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Kinetics of Na^+-ATPase: influence of Na^+ and K^+ on substrate binding and hydrolysis

Liselotte Plesner [a] and Igor W. Plesner [b]

[a] *Institute of Biophysics and* [b] *Department of Chemistry, Physical Chemistry Division, University of Aarhus, DK-8000 Aarhus C (Denmark)*

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An analysis of the influence of Na^+ and K^+ on the kinetics of Na^+-ATPase in broken membrane preparations from bovine brain is presented with particular emphasis on the effect of the cations on the binding and splitting of the substrate MgATP and on the derivation of a detailed kinetic model for that interaction. It was found that the enzyme in the absence of Na^+ and K^+, but in the presence of 7 mM free Mg^{2+}, at pH 7.4 (37°C) exhibits an ouabain-sensitive ATPase activity. The simplest model quantitatively compatible with all the data involves two different, interconvertible (conformational) forms of the enzyme, E_1 and E'_1, with the following properties: The E_1 form does not bind K^+ but has three independent and equivalent high-affinity sites ($K_d = 5.6$ mM) for Na^+. It binds and hydrolyzes substrate only when two or three sodium ions are bound to it. The E'_1 form binds and hydrolyzes the substrate only in the absence of monovalent cations. It is competitively inhibited by K^+ ($K_d = 0.23$ mM), and this inhibition is further enhanced by binding of Na^+ to the K^+-bound form at two equivalent, independent sites ($K_d = 12$ mM). It is suggested that the E'_1 form is the Mg^{2+}-induced conformational state of the enzyme observed by others, which differs from the usually encountered E_1 and E_2 forms. The model allows the calculation of ATP-binding and ADP-releasing rate constants for the E_1-form for later comparison with corresponding rate constants for the ($Na^+ + K^+$)-ATPase (following paper).

Introduction

One of the still unsettled questions concerning the action of the sodium pump responsible for the active transport of Na^+ and K^+ through cell membranes is the type of transport mechanism involved: are the sodium and potassium ions transported sequentially (a 'ping-pong' mechanism), simultaneously (an ordered mechanism), or is it a hybrid mechanism, in which the two ions in part of the reaction cycle are simultaneously bound to the transport system?

One way to study this question is to investigate the influence of the monovalent cations on the kinetics of the ($Na^+ + K^+$)-ATPase activity associated with the transport, in fragmented membrane preparations of the enzyme.

In recent years several articles have appeared [1–3] dealing with the influence of Na^+ and K^+ on the part of the enzymatic cycle involving phosphorylated enzyme intermediates. The work to be reported here is complementary to these articles in the sense that we shall be particularly concerned with the influence of the monovalent cations on the initial stages of the enzymatic process, i.e. with their influence on the binding and hydrolysis steps of the substrate, for the 'Na enzyme' (i.e., the activity observed at micromolar substrate concentrations) prepared from bovine brain.

The most widely accepted scheme for the (Na^+

$+ K^+$)-ATPase kinetics is that due to Albers [4] and Post et al. [5]. However, a few years ago we [6,7] presented kinetic results which could not be explained solely on the basis of that scheme. This led to our proposing a more extended model, containing the Albers-Post scheme as a special case. One noteworthy difference between our 'bi-cyclic' model and the Albers-Post scheme is that while in the bi-cyclic model the hydrolysis cycles corresponding to $(Na^+ + K^+)$-ATPase (at millimolar substrate and $Na^+ + K^+$) and Na^+-ATPase (at micromolar substrate) are completely distinct (but doubly connected), in the Albers-Post scheme the two cycles are partially overlapping, i.e. some intermediates are common to both activities.

The main objective in the present work is to extract, from kinetic studies, values of rate constants for the two activities that describe similar steps. A comparison of these values should then illuminate the question of identity or not of corresponding intermediates.

A necessary requirement for the estimation of intrinsic rate constants, independent of cation concentrations, is a detailed model for the interaction of cations with the enzyme. The present article is concerned with the analysis of kinetic data for the Na^+-ATPase resulting in such a model. In the following article [8] the $(Na^+ + K^+)$-ATPase is treated in an analogous way. Some preliminary data pertinent to the work described here have appeared [9].

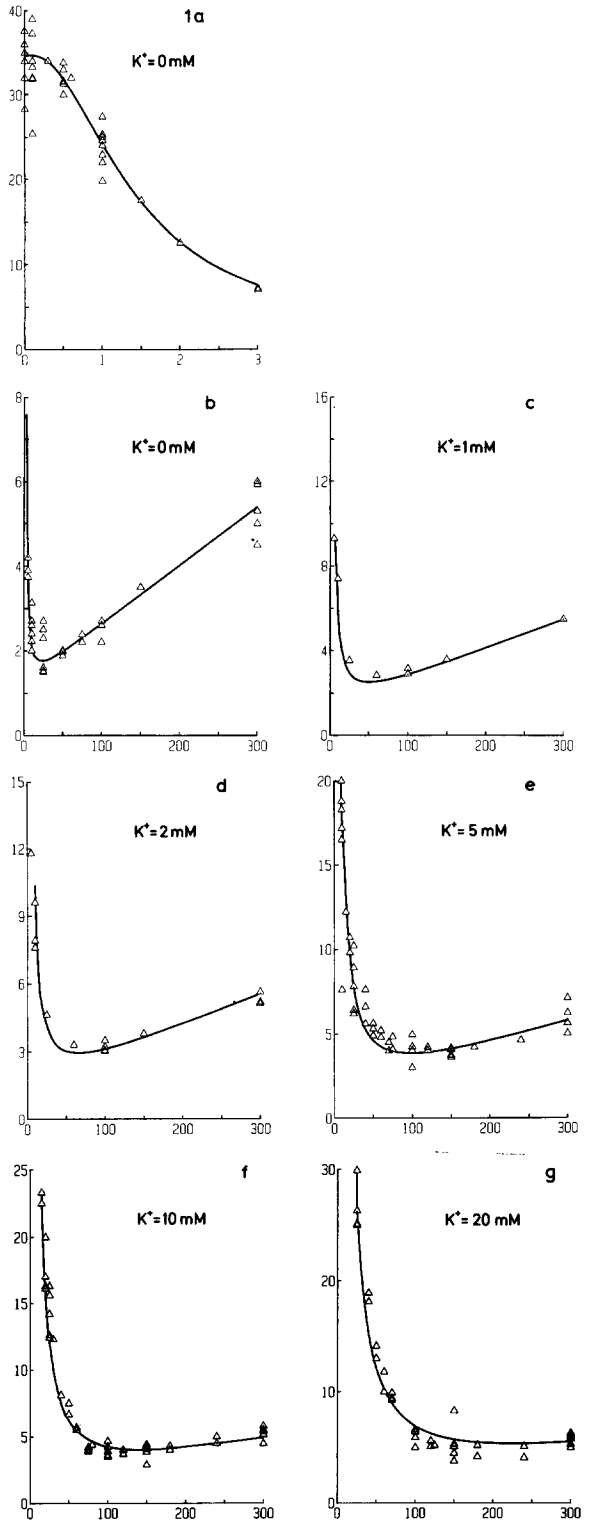
Materials and Methods

Enzyme. The $(Na^+ + K^+)$-ATPase was prepared from ox brain as described by Klodos et al. [10]. The activity of the enzyme measured with 3 mM ATP, 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 30 mM histidine buffer, pH 7.4 at 37°C, was 2.6 μmol ATP hydrolyzed/mg protein per min. Under these conditions less than 1% of the activity was not inhibited by 1 mM G-strophantin (ouabain). Gel electrophoresis performed by Peter L. Jørgensen in SDS at pH 2.4 [11] of this brain enzyme after phosphorylation from [γ-^{32}P]-ATP using 5–15 T% gradient slab gels showed only a single band of ^{32}P-labelled protein. This band comigrated with the ^{32}P-labelled α-subunit of pure renal $(Na^+ + K^+)$-ATPase (cf. Jørgensen et al. [12]).

Assay of ATP hydrolysis rate. The enzyme preparation containing 2 mg protein per ml was diluted 100–4000-times for the kinetic experiments. A further dilution of 10-times took place in the assay which was performed in histidine, 30 mM, pH 7.4 at 37°C. Incubation time was 5–10 min. Substrate concentrations were between 0.14 and 2 μM MgATP at constant $Mg^{2+}_{free} = 7$ mM. P_i was determined according to Lindberg and Ernster [13] with a specific activity of the substrate of $1 \cdot 10^6$–$3 \cdot 10^7$ cpm $\cdot$ nmol^{-1}. The ouabain-insensitive ATPase activity was measured as a blind corresponding to each assay tube, containing 1 mM ouabain, when $[Na^+] < 5$–10 mM, depending on $[K^+]$. At higher Na^+ concentrations the ouabain blind never exceeded the hydrolysis blind run for each assay tube. It was assured that under each set of conditions, the reaction rate was constant within the assay time. The Na^+ concentration in the absence of added Na^+ was measured in an Eppendorf flame photometer in the assay (plastic tubes). It depended on the ATP and enzyme concentrations, but never exceeded 6.3 μM. The ionic strength varied with the Na^+ and the K^+ concentrations and no attempt was made to keep it constant. In the reported experiments the ionic strength was varied from 0.25- to 2-times the ionic strength present in the solutions used for the determination of K_{diss} for MgATP [35]. This does cause a considerable change in the dissociation constant [14,38,39], but at the concentration of Mg^{2+}_{free} used (7 mM in all experiments) the effects in the relevant calculations are immaterial.

Adenosine 5'-triphosphate, diethanolamine salt from equine muscle, 'vanadium free', was from Sigma. (γ-^{32}P)-labelled adenosine 5'-triphosphate, triethylammonium salt, was from Amersham (Code Pb.132). In this product the amount of counts present as inorganic phosphate never exceeded 0.7% of the total amount of counts. In the equivalent product from New England Nuclear this fraction could amount to 3 to 5% in some batches of ATP rendering these batches difficult to use in kinetic experiments, where the total amount of ATP hydrolyzed in the assay should never exceed 10% of the ATP present.

In all kinetic experiments the measured ATP hydrolysis rates were standardized so as to correspond to the undiluted enzyme, the concentration



of which, measured as ATP binding sites (one site per molecule), was 0.66 μM (2 mg/ml).

Experimental results

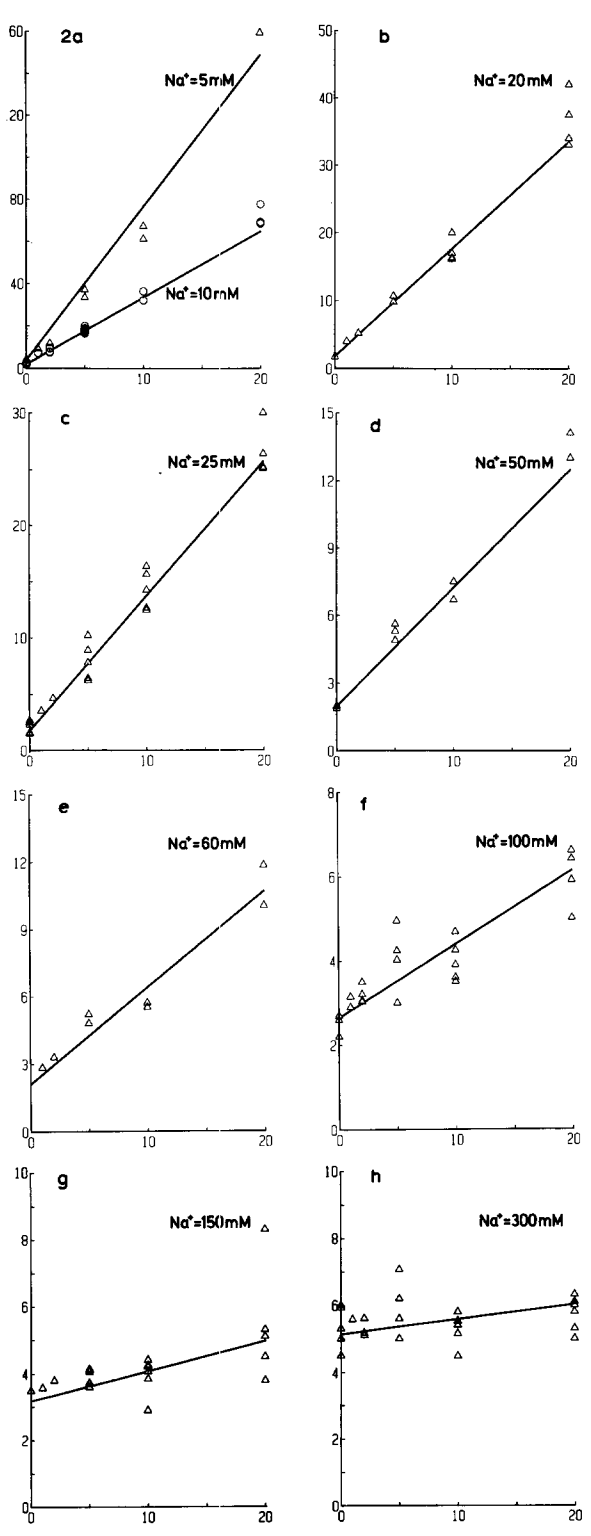
The data from a large number of kinetic runs are presented in the form of slopes from double reciprocal plots as a function of $[Na^+]$ at various K^+ concentrations (Fig. 1), and as a function of $[K^+]$ at various Na^+ concentrations in Fig. 2, the latter being a replot of the data from Fig. 1 to exhibit more clearly that the inhibition observed by K^+ appears linear within the range investigated.

We note from Fig. 1a that Na^+ in the range 0–0.5 mM has no appreciable influence on the value of the slope. Hence the finite value of the slope found in the absence of added Na^+ cannot be explained by the presence of endogenous Na^+, since, as detailed in Materials and Methods, this was present at concentrations less than 7 μM. Na^+, on the other hand, shows strong activation in the range 0.5–5 mM. At still larger values, above 50 mM, a weak Na^+ inhibition was observed.

The linear K^+ inhibition observed (Fig. 2) implies that only a single potassium ion interferes with the initial steps of the Na^+-ATPase reaction. From Fig. 2 it is seen that Na^+ appears to counteract the K^+ inhibition: at increasing Na^+ concentrations the slope of the lines in Fig. 2 is decreased.

In Fig. 3 is shown the apparent maximal velocity derived from the primary kinetic data as a function of $[Na^+]$ at various K^+ concentrations. When $[Na^+] = 0$, V_{max} is independent of $[K^+]$ in accordance with the purely competitive K^+ inhibition of '0-ATPase' [18]. For $[K^+] = 0$, the results are in general agreement with those reported by other investigators (see, for example, Ref. 15): there is a 'lag' at very small Na^+ concentrations and subsequently a strong activating effect of Na^+, with saturation obtained at $[Na^+] = 150$ mM. For

Fig. 1. The experimental values obtained for the slope from double-reciprocal plots of the primary kinetic data (v^{-1} vs. $[MgATP]^{-1}$) as a function of the Na^+ cocnentration. The K^+ concentrations are indicated. $[Mg^{2+}_{free}] = 7$ mM, pH = 7.4 at 37°C (30 mM histidine buffer). The curves are the theoretical curves calculated using Eqn. 1 and Table I. Ordinate: slope in 10^{-3} min; abscissa: $[Na^+]$ in mM.



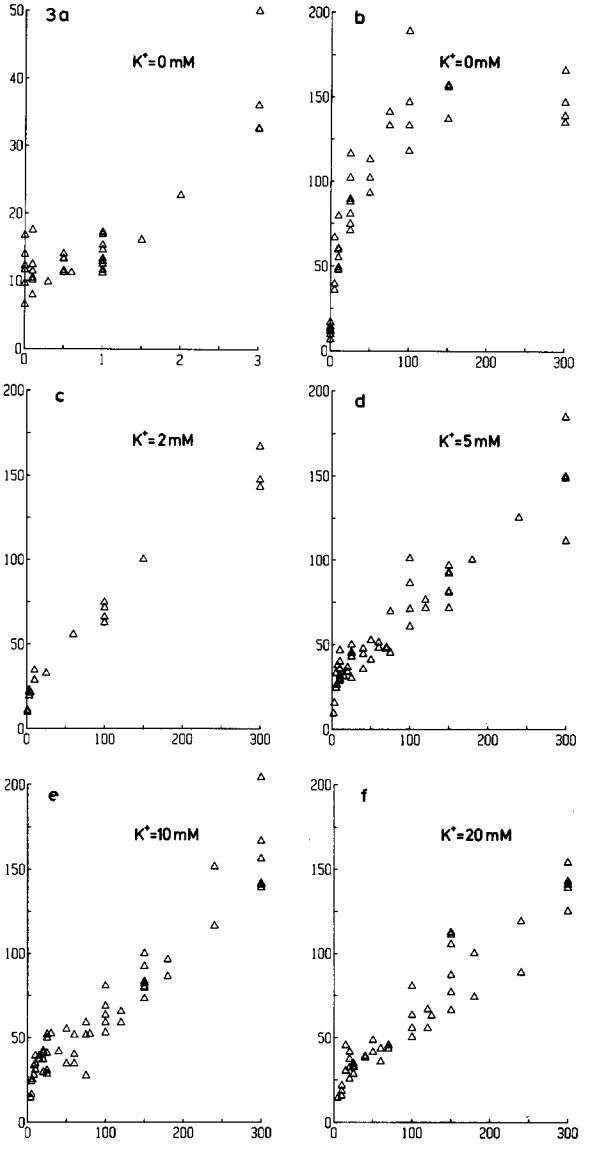


Fig. 3. The apparent maximal velocities, determined from the double-reciprocal plots, as a function of the Na^+ concentration at various constant K^+ concentrations as indicated. The experimental conditions were as in Fig. 1. Ordinate: V_{max} in nmol·ml^{-1}·min^{-1}; abscissa: [Na^+] in mM.

Fig. 2. The data from Fig. 1 plotted versus the K^+ concentration, at the various constant Na^+ concentrations indicated, to show the apparently linear slope inhibition exhibited by K^+. The lines are the theoretical lines calculated using Eqn. 1 and Table I. Ordinate: slope in 10^{-3} min; abscissa: [K^+] in mM.

$[K^+] \neq 0$ a distinct biphasic response is observed. Biphasic activity curves have on occasion been interpreted as representing a sum of two terms, each having the same mathematical form [15,16]. Although this is possible as a curve-fitting procedure, no physical meaning can be attached to the individual terms when the data represent the action of a single enzyme, as has recently been pointed out by Rossi and Garrahan [17].

It is noted that when $[Na^+] < 300$ mM, K^+ is inhibitory. The inhibition is overcome by Na^+ when $[Na^+] = 300$ mM. This is in contrst to results reported for the dog kidney enzyme, which was activated (at micromolar substrate concentrations) by K^+ at $[Na^+] = 130$ mM [16]. However, these latter experiments were carried out at $[Mg^{2+}] = 0.1$ mM whereas we have maintained $[Mg^{2+}] = 7$ mM in all experiments.

The results shown in Fig. 3 indicate a rather complex dependence of V_{max} on Na^+ and K^+. When it is taken into consideration that for Na^+-ATPase at least three forms of phosphoenzyme exist [3], each of which may spontaneously dephosphorylate, the expression that can be derived for V_{max} in terms of rate constants (and hence of Na^+ and K^+ concentrations) will be extremely complex. With the data at hand, a detailed analysis of such an expression would be a hopeless task. In the following section we therefore confine our interest to the slope data and the construction of a model for the initial steps in the ATPase reaction.

Discussion

It was stated above (see Materials and Methods) that the enzyme preparation, when subjected to gel electrophoresis after phosphorylation with [^{32}P]ATP exhibited only a single band of ^{32}P-labelled protein coinciding with the ^{32}P-labelled α subunit of pure renal ($Na^+ + K^+$)-ATPase. Probably, therefore, the properties exhibited in Figs. 1–3 represent the action of a single enzyme under different conditions. A model for the enzyme and its interaction with Na^+ and K^+ must accordingly predict the following:

1. The enzyme is active in the absence of added Na^+ and K^+ ('0-ATPase').

2. Concentrations of Na^+ up to about 0.5 mM have no measurable effect on the slope.

3. Larger concentrations of Na^+ activate strongly at high-affinity sites, such that 5–10 mM Na^+ decreases the slope to less than 10% of the value found with no Na^+ added.

4. At large (> 50 mM) Na^+ concentrations, an apparently linear increase of the slope is observed, indicating low-affinity Na^+-inhibition.

5. K^+ shows linear inhibition on the slope (Fig. 2), indicating the involvement of only one potassium ion.

6. Na^+, at increasing concentrations, counteracts the K^+ inhibition.

7. The '0-ATPase' activity is inhibited competitively by K^+ acting at a high-affinity site.

The last of these properties is supported by kinetic results reported in detail elsewhere [18]. In summary, the results obtained were that the '0-ATPase' has a K_m of 0.4 μM and a V_{max} of about 1/15 of that found for Na^+-ATPase. K^+ and ADP are competitive inhibitors of the hydrolysis, and are also mutually exclusive.

In a recent study of nucleotide binding to ATPase and the influence of Na^+ and K^+ thereupon, Jensen et al. [19] showed that Na^+ binds to independent and equivalent sites. Their results allow us to limit our interest to models satisfying the following criteria:

(i) multiple cation sites within a pool are independent and equivalent;

(ii) the affinity for Na^+ is independent of ATP; and

(iii) the intrinsic rate constants characterizing the conversion of the species in a pool to their counterparts in a neighboring pool are all identical.

The criterion (iii) follows from the first two and the principle of detailed balance at equilibrium, requiring that the product of the rate constants around any cycle in the clockwise direction equals that in the opposite direction.

A priori one would expect that the simple scheme $E_1 \rightleftarrows E_1ATP \rightarrow (EP) \rightarrow E_1$ (where the parenthesis around EP signifies several distinguishable phosphointermediates) should be able to account for the slope effects in Na^+-ATPase providing that each state is considered as an equilibrium pool with several Na^+-bound species. However, when evaluating the slope as a function

of the Na^+ concentration (see Appendix, section A), it is clear that such a model cannot satisfy the data in Fig. 1, with $[K^+] = 0$, when also the criteria (i)–(iii) above are incorporated.

To arrive at a model capable of explaining the observed slope variations, we note the following properties of the enzyme. When enzyme, in the absence of added cations, is suspended in histidine buffer at pH = 7.4, it is in the E_2 conformation as judged by the fluorescence signal of eosin [20,21]. Addition of Mg^{2+} to the enzyme, i.e. the conditions corresponding to the '0-ATPase' activity, produces a Mg^{2+}-form [22,23,24] which differs from the Na^+-form (usually denoted E_1) obtained by the addition of Na^+. Addition of K^+ to the Mg^{2+}-form rapidly produces the K^+-form [23], the E_2 conformation. Titration of the 'native' E_2-conformation of the enzyme with Na^+ suggests the involvement of more than one sodium ion [20]. These observations suggest that the hydrolytically active enzyme form in the absence of added Na^+, but in the presence of Mg^{2+}, (the '0-ATPase') is different from, but convertible to, that responsible for hydrolysis in the presence of Na^+.

The simplest formal kinetic scheme having these properties is shown in Fig. 4. Derivation of the rate equation (see Appendix) permits calculation of the slope (in terms of rate constants) of a double-reciprocal plot, which may conveniently be written in terms of 'intrinsic slope functions', R'_2 and R'_1 for the two interconvertible enzyme forms (the '0-ATPase' and the Na^+-ATPase), together with the equilibrium constant, $L' = k'_{-0}/k'_0$ governing their interconversion. These are defined in Eqns. (A12) (Appendix). Analysis of the data in terms of this model again involves assumptions concerning the contents of the equilibrium pools and investigating the properties of the resulting slope function. Following this procedure, the detailed model shown in Fig. 5 is obtained. As shown in the Appendix, the slope function for this scheme is:

$$\text{slope} \equiv Y = \frac{R_1^0 R_2^0 \left(1+\frac{Na}{K_1}\right)^3 \left(1+\frac{Na}{K_i}\right)}{R_2^0 \left(\frac{Na}{K_1}\right)^2 \left(3+\frac{Na}{K_1}\right) + LR_1^0} \times \left\{ 1 + \frac{L\left[1+\frac{K}{K_K}\left(1+\frac{Na}{K_2}\right)^2\right]}{\left(1+\frac{Na}{K_i}\right)^3 \left(1+\frac{Na}{K_i}\right)} \right\} \qquad (1)$$

where the expressions for the kinetic parameters R_1^0, R_2^0, and L are given in Eqns. A21–A23 and the dissociation constants are those shown in Fig. 5. It is seen that for intermediate and large Na^+ concentrations, and with $[K^+] = 0$, the slope re-

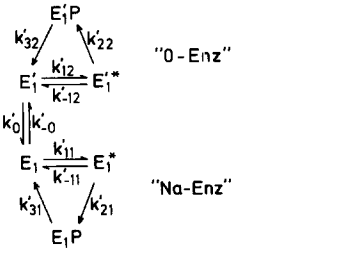


Fig. 4. A schematic model for the Na^+-ATPase and the '0-ATPase' activity showing only the kinetic intermediates not in internal equilibrium. The primes on the rate constants indicate 'effective', cation-dependent, rate constants. The enzyme-substrate adducts are indicated by asterisks.

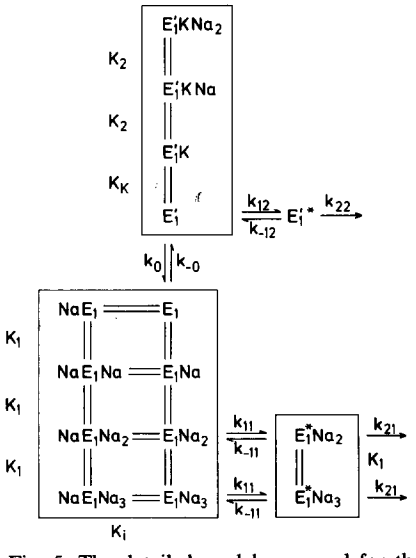


Fig. 5. The detailed model proposed for the first two kinetic intermediates (in each cycle) of Fig. 4. The rate constants indicated along the arrows are intrinsic rate constants. Within boxes, rapid internal equilibrium among the species is indicated by double lines. The assignment of dissociation constants used in the calculations is indicated along the appropriate equilibria in the figure. The presence on the enzyme of the substrate MgATP is indicated by an asterisk.

duces to

$$\text{slope} = R_1^0\left(1+\frac{\text{Na}}{K_i}\right) \qquad (2)$$

Hence, using the data from Fig. 1, at $[Na^+] > 50$ mM and $[K^+] = 0$, R_1^0 and K_i may be determined.

To determine the number of Na^+ sites on the E_1-form, the data from Fig. 1 (with $[K^+] = 0$) were analyzed using a non-linear curve-fitting routine [25] to determine the quantities L, R_2^0, and K_1. The following possibilities were tested:

(a) Two Na^+ sites on E_1, none on E_1' (with no K^+). Kinetically active species: E_1', E_1Na, E_1Na_2.

(b) as (a), but only E_1' and E_1Na_2 are kinetically active.

(c) Three Na^+ sites on E_1, none on E_1', (with no K^+). Kinetically active species: E_1', E_1Na_3.

(d) as (c), but with E_1', E_1Na, E_1Na_2, E_1Na_3 kinetically active.

(e) as (c), but with E_1', E_1Na_2, and E_1Na_3 kinetically active (as in Fig. 5).

Based on the calculated reduced χ^2 (which should be close to 1, see Ref. 25) and on the sign distribution of the absolute deviation of the fit from the data, it was found that the possibility (e), shown in Fig. 5, gives the best fit. The values of the parameters obtained are given in Table I.

The way in which K^+ interferes can be inferred from the kinetic study of the K^+ inhibition of the '0-ATPase' [18], and from the slope data vs. $[K^+]$ in Fig. 2. Clearly, if '0-ATPase' is the activity of the E_1'-form which is competitively inhibited by K^+, K^+ must bind to E_1' in a dead-end fashion to prevent ATP binding. Also, fitting straight lines to the data in Fig. 2 by linear regression, the slope of these lines decreases when $[Na^+]$ is increased, i.e. the apparent K^+ inhibition constant increases with $[Na^+]$. This increase is linear, not as $[Na^+]^3$ as would be expected if only E_1 could bind Na^+ (see below and Fig. 6). Therefore two Na^+ may bind to the K^+-bound form $E_1'K$ yielding kinetically inactive adducts. On the other hand, $E_1'K$ cannot be converted to E_1K: if K^+-bound forms are present also in the E_1-pool, the resulting slope function, calculated along the lines indicated in the Appendix, would not be linear in the K^+ concentration, as is found experimentally. This point is supported also by the observation [23] that addition of K^+ to the Mg^{2+}-form rapidly produces an E_2-form.

TABLE I

VALUES OF KINETIC PARAMETERS IN EQN. 1 DETERMINED AS DESCRIBED IN THE TEXT

R_1^0 (min)	R_2^0 (min)	K_1 (mM)	K_2 (mM)	K_i (mM)	K_K (mM)	L
$1.22\cdot10^{-3}$	$26.8\cdot10^{-3}$	5.6	12	89	0.23	3.5

The values of K_K and K_2, characterizing the cation sites on the E_1'-form, were obtained as follows:

The model in Fig. 5 predicts that, with two independent and equivalent sites for Na^+ on the $E_1'K$-form, the slope, b, of the lines Y vs. $[K^+]$ Eqn. 1 and Fig. 2 is

$$b = \frac{R_1^0R_2^0L\left(1+\frac{\text{Na}}{K_2}\right)^2}{R_2^0\left(\frac{\text{Na}}{K_1}\right)^2\left(3+\frac{\text{Na}}{K_1}\right)+LR_1^0}\cdot\frac{1}{K_K} \qquad (3)$$

i.e. b should vary as $1/[Na^+]$. This is borne out by the experimentally determined values, as seen from Fig. 6. Using Eqn. 3, we then have

$$C \equiv \left\{\frac{b\left[R_2^0\left(\frac{\text{Na}}{K_1}\right)^2\left(3+\frac{\text{Na}}{K_1}\right)+LR_1^0\right]}{R_1^0R_2^0L}\right\}^{1/2}$$

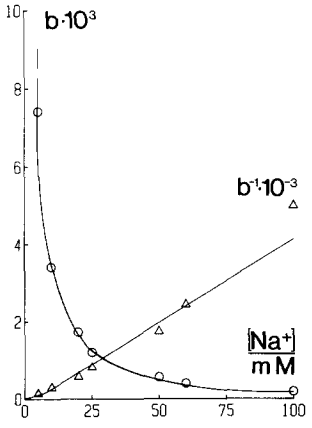


Fig. 6. The slope, b, and reciprocal slope, b^{-1}, determined by linear regression of the data shown in Fig. 2, as a function of the Na^+ concentration. The lines in the figure are drawn between the points as a guide. The values of b obtained were used to calculate the quantity C (Fig. 7). For $[Na^+] > 100$ mM the values of b are statistically ill-determined (they are close to zero) and have not been used in the calculations.

$$= \left(\frac{1}{K_K}\right)^{1/2}\left(1+\frac{Na}{K_2}\right) \quad (4)$$

The quantity C, calculated from the data, b, Fig. 6 and using Table I, is shown as a function of $[Na^+]$ in Fig. 7, which has been used to determine K_K and K_2 (Table I). The values of the kinetic parameters in Table I have been used in Eqn. 1 to obtain the theoretical curves in Figs. 1 and 2.

The value of the parameter R_1^0 (Table I), in conjunction with our previously reported kinetic study of Na^+-ATPase [7], allows an estimate of the intrinsic rate constants k_{11}, k_{-11}, and k_{21} characterizing the E_1-form of the enzyme (see Fig. 5). From the equation defining R_1^0, Eqn. A21, we have

$$E_0 R_1^0 = \frac{K_{d,ATP}}{k_{21}} + \frac{1}{k_{11}} \quad (5)$$

where $K_{d,ATP} \equiv k_{-11}/k_{11}$ is the intrinsic ATP-dissociation constant. In our previous work [7], with $[Na^+] = 150$ mM, we found

$$k'_{21} = 2.2 \cdot 10^3 \text{ min}^{-1}$$

$$K'_d = \frac{k'_{-11}}{k'_{11}} = 0.76\ \mu M$$

where the primes indicate apparent values, i.e. a possible dependence on the Na^+ concentration. From the model in Fig. 5 it is clear that the apparent ATP-splitting constant, k'_{21}, is independent of $[Na^+]$, and hence $k_{21} = 2.2 \cdot 1010^3$ min^{-1}. For K'_d we have, using Eqns. A15 and A16:

$$K'_d(Na^+ = 150 \text{ mM}) = \frac{k_{-11}\left(1+\frac{150}{K_1}\right)^3\left(1+\frac{150}{K_i}\right)}{k_{11}\left(\frac{150}{K_1}\right)^2\left(3+\frac{150}{K_1}\right)}$$

$$= K_{d,ATP} \cdot \tfrac{1}{0.37} = 0.76\ \mu M \quad (6)$$

where Table I has been used. This equation yields $K_{d,ATP} = 0.28\ \mu$M. From Eqn. 5 and the defining equation for $K_{d,ATP}$ the rate constants k_{11} and k_{-11} can be calculated. These values are collected in Table II.

A similar calculation for the form E'_1 cannot be carried out because of lack of knowledge of k_{22} and of the intrinsic affinity for ATP for this enzyme form. Although the similarity of the value of K_m found for '0-ATPase' [18], $K_m = 0.4\ \mu$M to that found above for $K_{d,ATP}$ (0.28 μM), is suggestive, it cannot be concluded (nor, of course, excluded) that the two forms have similar affinities for ATP. It is entirely possible to have an intrinsic affinity of ATP for E'_1 similar to that found for $(Na^+ + K^+)$-ATPase, K_d approx. 200 μM, and still find a very small K_m, provided that the dephosphorylation rate constant (and/or the ATP-splitting rate k_{22}) is very small. A kinetic study of the dephosphorylation of the E'_1-form would shed light on this question.

Our finding that two or three sodium ions must be bound to E_1 before hydrolysis of ATP can occur is gratifying in view of the Na : ATP stoichiometry observed in ADP-ATP exchange [26] and Na^+ efflux [27,28], since both these activities

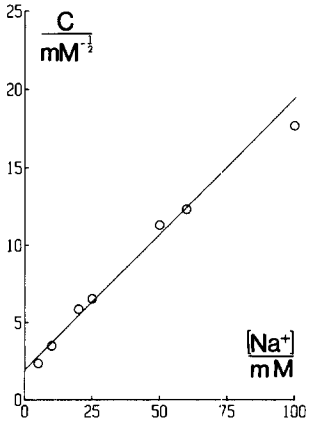


Fig. 7. The quantity C, Eqn. 4, as a function of $[Na^+]$, used for estimating K_K and K_2 (see Fig. 5). The line is the least-squares regression line $C = 2.1 + 0.175\ [Na^+]$.

TABLE II

INTRINSIC RATE CONSTANTS AND ATP-DISSOCIATION CONSTANT ESTIMATED USING TABLE I AND PREVIOUSLY REPORTED RESULTS [7], FOR THE E_1 CONFORMATION (SEE TEXT)

k_{11}	$2.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$
k_{-11}	$6.8\ s^{-1}$
k_{21}	$36.7\ s^{-1}$
$K_{d,ATP}$	$0.28 \cdot 10^{-6}$ M

are believed to be associated with Na^+-ATPase. Similarly, this result is consistent with previous kinetic analyses using fragmented membrane preparations [29,15]. Those results are not directly comparable to ours, however, since in both cases the influence of Na^+ on the activity observed at saturating substrate concentrations, i.e. Na^+ influence on the apparent V_{max}, was studied. Since V_{max} depends on (apparent) rate constants characterizing steps other than those in which the substrate is bound or released (see Appendix), no information pertaining to the cation-enzyme interaction prior to substrate addition can be obtained. This interaction is reflected, for steady-state systems, only in the slope of double-reciprocal plots, which, as seen from Eqns. A2, is just the reciprocal apparent first order rate constant characterizing the rate $v = -da/dt$ when $a \ll K_m$ (a is the substrate concentration).

Our finding that binding of two or three sodium ions leads to phosphorylation of the enzyme is in accord with the recent study of the dephosphorylation kinetics of Na^+-ATPase by Nørby et al. [3]. They found that at least three different phosphoenzyme forms were necessary to explain their data, and that the kinetic intermediate obtained immediately after ADP release in the forward reaction, the 'ADP-sensitive' E_1P, should be regarded as an equilibrium pool consisting of two forms of phosphoenzyme, with two and three sodium ions bound, respectively.

The E_1'-form of the enzyme which we propose to be identical to the conformation induced by Mg^{2+}, has not to our knowledge been incorporated in previous models describing ($Na^+ + K^+$)-ATPase, but is presently receiving some attention. It was first described some years ago by Nakao and co-workers [30,31]. It can be phosphorylated by ATP [31–33], but apparently resists digestion by trypsin [24]. Our finding that it, in contrast to the E_1-form, binds K^+ with high affinity, resulting in competitive inhibition of the ATPase reaction [18], coupled with the observation, using eosin fluorescence, that the Mg^{2+}-form upon addition of K^+ rapidly converts to an E_2 conformation [23], suggests that its importance resides in its being a conformational intermediate between the Na^+-form, E_1, and the K^+-form, E_2. If so, its existence may explain the apparent discrepancy between our result, that E_1 does not bind K^+ while E_1' does (with high affinity), and the work of Jørgensen and Petersen [34], in which it is suggested, on the basis of measurements of fluorescence and Rb^+ binding, that the Na^+-form does exhibit high-affinity Rb^+ (and therefore presumably also K^+) binding sites. Since the change in the eosin fluorescence curve induced by adding Mg^{2+} to the enzyme is the same as that induced by adding Na^+ [23] (except that the optimal curve in the former case is always above that in the latter), it seems plausible to suggest that the form observed by Jørgensen and Petersen to expose high-affinity binding sites for Rb^+ may in fact be the E_1'-form described in the present work, maintaining conformational equilibrium with the E_1-form. The position of this equilibrium would of course depend on the conditions. According to Table I, the equilibrium constant is $L = [E_1']/[E_1] = 3.5$ at pH 7.4 (37°C) and $[Mg^{2+}] = 7$ mM, and thus about 80% of the total amount of enzyme would be found in the E_1'-form in the absence of other ligands.

It might be thought from the model in Fig. 5 that Mg^{2+}, if indeed the E_1' form is favored by Mg^{2+}, should be a competitive inhibitor of Na^+-ATPase. In earlier work [35] we found that this was not the case. However, the kinetic experiments just referred to were carried out at a (constant) Na^+ concentration of 150 mM, and thus, according to the model in Fig. 5, the E_1-form would be strongly favored due to the relatively high affinity of this form for Na^+. In contrast, such competitive Mg^{2+} inhibition, with a $K_i = 7.6$ mM, was observed for ($Na^+ + K^+$)-ATPase [35], determined with $[K^+] = 20$ mM and $[Na^+] = 130$ mM.

It is clear from the derivation of the values of the rate constants in Table II, using Eqns. 5 and 6, that they are independent of the Na^+ concentration. They are also independent of the Mg^{2+} concentration, even though the influence of Mg^{2+} has not been explicitly considered here. We have previously found [35] that Na^+-ATPase is uncompetitively inhibited by Mg^{2+}_{free}: the enzyme-substrate pool E_1^* (Fig. 5) contains a dead-end Mg-complex with a dissociation constant of 4.9 mM. However, the values used in Eqn. 5 for $K_{d,ATP}$ and k_{21}, are the intrinsic values (see Ref. 7 for details), both of which are changed by the same factor, (1 +

$Mg^{2+}/K_{i,Mg})^{-1}$, if the Mg^{2+}-inhibition is included. Thus the value for k_{11} obtained using Eqn. 5 (and hence for k_{-11}) is unchanged. The value obtained for the apparent binding rate constant for ATP, valid at $[Na^+] = 150$ mM, is, according to the model in Fig. 5 (see Eqn. A15) and with the parameters obtained in Table I,

$$k_{11}(Na^+ = 150\ mM) = 5.5 \cdot 10^8\ M^{-1} \cdot min^{-1}$$

in good agreement with the value $4.8 \cdot 10^8\ M^{-1} \cdot min^{-1}$ obtained earlier [7]. k_{-11} is independent of $[Na^+]$, and the value $4.1 \cdot 10^2\ min^{-1}$ is directly comparable to the earlier determination of $3.6 \cdot 10^2\ min^{-1}$ (Table V of Ref. 7).

It was stated in the Introduction that one of the main objectives of the present kinetic investigation was the derivation of a model from which the ATP-splitting constant could be estimated and compared with the corresponding rate constant for $(Na^+ + K^+)$-ATPase, in order to see whether or not the ATP-hydrolysis cycles are partially overlapping, as they are in the Albers-Post scheme. In the following paper [8] we discuss the kinetic evidence obtained for $(Na^+ + K^+)$-ATPase which allows us to calculate the corresponding rate constant for that activity.

Appendix

We summarize here a few properties of the kinetics parameters derived from simple models of Na^+-ATPase action. The discussion is limited to the properties of the slope from double-reciprocal plots, $1/v$ vs. $[MgATP]^{-1}$, and its dependence on the concentrations of Na^+ and K^+. Rapid equilibrium of these ligands with the enzyme forms are assumed throughout. Also, multiple sites for a ligand are assumed to be equivalent and independent, and the intrinsic (site) dissociation constants are independent of whether or not substrate is bound to the enzyme.

A. Single-cycle model

For the kinetic scheme

$$E \underset{-1}{\overset{1}{\rightleftharpoons}} EATP \overset{2}{\rightarrow} EP \overset{3}{\rightarrow} E \qquad (A1)$$

the steady-state rate in the absence of products is

$$v = \frac{V \cdot a}{K_m + a} \rightarrow v^{-1} = \frac{K_m}{V} \cdot \frac{1}{a} + \frac{1}{V} \qquad (A2)$$

where a is the substrate concentration, [MgATP], and

$$\text{slope} = \frac{K_m}{V} = \frac{1}{E_0} \frac{k'_{-1} + k'_2}{k'_1 k'_2} \qquad (A3)$$

$$\text{intercept} = \frac{1}{V} = \frac{1}{E_0}\left(\frac{1}{k'_2} + \frac{1}{k'_3}\right) \qquad (A4)$$

$$K_m = \frac{k'_3(k'_{-1} + k'_2)}{k'_1(k'_2 + k'_3)} \qquad (A5)$$

To obtain the slope, only the rate constants characterizing the first two steps in the mechanism need to be specified. This is true irrespective of the complexity of the mechanism subsequent to the state EP in Eqn. A1.

If a ligand, Na^+, influences the kinetics, under the conditions set forth above, each of the states in Eqn. A1 is to be considered an equilibrium pool with ligand bound to various degrees, and the apparent rate constants appearing in Eqns. A3–A5 are functions of the ligand concentration. These functions are calculated using the procedure of Cha [36].

We consider the situation with three sites for Na^+ on the enzyme. Generalization of the arguments to any number of sites is straight forward. The possible contents of the first two pools in the scheme A1 is shown in Fig. 8, where an asterisk is used to indicate the presence on the enzyme of substrate. Since the site constants for Na^+ are equal in the two pools, the requirement for detailed balance at equilibrium demands that the ratios of forward and backward rate constants connecting corresponding species in the two pools are also equal. The simplest possibility is to assume that all forward rate constants are equal, as are the back-

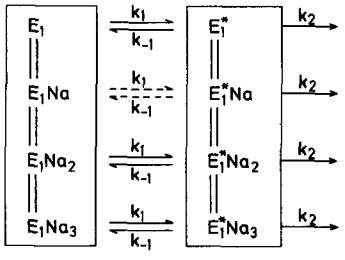


Fig. 8. The possible contents of the first two equilibrium pools with ligand (Na^+) bound to various degrees.

ward rate constants. This is indicated in the figure.

If all the species in pool 1 are kinetically competent, i.e. accept substrate (the dotted arrows in Fig. 8 are included), then the effective rate constants are all equal to the intrinsic constants. Hence, when these are used in Eqn. A3, the slope function becomes independent of Na^+. The experimental values shown in Fig. 1a thus rule out this possibility.

As an alternative, we consider, as an example, the case where E, ENa_2, and ENa_3 all accept ATP, but ENa does not. This corresponds to omitting the dotted arrows in Fig. 8. Using Cha's procedure, the apparent rate constants are

$$k'_1 = k_1 \frac{(1+3x^2+x^3)}{(1+x)^3} \tag{A6}$$

$$k'_{-1} = k_{-1} \tag{A7}$$

$$k'_2 = k_2 \tag{A8}$$

where the unprimed k values are the intrinsic values, and $x \equiv [Na^+]/K_{Na}$; K_{Na} is the site dissociation constant for Na^+. Inserting Eqns. A6–A8 in Eqn. A3 we obtain

$$\text{slope} = \frac{1}{E_0} \frac{k_{-1}+k_2}{k_1 k_2} \cdot \frac{(1+x)^3}{(1+3x^2+x^3)} \tag{A9}$$

From this equation it is clear that

$$\text{slope}(x=0) = \text{slope}(x \to \infty) = \frac{1}{E_0} \frac{k_{-1}+k_2}{k_1 k_2} \tag{A10}$$

Since experimentally it is found that slope ($x = 0$) is finite (and different from the slope at high Na^+), requiring retainment of the constant term in the denominator of Eqn. A9, and bearing in mind that ENa_3 must be kinetically competent to account for the well known stoichiometry Na : ATP = 3 : 1, it is seen that the property expressed in Eqn. A10 characterizes all models of the type in Fig. 8, which therefore cannot explain the experimental results, Fig. 1.

B. Two-cycle model

The simplest formal kinetic scheme of this type is seen in Fig. 4. The enzyme may exist in two interconvertible conformations, each of which may independently hydrolyze the substrate. Due to the single-step connection between the two cycles, this scheme also exhibits simple Michaelis-Menten kinetics, as in Eqn. A2. Using standard procedures to derive the rate equation [37], it is found that the slope of a double reciprocal plot in this case may be written

$$\text{slope} = \frac{R'_1 R'_2 (1+L')}{R'_2 + L' R'_1} \tag{A11}$$

where

$$R'_1 = \frac{1}{E_0} \frac{k'_{-11}+k'_{21}}{k'_{11}k'_{21}}$$

$$R'_2 = \frac{1}{E_0} \frac{k'_{-12}+k'_{22}}{k'_{12}k'_{22}} \tag{A12}$$

$$L' = \frac{k'_{-0}}{k'_0}$$

It is noted that, apart from the interconversion rate constants k'_0 and k'_{-0}, the slope, in analogy to the simpler case A above, contains only the rate constants characterizing the first two steps in each cycle. For the purpose of slope derivation, therefore, the number of formally isomeric intermediates subsequent to the enzyme-substrate adduct in each cycle is irrelevant.

The procedure for obtaining the slope as a function of Na^+ (K^+ where relevant) is then as before to assume a structure for the kinetic pools, derive, using Cha's procedure, the resulting apparent rate constants for insertion in Eqn. A11, and investigate the qualitative and quantitative properties of the resulting function. For the model arrived at, based on arguments presented in the main text, and shown in Fig. 5, the apparent rate constants are:

$$k'_{12} = \frac{k_{12}}{1+y(1+x_2)^2} \tag{A13}$$

$$k'_{-12} = k_{-12};\ k'_{22} = k_{22} \tag{A14}$$

$$k'_{11} = \frac{k_{11}x_1^2(3+x_1)}{(1+x_1)^3(1+x_i)} \tag{A15}$$

$$k'_{-11} = k_{-11};\ k'_{21} = k_{21} \tag{A16}$$

$$k'_0 = \frac{k_0}{1+y(1+x_2)^2} \tag{A17}$$

$$k'_{-0} = \frac{k_{-0}}{(1+x_1)^3(1+x_i)} \tag{A18}$$

$$x_1 = \frac{[Na^+]}{K_1};\ x_2 \equiv \frac{[Na^+]}{K_2};\ x_i \equiv \frac{[Na^+]}{K_{iNa}} \tag{A19}$$

$$y \equiv \frac{[K^+]}{K_K} \tag{A20}$$

When the expressions are inserted in Eqns. A12 we obtain for the quantities R'_1 and R'_2:

$$R'_1 = \frac{1}{E_0}\frac{k_{-11}+k_{21}}{k_{11}k_{21}} \cdot \frac{(1+x_1)^3(1+x_i)}{x_1^2(3+x_1)}$$

$$\equiv R_1^0 \frac{(1+x_1)^3(1+x_i)}{x_1^2(3+x_1)} \tag{A21}$$

$$R'_2 = \frac{1}{E_0}\frac{k_{-12}+k_{22}}{k_{12}k_{22}}\left[1+y(1+x_2)^2\right] \equiv R_2^0\left[1+y(1+x_2)^2\right] \tag{A22}$$

and for the apparent equilibrium constant L':

$$L' = \frac{k_{-0}}{k_0}\frac{\left(1+y(1+x_2)^2\right)}{(1+x_1)^3(1+x_i)} \equiv L\frac{\left(1+y(1+x_2)^2\right)}{(1+x_1)^3(1+x_i)} \tag{A23}$$

Finally, the slope function, Eqn. A11, becomes, after rearrangement:

$$\text{slope} = \frac{R_1^0 R_2^0(1+x_1)^3(1+x_i)}{R_2^0 x_1^2(3+x_1)+LR_1^0} \times \left\{1+\frac{L\left[1+y(1+x_2)^2\right]}{(1+x_1)^3(1+x_i)}\right\} \tag{A24}$$

which is identical to Eqn. 1 in the main text.

The following properties of Eqn. A24 may be derived by inspection:

(a) If $[Na^+]=[K^+]=0$, i.e. $x_1 = x_2 = x_i = y = 0$ we have

$$\text{slope}\,(0,0) = R_2^0\frac{1+L}{L} \tag{A25}$$

(b) At $[K^+]=0$ (i.e. $y=0$), and if (cf. Fig. 5) $K_1 \approx K_2 \ll K_i$, then for $K_1,\ K_2 \ll [Na^+] \approx K_i$:

$$\text{slope} \simeq R_1^0(1+x_i) \tag{A26}$$

i.e. the slope is a linear function of $[Na^+]$.

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