Cation Activation of the Basolateral Sodium-Potassium Pump in Turtle Colon

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ABSTRACT The current generated by electrogenic sodium-potassium exchange at the basolateral membrane of the turtle colon can be measured directly in tissues that have been treated with serosal barium (to block the basolateral potassium conductance) and mucosal amphotericin B (to reduce the cation selectivity of the apical membrane). We studied the activation of this pump current by mucosal sodium and serosal potassium, rubidium, cesium, and ammonium. The kinetics of sodium activation were consistent with binding to three independent sites on the cytoplasmic side of the pump. The pump was not activated by cellular lithium ions. The kinetics of serosal cation activation were consistent with binding to two independent sites with the selectivity Rb > K > Cs > NH₄. The properties and kinetics of the basolateral Na/K pump in the turtle colon are at least qualitatively similar to those of the well-characterized Na/K-ATPase of the human red blood cell.

INTRODUCTION

Recently (15), we showed that the basolateral membrane of the isolated turtle colon contains an electrogenic, sodium-potassium exchange pump. The current generated by obligatory sodium-potassium exchange can be measured directly in portions of colon in which (a) the apical membrane has been rendered highly permeable to both sodium and potassium by treatment with the polyene antibiotic amphotericin B, and (b) the basolateral potassium conductance has been blocked by barium. This combination of treatments appears to eliminate “recycling” of potassium at the basolateral membrane so that the cation fluxes and electrical currents generated by the activity of the pump are expressed as transmural flows. Simultaneous measurement of ouabain-sensitive “pump current” and the flow of either sodium or potassium ion indicated that the stoichiometry of the pump is approximately 3Na:2K.

An alternative approach to determining the properties of the pump is to measure the kinetics of cation activation of sodium-potassium exchange.
Because of the relative ease with which the pump current can be determined in the polyene-treated colon, we studied the activation of ouabain-sensitive pump current by mucosal (cellular) and serosal cations. The kinetics of cation exchange can be fit to either of two schemes, one involving independent binding and the other assuming highly cooperative binding. The kinetic parameters obtained from the former, however, conform to the measured stoichiometry of cation exchange, whereas those obtained from the latter do not, which suggests that multiple ion binding to independent sites is an adequate description of pump kinetics. The parameters obtained from this analysis suggest several similarities between the basolateral Na/K pump and that of the red blood cell. The selectivity of cation activation of the potassium site was identical to that of the human red blood cell, i.e., Rb > K > Cs > NH₄. The sodium site did not show any detectable affinity for lithium, i.e., mucosal (cellular) lithium did not activate the pump current.

METHODS

Colons were removed from turtles (Pseudemys scripta; Lemberger Co., Germantown, WI), stripped of serosal musculature, and mounted in Ussing chambers (A = 5.2 cm²) as previously described (4). Provisions for measurement of transepithelial potential and voltage-clamping were identical to those described previously (4). Both sides of the tissue were initially bathed by identical Ringers solutions containing (mM) 114 Na, 114 Cl, 2.5 K, 2.5 HCO₃, 1.0 Ca, 5.0 D-glucose, 5.0 D-mannitol, and 2.0 pyruvate. The solutions were bubbled with air at room temperature so that the pH was ~8.3. After 30–60 min of preincubation, both solutions were replaced with a Ringers solution in which the predominant anion was benzenesulfonate. The replacement of chloride ion was necessary to prohibit osmotic lysis of the epithelial cells after addition of polyene to the mucosal bath (15). For measurement of pump activation by mucosal sodium or lithium, a sodium-free, potassium benzenesulfonate Ringers was used that contained (mM): 112 K, 109.5 benzenesulfonate, 2.0 Cl, 1.0 Ca, 2.5 HCO₃, 5.0 D-glucose, 5.0 D-mannitol, and 2.0 pyruvate. For measurement of serosal cation activation, tissues were bathed on both sides by a potassium-free, sodium benzenesulfonate Ringers that was identical to the chloride Ringers except that all but 2 mM of the Cl was replaced by benzenesulfonate. Amphotericin B (E. R. Squibb & Son, Inc., Princeton, NJ) was added as a small volume of a concentrated stock solution dissolved in dimethylsulfoxide (DMSO). All cation additions were made after the addition of amphotericin B by the nonisosmotic addition of a small volume of the appropriate salt. Although this procedure resulted in small osmotic gradients across the tissue, it had the advantage of resulting in only one cation gradient and also allowed us to establish ion gradients without otherwise disturbing the bathing solutions. In preliminary experiments on paired tissues, addition of the chloride, methysulfate, or nitrate salts of sodium or potassium produced identical results, i.e., activation of pump current by mucosal sodium or serosal potassium was not anion dependent.

RESULTS

Induction of Pump Current by Amphotericin B

The short-circuit current induced by mucosal amphotericin B in the presence of mucosal sodium and serosal potassium (with serosal barium) can be equated
with the “pump current” produced by ouabain-sensitive, electrogenic sodium-potassium exchange (15). In the present experiments, we wished, as nearly as possible, to eliminate sodium gradients between the mucosal bathing solution and the cell and so used maximal doses of the polyene. Fig. 1 shows the relationship between the pump current ($I_p$) and the mucosal concentration of amphotericin B. The steep dependence of $I_p$ on polyene concentration is consistent with kinetics of an order $> 1$. This is in accord with previous studies on lipid bilayers, which suggested that the polyene pore is an oligomer of several polyene molecules (20). All experiments were conducted at concentrations of amphotericin B of $\geq 8 \mu M$.

![Figure 1](image)

**Figure 1.** Plot of pump current normalized to the maximum value vs. the concentration of amphotericin B in the mucosal bath. Values are mean ± SD for three tissues. Bathing solutions were both sodium benzenesulfonate Ringers containing 2.5 mM potassium.

**Activation of $I_p$ by Mucosal Na**

Fig. 2 shows the results of a representative experiment in which a portion of isolated colon was initially bathed on both sides by sodium-free, potassium benzenesulfonate Ringers. Mucosal and serosal potassium concentrations of 114 mM were chosen to reduce polyene-induced loss of cellular potassium and to minimize diffusional emfs across the apical polyene channel. Under these conditions, with 0.1 mM amiloride in the mucosal solution and 5 mM
barium in the serosal solution, the transmural potential and short-circuit current were near zero. The addition of amphotericin B to the mucosal solution produced a slight increase in conductance (not shown), but did not cause any change in $I_c$. As shown in the figure, the sequential addition of aliquots of sodium chloride to the mucosal bathing solution activated a current, positive in the M-to-S direction, that was abolished by ouabain. In a previous study (15), we showed that the ouabain-sensitive current evoked by mucosal sodium in the polyene-treated colon exhibits an obligatory dependence on serosal potassium.

Fig. 3 shows the average values from four such experiments plotted vs. the mucosal sodium concentration. In all of these experiments, the current induced by mucosal sodium was abolished by ouabain so that the sodium-induced value of $I_c$ and ouabain-sensitive value of $I_c$ were identical. The curve has the sigmoidal shape expected if activation of the pump requires

\[ I_c \text{ (} \mu A/5.2 \text{ cm}^2 \text{)} \]

**Figure 2.** Representative experiment showing the activation of pump current by mucosal sodium in a tissue bathed on both sides by sodium-free, potassium benzenesulfonate Ringers and treated with 10 $\mu$M amphotericin B. The indicated mucosal sodium concentrations were achieved by sequential additions of small aliquots of sodium chloride to the mucosal bathing solutions.

the binding of more than one sodium ion. Winne (32), however, pointed out that unstirred layers between the bulk solution and the transport site can cause simple hyperbolic (Michaelis-Menten) kinetics to appear to be sigmoidal. This distortion arises because, in the presence of a series diffusion barrier, the concentration of transported substrate at the active site is a nonlinear function of the bath concentration. The apparent thickness of the unstirred layer in the present experiments was estimated from the half-time in the rise of pump current after the addition of sodium to the mucosal bath (24). As shown in Appendix A, this unstirred layer has an apparent thickness (neglecting tortuosity and area restrictions) of $\sim 300$ $\mu$m, but would cause, at most, a 7% error in the estimate of the apparent dissociation constant. Eaton et al. (9) recently determined sodium fluxes across nystatin-treated rabbit urinary bladder and concluded that unstirred layers did not affect the kinetics of transport in the polyene-treated preparation.
Kinetic Analysis of Pump Activation by Mucosal Sodium

We explored two approaches to describing the kinetics of the pump current, both of which have been used to describe apparent pump activity in other systems. As shown in Appendix B, both of these formulations arise from the analysis of multiple equilibria and differ only with regard to the assumption about cooperativity of individual binding sites. One extreme is represented by Eq. 1, in which $I_p$ is given by an expression of the familiar Michaelis-Menten form with the concentration-dependent portion raised to a power $n$, where $n$ represents the number of sodium ions that must bind to activate the pump:

$$I_p = I_m/(1 + K_{Na}/[Na])^n.$$  

(1)

This description results from assuming that $n$ ions must bind to activate the pump but that the binding sites are completely independent, i.e., the binding of one ion does not alter the apparent affinity of the remaining sites. A linear transformation of this equation that is useful in determining the best fit to the data can be obtained by using an Eadie-Hofstee plot in which $I_p$ is raised to the $(1/n)$th power.

Eq. 2, a variant of the Hill equation, represents the opposite extreme with regard to the assumption about cooperativity. Here it is assumed that $n$ ions must bind to activate the pump but that the sites exhibit a high degree of positive cooperativity. As shown in Appendix B, this assumption means that the pump will exist in only two configurations, no sodium bound, or all sodium bound. This equation can be linearized on a logarithmic plot of the Hill type.

$$I_p = I_m/[1 + (K_{Na}/[Na])^n].$$  

(2)
To obtain estimates of the kinetic parameters, the average data and the data from individual experiments were treated in the following way. The points were plotted on an Eadie-Hofstee plot using various values of $n$ to derive an estimate of the maximal pump current, $I_m$. This value of $I_m$ was then used to compare the fit of the points with the predictions of independent or highly cooperative binding using the appropriate linear transformation.

Fig. 4 shows the average values of $I_p$ and $[\text{Na}^+]_m$ plotted on a normalized Eadie-Hofstee plot using a value for $I_m$ of $11 \mu A/cm^2$. The data points were plotted for three assumed values of $n$: 1, 2 or 3; an $n$ of 3 provided the best description of the data. The average kinetic constants were $11 \mu A/cm^2$ for the maximal pump current and 4 mM for $K_{Na}$ with $n = 3$.

Fig. 5 shows the same data represented on a Hill plot, which also provided a satisfactory straight line fit, yielding a value of 16 mM for the effective sodium dissociation constant and a Hill coefficient (slope) of 1.5.

A comparison of Figs. 4 and 5 shows that such kinetic analyses alone provided little guidance in choosing an appropriate description of pump activation. However, the agreement between the values for $n$ obtained in the present study assuming multiple, independent binding and that obtained in a previous study by direct measurement of cation fluxes suggested that Eq. 1 was more appropriate for analyzing pump current activation. This comparison also illustrates that the choice of kinetic scheme was nontrivial, because the resulting values for the apparent affinities differed appreciably.

*Mucosal Lithium Does Not Activate the Pump*

The possibility that mucosal lithium could activate the pump was of interest because the isolated turtle colon actively absorbs lithium by a ouabain-sensitive mechanism. Fig. 6 shows the results of a representative experiment, conducted as described above, in which tissues were bathed initially on both sides by sodium-free, potassium benzenesulfonate Ringers solutions. After
the addition of mucosal amphotericin B, either sodium or lithium chloride was added to the mucosal bath. Trace A shows activation of a ouabain-sensitive current by 20 mM mucosal sodium. Curve B shows that 20 mM mucosal lithium produced a small increase in $I_{sc}$ that was either slightly inhibited, as shown, or was unaffected in other experiments by the addition of ouabain. Increasing the concentration of the lithium to 40 mM in other experiments did not increase the ouabain-sensitive current. Trace C shows that after the addition of mucosal lithium, the subsequent addition of sodium produced the typical activation of $I_p$.

**Activation of $I_p$ by Serosal Cations**

In a previous study (15) we showed that when both sides of the tissue were bathed by potassium-free, sodium benzenesulfonate Ringers, the addition of potassium to the serosal bath activated a ouabain-sensitive current. The fact that increasing serosal potassium by only 2.5 mM under these conditions activates a current that is positive in the M-to-S direction emphasizes that these currents cannot be attributed to diffusional emfs. Fig. 7 compares the activation of $I_p$ by potassium, rubidium, cesium, and ammonium on a modified Eadie-Hofstee plot (see above). In each case the activation was best described by an $n$ of 2. The sequence of apparent affinities thus obtained was $Rb > K > Cs > NH_4$, identical to that for the Na/K-ATPase in the human red blood cell (12, 14).

Serosal lithium did not activate $I_p$ in the presence or absence of serosal potassium or sodium and did not prevent the subsequent activation of $I_p$ by serosal potassium.

**DISCUSSION**

The results presented here represent a direct determination of Na/K pump kinetics in the basolateral membranes of an epithelium. Taken together with
Figure 6. Representative experiments comparing the activation of pump current by mucosal sodium and lithium. Tissues were bathed on both sides by sodium-free, potassium benzenesulfonate Ringers and basolateral potassium conductance was blocked by adding 5 mM barium to the serosal bath.

Figure 7. Normalized Eadie-Hofstee plot showing activation of the Na/K pump by serosal addition of rubidium, potassium, cesium, and ammonium.
data from a previous study (15), the present results suggest that this transport element is remarkably similar to the well-characterized Na/K-ATPase in the plasma membrane of the human red blood cell (12). Kinetic analysis and direct measurement of sodium and potassium fluxes (15) both lead to the conclusion that the basolateral pump produces a current because of the exchange of intracellular sodium for extracellular potassium in the ratio of approximately 3 Na for 2 K. The value of $K_{Na}$ for sodium activation is $\sim 4$ mM in the presence of high potassium, whereas $K_{Na}$ under similar conditions for the red blood cell pump is $3.2$ mM (10). The selectivity of pump activation by serosal cations is identical for the colon and the human red blood cell and the apparent $K_m$ for potassium activation was $3.2$ mM for colon and between 2 and 3 mM for the human red blood cell in the presence of high external sodium concentrations (11). An analysis of the half-time of the response of pump current to serosal potassium (data not shown) ruled out the unstirred layer as the source of this discrepancy.

**Analysis of Pump Kinetics**

In the course of analyzing the results of these experiments, we were forced to address the issue of what type of kinetic analysis is most appropriate for a description of cation activation of the basolateral Na/K pump. We considered two extremes based on simple equilibrium binding considerations, one in which sites are independent and another in which binding is highly cooperative (Appendix B). Two results emerge from this analysis. First, both types of models can fit the data, so that cation activation kinetics alone could not resolve issues of stoichiometry or apparent dissociation constants. Second, the choice of kinetic scheme is nontrivial, because choosing one or the other would lead to markedly different interpretations of the underlying events. Direct measurements of transport rates indicated a stoichiometry for basolateral Na/K exchange in the turtle colon of approximately 3Na:2K (15). The correspondence of these values with those obtained using a modified Eadie-Hofstee analysis strongly suggests that a kinetic scheme based on multiple cation binding to independent sites is an appropriate description of the cation exchange process. A similar analysis was used by Garay and Garrahan (10) to describe the kinetics of Na/K exchange in the human red blood cell. The importance of choosing the appropriate kinetic scheme is well illustrated by the disparity in the values of $n$ and $K_{Na}$ obtained in Figs. 4 and 5. It should be noted that, in principle, a relation of the form of Eq. 1 (with $n = 1$) will produce a curvilinear Hill plot, but the range and scatter of the data points may preclude this discrimination (see also Fig. 6).

Lewis and Wills (18, 19) and Eaton (8) studied the kinetics of the basolateral pump in rabbit urinary bladder by using intracellular electrodes to estimate cell sodium activity and using either an electrophysiologically derived value of $I_p$ or simply $I_c$ as a measure of pump rate. Eaton (8) fit the data using the model appropriate for independent multiple binding and obtained results similar to those reported here with an $n$ for sodium of $\sim 3$. The Hill coefficient was not reported. In contrast, Lewis and Wills (18, 19) fit their results to the Hill equation, which is most appropriate for highly cooperative,
multiple-site binding, and obtained a Hill coefficient of 2.9 for sodium activation. It must be stressed that this coefficient can be interpreted in terms of numbers of sodium ions binding only if a high degree of cooperativity exists between individual sites. In general, the Hill coefficient is simply a measure of the departure of the kinetics from a rectangular hyperbola. It should be clear that although kinetic data can be linearized by either approach, the same data cannot yield similar coefficients for both equations. Thus, a major discrepancy exists between the kinetics of the pump in rabbit urinary bladders reported by these two groups. Recently, Eaton et al. (9) reported an analysis of the ouabain-sensitive current and sodium flux across nystatin-treated rabbit urinary bladder, in which they also found both current and sodium flux to be best described by a model based on three noninteracting sites.

Stoichiometry of Epithelial Na/K Exchange

It is generally recognized that the inhibitory effect of serosal ouabain and removal of serosal potassium on epithelial sodium absorption, as well as cytochemical localization of Na/K-ATPase, are consistent with the notion that active sodium absorption is driven by a sodium-potassium exchange pump located in the basolateral membranes of the epithelial cells. Attempts to demonstrate directly the expected obligatory coupling between the movements of sodium and potassium, however, have not produced consonant results (27). In the majority of these studies, potassium uptake from the serosal bathing solution has been compared with the rate of active sodium transport, usually measured as the short-circuit current. Although it has been uniformly found that potassium uptake is ouabain sensitive, it has not been possible to obtain unambiguous evidence for a fixed, stoichiometric coupling between potassium influx and sodium efflux. In experiments on isolated frog skin (1, 2) or rabbit ileum (21), for instance, it was possible to effect dramatic changes in the rate of sodium absorption using amiloride (to inhibit) or amino acids (to stimulate), respectively, but in neither case did these maneuvers affect the rate of potassium uptake from the serosal bath. Similar observations in a variety of epithelia led to the suggestion that there may be separate Na and K pumps or one pump with a variable coupling ratio (27).

In contrast, the present results suggest that in the isolated turtle colon there is a simple, fixed, stoichiometric coupling between pump-mediated sodium extrusion and potassium entry, which is reflected in transmural flows of sodium and potassium across tissues treated with mucosal amphotericin B and serosal barium. Nielsen (22) and Eaton (9) also reported apparent pump stoichiometries of about 3Na:2K in polyene-treated preparations of frog skin and rabbit urinary bladder, respectively. Although it is not possible to resolve completely the discrepancy between the present studies and potassium uptake experiments, several possibilities merit consideration. First, it must be recognized that potassium uptake across the basolateral membranes of epithelial cells must occur by at least two parallel pathways, the Na/K-ATPase and the basolateral potassium leak. Since the properties of the basolateral potassium
channel are largely unknown, it would seem difficult or impossible to predict the effects on tracer influx brought about by modulating the rate of sodium transport. Several observations suggest that basolateral potassium conductance may change in parallel with Na/K pump rate (3, 13, 28), an effect that should, in principle, accentuate transport-related potassium uptake. Ouabain, however, might by the same logic be expected to reduce basolateral K conductance so that the ouabain-sensitive component of the potassium influx may not be an appropriate operational definition of the potassium influx via the pump. On the other hand, it is also necessary to recognize that the polyene-treated colon may represent some limiting case of the full range of behaviors of the Na/K pump, and that 3Na:2K is one point on a continuum of possible operating modes.

**Implications of Na/K Pump Kinetics for Transepithelial Transport**

In the presence of mucosal amphotericin B and serosal barium, the basolateral Na/K pump was characterized by well-defined dissociation constants for cellular sodium and serosal potassium and a constant value of the maximal pump current, Im. A question not directly addressed by this study is how these parameters relate to the normal process of active sodium absorption, specifically the nature of the coupling between sodium entry across the apical membrane and active sodium exit. Simple kinetic coupling, whereby increases in sodium entry lead to increases in cell sodium that then stimulate the pump, is favored by sigmoidal kinetics. For sigmoidal kinetics, the value of $K_{Na}$ is not the concentration for half-maximal rate, but rather a lower value at which the slope of $I_p$ on $[Na]_c$ is maximal. Kinetic coupling would be most efficient in this region of the curve because changes in pump rate would require the smallest changes in cellular sodium concentration. The $K_{Na}$ for the basolateral pump of the turtle colon in the presence of high potassium is $\sim 4$ mM. This value can be compared with values for intracellular sodium measured in another tight epithelium, the rabbit urinary bladder. Wills et al. (31) reported that in this epithelium cellular sodium activity averaged $\sim 7$ mM and was independent of transport rate, while Eaton (8) reported for the same tissue that cell sodium ranged from $\sim 2$ to $>60$ mM and could be related to transport rate by a sigmoidal function with an $n$ of 3 and an apparent $K_{Na}$ of 3.5 mM. Thompson et al. (30) estimated intracellular sodium in the rabbit colon using electrophysiological techniques and found an average value of $\sim 10$ mM over a range of sodium transport rates from 37 to 124 $\mu$A/cm².

The value of $K_{Na}$ for the basolateral pump in turtle colon is appropriate for kinetic coupling or substrate regulation of pump rate by cell sodium in the range of $\sim 2$–10 mM. To attain maximal pump rates, however, cell sodium would have to exceed 40 mM. Although the data of Eaton (8) suggest that intracellular sodium concentrations as high as 60 mM are observed in rabbit urinary bladder, other studies have detected only minor changes in cell sodium over a wide range of transport rates (30, 31).

An attractive alternative to simple kinetic coupling, particularly in view of
the emerging evidence for the regulation of conductive properties of the apical and basolateral membranes of epithelial cells \(3, 15, 28\), is that the maximal pump rate can be varied, perhaps by the recruitment of new pump units so that the pumps are always operating far from saturation. It may be that the amphotericin-treated turtle colon represents a “ground state” where regulatory mechanisms have been subverted because of the unusual treatment of the cell.

**Epithelial Lithium Transport**

The failure of intracellular lithium to activate the basolateral Na/K pump confirms observations in a variety of ATPase preparations that lithium is at best a poor substitute for sodium \(7, 25\). Thus, the active absorption of lithium, which has been documented in turtle colon \(26\) and frog skin \(33\), must arise from some other mechanism. A likely candidate would be a basolateral Na/Li exchange mechanism, such as that found in the human red blood cell \(5, 6, 23\), that would use the energy of the sodium pump indirectly by coupling lithium efflux to an inwardly directed sodium gradient. Kirk and Dawson \(16\) obtained direct evidence for basolateral Na/Li countertransport in ouabain-treated turtle colons.

**APPENDIX A**

**Effect of Unstirred Layer on Pump Kinetics**

Let the pump current, \(I_p\), be given by:

\[
I_p = I_m/(1 + K_{Na}/[Na]_c),
\]

where \([Na]_c\) is the cellular sodium concentration. At the apical membrane assume that the membrane potential is zero so that the sodium flow can be written as:

\[
I_{Na} = FJ_{Na} = FP_{Na}([Na]_m - [Na]_c),
\]

where \(I_{Na}, J_{Na}, \text{ and } P_{Na}\) are the sodium current, ion flux, and permeability at the apical membrane. Solving for \([Na]_c\) and noting that \(I_{Na} = nI_p\), we obtain:

\[
[Na]_c/[Na]_m = 1 - nI_p/(FP_{Na}[Na]_m).
\]

To estimate the discrepancy between \([Na]_c\) and \([Na]_m\) at any values of \(n, I_p, \text{ and } [Na]_m\), we need a value for \(P_{Na}\). The effective permeability of series layers can be obtained from the half-time of the rise in \(I_p\) after a step increase in mucosal sodium concentration where the half-time is given by \(24\):

\[
t_{1/2} = 0.385\delta/D,
\]

where \(\delta\) and \(D\) are the effective path length and diffusion coefficient, so that the effective permeability is defined by:

\[
P_{Na} = D/\delta.
\]

Using a value of 30 s for \(t_{1/2}\) \((\text{Fig. 2})\), 10–5 cm\(^2\)/s for \(D_{Na}\), \(n = 3\), and representative values for \(I_p\) and \([Na]_m\), the effective thickness of the mucosal unstirred layer is of the order of 300 \(\mu\)m, but the maximum deviation of cell sodium from mucosal sodium is of the order of 7\%. 

APPENDIX B

Kinetic Equations for Pump Activation

To obtain a simple expression for the kinetics of pump activation by multiple ion binding, we assume that a single pump unit operates only if \( n \) ions bind, so that the total transport rate is determined by the number of pump units that have each bound \( n \) ions. We express the total pump current by:

\[
I_p = V_n I_m,
\]

where \( I_p \) is the pump current, \( I_m \) is the maximal pump current, and \( V_n \) is the fraction of pump units that have been activated by binding the requisite number of transported ions, \( n \).

Let the binding be described by a simple multiple equilibrium between the transported substrate, say Na, and the pump macromolecules, \( P \). The fraction of pump units that bind \( n \) ions, \( V_n \), can be written:

\[
V_n = \frac{[P(\text{Na})_n]}{[P] + [P(\text{Na})] + \ldots + [P(\text{Na})_n]},
\]

where \([P]\), \([P(\text{Na})]\), etc., are the concentrations of the various forms. The association constant for the formation of each complex, \( k_i \), can be defined as:

\[
k_i = \frac{[P(\text{Na})_i]}{[P(\text{Na})_{i-1}][\text{Na}]}.
\]

Substituting into Eq. B2 yields a general expression for \( V_n \) as a function of the \( k \)'s and ion concentration.

\[
V_n = \frac{(k_1 k_2 \ldots k_n)[\text{Na}]^n}{1 + k_1[\text{Na}] + k_1 k_2[\text{Na}]^2 + \ldots + (k_1 k_2 \ldots k_n)[\text{Na}]^n}.
\]

We now consider two limiting cases—-independent, identical sites and highly cooperative binding.

As shown by Tanford (29) for \( n \) identical and completely independent sites, it is possible to relate the \( n \) conventional equilibrium constants defined by Eq. B3 to a single equilibrium constant, \( k \). Note that for completely equivalent sites the association constants as defined by Eq. B3 are not equal because this expression takes into account only how many ions are bound and not to what sites they are bound. The \( n \) conventional equilibrium constants can, however, be expressed as (29):

\[
k_i = k(n - i + 1)/i,
\]

where \( k_i \) is the \( i \)th conventional association constant and \( k \) is the equilibrium constant for any single equivalent site. Combining with Eq. B4 and simplifying according to Tanford (29), we obtain for \( V_n \):

\[
V_n = (k[\text{Na}])^n/(1 + k[\text{Na}])^n,
\]

or, substituting into Eq. B1,

\[
I_p = I_m/(1 + K_{Na}[\text{Na}])^n,
\]

where \( K_{Na} = 1/k \).

Alternatively, we can assume a high degree of positive cooperativity between sites such that the pump units can exist only in two states, no ions bound or all \( n \) ions bound.
bound. The binding of a single ion ensures the binding of all the ions. With the assumption of high cooperativity, the expression for $V_\alpha$ becomes:

$$V_\alpha = \frac{[P(Na)_\alpha]}{[P] + [P(Na)_\alpha]} = \left[1 + \frac{[P]}{[P(Na)_\alpha]}\right]^{-1}.$$  \hfill (B8)

Using Eq. B3 we obtain:

$$V_\alpha = \left[1 + \frac{1}{(k_1 k_2 \ldots k_\alpha)[Na]^\alpha}\right]^{-1},$$  \hfill (B9)

which yields for $I_p$ using Eq. B1

$$I_p = I_m/[1 + (K_{Na}/[Na])^\alpha].$$  \hfill (B10)

where

$$K_{Na}^\alpha = (k_1 k_2 \ldots k_\alpha)^{-1}.$$  \hfill (B11)

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