $-16 \pm 7$ , allowing for a driving force ratio of  $77/43 = 1.79$ , this suggests a conductance ratio  $g_{Na^+}/g_{Cl^-}$  of  $9 \pm 4$ . Inverting this ratio gives a value of  $g_{Cl^-}/g_{Na^+}$  of  $0.11 \pm 0.09/-0.03$ . Given the relative concentrations of  $Cl^-$  in the external

solution (for an inward movement of  $\text{Cl}^-$ ) to that of  $\text{Na}^+$  in the internal solution (for an outward movement of  $\text{Na}^+$ ) of 71/148 (= 0.48) from the NaCl concentration gradient across the membrane, this would predict a  $P_{\text{Cl}}/P_{\text{Na}}$  ratio of  $0.23 + 0.19/-0.06$ , which is not too different from the experimentally determined value of  $0.34 \pm 0.04$  (7). This correction assumes that the conductance is essentially proportional to the ion concentration, on the solution side from which the ions are leaving, times the permeability.

Given all the assumptions in these calculations and errors involved in both simulation and experimental data, there is a reasonable agreement between the simulated permeabilities from the second BD study (13) and the experimental data (6,7). In particular in that study, the presence of counter-ion permeation has now been clearly demonstrated through both the WT and cation-selective GlyR channels in BD simulations of currents.

Differences between the permeation predictions of the two BD studies presumably arise from the differences in parameters chosen by each study. For example, the dielectric constant of both the channel lumen and protein-membrane were chosen to be higher in the Cheng et al. study (13), with resultant smaller magnitudes for the energy barriers and wells within the channel than in the O'Mara et al. study (11). On the other hand, some of the differences relating to the correspondence, or lack of it, between reversal potentials and ion fluxes may also arise from the precise way in which the boundary conditions were implemented.

One major difficulty in accurately determining reversal potentials with BD is the correct implementation of the boundary conditions at each interface of the channel with the bulk solution. For example, consider a membrane separating two different concentrations of a salt (e.g., NaCl) with the membrane channels potentially permeable to both ions and consider the case in which there is zero current flow through the system. In a real situation with well-stirred and large solutions with relatively small ion fluxes, the movement of ions through an ion channel is not going to significantly change the concentrations on both sides of the membrane; therefore, the concentrations of ions on both sides of the membrane will stay constant over a reasonable period. However, unless there is equal movement of both anions and cations down their energy gradients, a slight separation between cations and anions will occur through the channel and across the membrane, which will result in a potential difference across it, known as a reversal potential when the net current flow is zero. This situation can be easily handled in simple analyses such as GHK derivation or various electrodiffusion analyses. However, in a BD simulation, two finite reservoirs are

considered—one on each side of the channel openings to simulate the two solutions bathing the membrane. Within these reservoirs, the ions are treated as discrete particles. Because of computational time constraints, the size and hence number of ions in each reservoir cannot be too large. Hence as an ion crosses the channel (e.g., from left to right), the number of those ions in the left reservoir will have decreased by one and the number of those ions in the right reservoir will have increased by one, significantly changing the concentration of that ion in both reservoirs. Ideally, the ion concentrations in the two bathing solution reservoirs should remain constant. A simple way to do this would be to artificially add one ion to the left reservoir and subtract one from the right reservoir whenever an ion crosses from left to right to maintain the ion concentrations in the two reservoirs (simple stochastic boundary). A more realistic boundary (known as a Grand canonical Monte Carlo stochastic boundary) includes a buffer zone within the outer part of this reservoir and allows some fluctuation of ions in this buffer zone. In addition, there is a need not to have too large a volume for each reservoir with too many ions for logistic computational reasons (e.g., eight pairs of  $\text{Na}^+$  and  $\text{Cl}^-$  ions were used by O'Mara et al. [11]). However, whatever the details of boundary reservoirs, the problem is to maintain the concentrations in the adjacent bulk solution with only a relatively small number of ions in each solution reservoir without disturbing the local changes in charge that would build up naturally in real membrane systems adjacent to the channel interfaces, for example, even under conditions of zero current flow. Such boundary conditions and their implementation are discussed at length by Im et al. (20). However, unless the compensation for ion movement through the channel is implemented in precisely the right way, it could completely annihilate or at least significantly upset the local charge separation across the membrane, which should develop naturally. An error in the local charge separation across the membrane would then give rise to an error in the reversal potential of the channel. It would be expected that the problem would be exacerbated under conditions of salt concentration gradients across a membrane, as in typical dilution situations. A clear indication of the incorrect implementation of the solution boundary conditions would be that the reversal potentials would not be consistent with the relative ion permeabilities, as determined by ion flux measurements. For example, for a simple salt gradient across a membrane, the (zero current) reversal potential should equal the equilibrium (Nernst) potential for the permeant ion, if only ions of one sign are permeating. This result is independent of the physical mechanism that underlies ion permeation through the channel; its

violation suggests a problem with the implementation of the boundary conditions.

## DISCUSSION

From considerations of both irreversible thermodynamics and discussions of equilibrium thermodynamics, this article has shown that there is a clear general relationship between relative permeabilities and reversal potentials even under conditions of solution dilution, which is completely independent of any underlying physical model. That is, for a simple electrolyte dilution, if the channel is permeable to only one of the ions the reversal potential should go to the equilibrium (Nernst) potential for that ion, whereas if it is equally permeable to both ions, then the reversal potential should go to zero.

It has been known for a long time that for bi-ionic potential measurements with *only permeant ions of the same sign* the form of the zero-current (reversal) membrane potential equation is essentially model independent (21–25). That is, very different models ranging from various types of electrodiffusion models with different underlying assumptions, including a model based on the Ussing flux equations, various rate theory models and a microscopic kinetic model, all produce an equation of identical form to the GHK equation. It should be noted that this equivalence is only true for the zero-current (reversal potential) values in the presence of permeant ions of one sign only and does not extend to the current-voltage curves at any other current. This article has indicated that the reversal potential equations should also be expressed in terms of activities rather than concentrations for dilution conditions and has included a new derivation of the GHK equation derived in terms of activities.

This article has shown that in the presence of a pair of permeant cations and anions, the zero current dilution potential is also broadly model-independent. A comparison was made between the GHK equation, dependent on a constant electrical field and independence of ionic fluxes, and the Planck equation, dependent on microscopic electroneutrality within the channel and therefore having a strong interaction between anions and cations. These two very different electrodiffusion equations each produce very similar relative permeabilities for the same reversal potential value over the whole spectrum of allowable reversal potential values (Table 2). We can therefore be very confident that the relative permeabilities derived from reversal potentials for such a situation determined with the GHK equation are giving us a good estimate of the relative permeabilities of those ions in that channel and that they are not seriously affected by the shortcomings

of the assumptions inherent in the derivation of the more general Goldman equation.

In contrast to the first BD simulation (11), the second BD simulation (13) of ion permeation through anion-selective WT GlyR channels and cation-selective mutant GlyR channels has clearly demonstrated that it is possible to simulate the presence of counter-ion permeation in such small channels. Presumably this occurs because of some slightly different parameters used in the two studies. However, the second BD simulation (13) has also demonstrated that such simulated currents can give rise to reversal potentials and relative permeabilities (determined from GHK fits to the reversal potentials) that are consistent with the approximate relative proportions of individual ion fluxes of both cations and anions passing through these channels.

This article also raises a question of the most appropriate handling of boundary conditions for the correct assessment of reversal potentials especially in dilution potential studies and suggests that any lack of appropriate correspondence between reversal potentials and relative permeabilities, as estimated from ion fluxes, implies a problem with the implementation of the boundary conditions used for the simulation.

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## APPENDIX A

### ***A Derivation of the Goldman–Hodgkin–Katz Equation, From the Ussing Flux Equation, in Terms of Activities Rather Than Concentrations.***

Although the Planck equation can be rigorously and correctly derived from the Nernst–Planck flux equations taking into account activities rather than concentrations (16), the solution of those equations with the Goldman assumptions is much complex. However, it is possible to derive the Goldman–Hodgkin–Katz equation quite differently using the basic approach of Patlak (21) who derived the concentration form of the GHK equation making use of the Ussing flux equation. The GHK equation will now be shown to more correctly be

derived from activities rather than concentrations, particularly where the ionic strength is different in both solutions.

Ussing (26) has shown that for an ion  $i$  passively diffusing across a membrane that with concentrations in solutions  $o$  and  $i$  of  $C_i^o$  and  $C_i^i$  and unidirectional fluxes  $j_i^{in}$  and  $j_i^{out}$ , and voltage,  $V$ , of the internal solution with respect to the external one, that

$$\frac{j_i^{in}}{j_i^{out}} = \frac{C_i^o}{C_i^i} \exp(-z_i \frac{FV}{RT}) \quad (A1)$$

in which  $R$ ,  $T$ , and  $F$  are the gas constant, temperature in K and Faraday constant, respectively, and  $z_i$  is the valency of the ion, where his original activities have been replaced by concentrations (e.g., ref. 21).

At equilibrium,  $j_i^{in} = j_i^{out}$  and hence Eq. A1 becomes

$$V_{eq} = \frac{RT}{z_i F} \ln \left( \frac{C_i^o}{C_i^i} \right) \quad (A2)$$

in which  $V_{eq}$  is the equilibrium potential for the ions. However, for situations in which ionic strength on both sides of the membrane is very different, we know that the Eq. A2, the Nernst equation, should really be:

$$V_{eq} = \frac{RT}{z_i F} \ln \left( \frac{a_i^o}{a_i^i} \right) \quad (A3)$$

in which  $a_i^o$  and  $a_i^i$  represent the activities of the ions in solutions  $o$  and  $i$  respectively. Hence, Eq. A1 should really be rewritten as in Ussing's original equation (26) as:

$$\frac{j_i^{in}}{j_i^{out}} = \frac{a_i^o}{a_i^i} \exp(-z_i \frac{FV}{RT}) \quad (A4)$$

Consider first of all positive ions  $i+$ , then Eq. A4 becomes:

$$\frac{j_{i+}^{in}}{j_{i+}^{out}} = \frac{a_{i+}^o}{a_{i+}^i} \exp(-\frac{FV}{RT}) \quad (A5)$$

Assuming that the influx and efflux are independent, then:

$$j_{i+}^{in} = k_{i+} a_{i+}^o \exp(-\frac{FV}{RT}) \quad (A6)$$

$$j_{i+}^{out} = k_{i+} a_{i+}^i \quad (A7)$$

in which  $k_{i+}$  is a rate constant or permeability term for these ions crossing the membrane. For negative ions, Eq. A5 can be written as:

$$\frac{j_{i-}^{in}}{j_{i-}^{out}} = \frac{a_{i-}^o}{a_{i-}^i} \exp(+\frac{FV}{RT}) \quad (A8)$$

and the influx and efflux can be again separated as:

$$j_{i-}^{in} = k_{i-} a_{i-}^o \quad (A9)$$

$$j_{i-}^{out} = k_{i-} a_{i-}^i \exp(-\frac{FV}{RT}) \quad (A10)$$

For a number of different anions and cations when the net current is zero, we will have that

$$\sum_i z_i (j_i^{in} - j_i^{out}) = 0 \quad (A11)$$

For a uni-univalent solution gradient, we will obtain:

$$k_{i+} [a_{i+}^o \exp(-\frac{FV}{RT}) - a_{i+}^i] - k_{i-} [a_{i-}^o - a_{i-}^i \exp(-\frac{FV}{RT})] = 0 \quad (A12)$$

$$[k_{i+} a_{i+}^o + k_{i-} a_{i-}^i] \exp(-\frac{FV}{RT}) = [k_{i+} a_{i+}^i + k_{i-} a_{i-}^o] \quad (A13)$$

Hence replacing the ks by Ps, we obtain

$$V = \frac{RT}{F} \ln \left[ \frac{[P_{i+} a_{i+}^o + P_{i-} a_{i-}^i]}{[P_{i+} a_{i+}^i + P_{i-} a_{i-}^o]} \right] \quad (A14)$$

Note that if one of the ions is impermeable, that the equation now correctly reduces to the Nernst equation (Eq. A3).

It can similarly be shown that for many different anions and cations Eq. A14 can be extended to:

$$V = \frac{RT}{F} \ln \left[ \frac{\sum_{i+} P_{i+} a_{i+}^o + \sum_{i-} P_{i-} a_{i-}^i}{\sum_{i+} P_{i+} a_{i+}^i + \sum_{i-} P_{i-} a_{i-}^o} \right] \quad (A15)$$

This is then equivalent to the Goldman-Hodgkin-Katz equation, with the concentrations now more correctly written as activities.

## APPENDIX B

### *The Use of Irreversible Thermodynamics to Analyze a Dilution Potential Situation*

For general principles, refer to the Onsager approach in Katchalsky and Curran (17). It will be assumed that there is a concentration gradient across a membrane: NaCl  $[C_1]$ :NaCl  $[C_2]$ . The irreversible flux:energy equations for the movement of  $Na^+$  and  $Cl^-$  can then be set up following the Onsager approach

$$J_{Na} = L_{Na} \cdot X_{Na} + L_{Na-Cl} \cdot X_{Cl} \quad (B1)$$

$$J_{Cl} = L_{Cl-Na} \cdot X_{Na} + L_{Cl} \cdot X_{Cl} \quad (B2)$$

in which  $L_{Na}$  is the coupling coefficient linking the flux of Na with the energy difference,  $X_{Na}$ , across the mem-

brane for  $\text{Na}^+$ , and  $L_{\text{Cl}}$  is the coupling coefficient linking the flux of  $\text{Cl}^-$  with the energy difference,  $X_{\text{Cl}}$ , across the membrane for  $\text{Cl}^-$ .  $L_{\text{Na-Cl}}$  and  $L_{\text{Cl-Na}}$  are cross-coupling coefficients.  $X_{\text{Na}}$  and  $X_{\text{Cl}}$  represent the electrochemical potential energy differences for  $\text{Na}^+$  and  $\text{Cl}^-$ , respectively, across a membrane and will be given by:

$$X_{\text{Na}} = F(V_m - V_{\text{Na}}) \quad (\text{B3})$$

$$X_{\text{Cl}} = -F(V_m - V_{\text{Cl}}) \quad (\text{B4})$$

in which  $F$  is the Faraday constant and  $V_{\text{Na}}$  and  $V_{\text{Cl}}$  represent the equilibrium (Nernst) potentials for  $\text{Na}^+$  and  $\text{Cl}^-$ , respectively, and given by:

$$V_{\text{Na}} = (RT/F) \ln (a_{\text{Na}}^o/a_{\text{Na}}^i) \quad (\text{B5})$$

$$V_{\text{Cl}} = (-RT/F) \ln (a_{\text{Cl}}^o/a_{\text{Cl}}^i) \quad (\text{B6})$$

and in which  $R$  and  $T$  are the gas constant and temperature in K, respectively.

Now there are three conditions relating the coupling coefficients:

- (1)  $L_{\text{Na}} \geq 0, L_{\text{Cl}} \geq 0$
- (2)  $L_{\text{Na-Cl}} = L_{\text{Cl-Na}}$
- (3)  $L_{\text{Na}} \cdot L_{\text{Cl}} \geq L_{\text{Na-Cl}}^2$

Consider the reversal potential case when  $I = 0$ , so that  $J_{\text{Na}} = J_{\text{Cl}}$ .

$$L_{\text{Na}} \cdot X_{\text{Na}} + L_{\text{Na-Cl}} \cdot X_{\text{Cl}} = L_{\text{Cl-Na}} \cdot X_{\text{Na}} + L_{\text{Cl}} \cdot X_{\text{Cl}} \quad (\text{B7})$$

$$X_{\text{Na}} = F(V_m - V_{\text{Na}}) \quad (\text{B8})$$

$$X_{\text{Cl}} = -F(V_m - V_{\text{Cl}}) \quad (\text{B9})$$

$$\begin{aligned} L_{\text{Na}} F(V_m - V_{\text{Na}}) + L_{\text{Na-Cl}} (-F)(V_m - V_{\text{Cl}}) \\ = L_{\text{Cl-Na}} F(V_m - V_{\text{Na}}) + L_{\text{Cl}} (-F)(V_m - V_{\text{Cl}}) \end{aligned} \quad (\text{B10})$$

Grouping terms in Eq. B10 and dividing throughout by  $F$ , we obtain:

$$\begin{aligned} L_{\text{Na}} (V_m - V_{\text{Na}}) + L_{\text{Na-Cl}} (-) (V_m - V_{\text{Cl}}) \\ = L_{\text{Cl-Na}} (V_m - V_{\text{Na}}) + L_{\text{Cl}} (-) (V_m - V_{\text{Cl}}) \end{aligned} \quad (\text{B11})$$

Reorganizing Eq. B11, we obtain:

$$\begin{aligned} V_m (L_{\text{Na}} - L_{\text{Na-Cl}} - L_{\text{Na-Cl}} + L_{\text{Cl}}) \\ = L_{\text{Na}} V_{\text{Na}} - L_{\text{Na-Cl}} V_{\text{Cl}} - L_{\text{Cl-Na}} V_{\text{Na}} + L_{\text{Cl}} V_{\text{Cl}} \end{aligned} \quad (\text{B12})$$

Hence

$$\begin{aligned} V_m (L_{\text{Na}} + L_{\text{Cl}} - 2L_{\text{Na-Cl}}) \\ = L_{\text{Na}} V_{\text{Na}} + L_{\text{Cl}} V_{\text{Cl}} - L_{\text{Na-Cl}} (V_{\text{Cl}} + V_{\text{Na}}) \end{aligned} \quad (\text{B13})$$

Defining  $V_m = V_{\text{rev}}$  for  $I = 0$ , we obtain:

$$V_{\text{rev}} = \{L_{\text{Na}} V_{\text{Na}} + L_{\text{Cl}} V_{\text{Cl}} - L_{\text{Na-Cl}} (V_{\text{Cl}} + V_{\text{Na}})\} / \{L_{\text{Na}} + L_{\text{Cl}} - 2L_{\text{Na-Cl}}\} \quad (\text{B14})$$

Consider three main cases.

### CASE 1. $L_{\text{Cl}} = 0$

$$\begin{aligned} \text{Then } L_{\text{Na-Cl}} = 0 \text{ and } V_{\text{rev}} = L_{\text{Na}} V_{\text{Na}} / L_{\text{Na}}. \\ \text{Hence, } V_{\text{rev}} = V_{\text{Na}} \end{aligned} \quad (\text{B15})$$

### CASE 2. $L_{\text{Na}} = 0$

$$\begin{aligned} \text{Then } L_{\text{Na-Cl}} = 0 \text{ and } V_{\text{rev}} = L_{\text{Cl}} V_{\text{Cl}} / L_{\text{Cl}}. \\ \text{Hence, } V_{\text{rev}} = V_{\text{Cl}} \end{aligned} \quad (\text{B16})$$

### CASE 3. $L_{\text{Na}} = L_{\text{Cl}}$

$$\begin{aligned} \text{Then } V_{\text{rev}} = \{(L_{\text{Na}} - L_{\text{Na-Cl}}) (V_{\text{Na}} + V_{\text{Cl}})\} / \\ \{2 (L_{\text{Na}} - L_{\text{Na-Cl}})\} \end{aligned} \quad (\text{B17})$$

$$\text{Hence, } V_{\text{rev}} = (V_{\text{Na}} + V_{\text{Cl}}) / 2 \quad (\text{B18})$$

### CASE 3A. THE TYPICAL SYMMETRICAL DILUTION CASE (E.G., A SIMPLE GRADIENT OF NaCl)

$$V_{\text{Na}} = -V_{\text{Cl}} \text{ Then } V_{\text{rev}} = 0 \quad (\text{B19})$$

### CASE 3B

For an ideal bi-ionic case (no other permeant ions present)

From Eq. B14,

$$V_{\text{Na}} = V_{\text{Cl}}. \text{ Then } V_{\text{rev}} = V_{\text{Na}} = V_{\text{Cl}} \quad (\text{B20})$$

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