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Electrogenic Properties of the Sodium-Alanine Cotransporter in Pancreatic Acinar Cells: II. Comparison with Transport Models

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Summary. In this paper, the results of the preceding electrophysiological study of sodium-alanine cotransport in pancreatic acinar cells are compared with kinetic models. Two different types of transport mechanisms are considered. In the "simultaneous" mechanism the cotransporter C forms a ternary complex NCS with Na^+ and the substrate S; coupled transport of Na^+ and S involves a conformational transition between states $NC'S$ and $NC''S$ with inward- and outward-facing binding sites. In the "consecutive" (or "ping-pong") mechanism, formation of a ternary complex is not required; coupled transport occurs by an alternating sequence of association-dissociation steps and conformational transitions. It is shown that the experimentally observed alanine- and sodium-concentration dependence of transport rates is consistent with the predictions of the "simultaneous" model, but incompatible with the "consecutive" mechanism. Assuming that the association-dissociation reactions are not rate-limiting, a number of kinetic parameters of the "simultaneous" model can be estimated from the experimental results. The equilibrium dissociation constants of Na^+ and alanine at the extracellular side are determined to be $K''_N \leq 64$ mM and $K''_S \leq 18$ mM. Furthermore, the ratio K''_N/K''^{S}_N of the dissociation constants of Na^+ from the binary (NC) and the ternary complex (NCS) at the extracellular side is estimated to be ≤ 6. This indicates that the binding sequence of Na^+ and S to the transporter is not ordered. The current-voltage behavior of the transporter is analyzed in terms of charge translocations associated with the single-reaction steps. The observed voltage-dependence of the half-saturation concentration of sodium is consistent with the assumption that a Na^+ ion that migrates from the extracellular medium to the binding site has to traverse part of the transmembrane voltage.

Key Words cotransport · electrogenic transport · sodium-coupled amino-acid transport · current-voltage characteristic

Introduction

In the accompanying paper (Jauch, Petersen & Läuger, 1986) an electrophysiological study of the sodium-alanine cotransport system in pancreatic acinar cells has been described. Using the method of tight-seal whole-cell recording with internal perfusion of the pipette, alanine-coupled sodium-currents could be measured as a function of intra- and extracellular concentrations of sodium and alanine. Since the sodium-alanine cotransport system contributes appreciably to the total membrane conductance, it has been possible to study the current-voltage characteristic of the cotransporter at different sodium concentrations. Both the sodium-concentration dependence of alanine-induced current as well as the reversal potential at a given concentration ratio of alanine indicate that the sodium/alanine coupling ratio is close to unity. In this second part of the paper we discuss the experimental results in terms of microscopic transport models based on a sodium/alanine stoichiometry of 1 : 1.

Microscopic Models for Ion-Driven Substrate Transport

At least two different classes of mechanisms of ion-coupled cotransport are feasible (Schultz & Curran, 1970; Stein, 1976; Crane & Dorando, 1980; Eddy, 1980; Hopfer & Groseclose, 1980; Turner & Silverman, 1980; Turner, 1981, 1983; Harrison, Rove, Lumsden & Silverman, 1984; Sanders, Hansen, Gradmann & Slayman, 1984; Semenza, Kessler, Hosang, Weber & Schmidt, 1984; Restrepo & Kimmich, 1985*a*; Sanders, 1986; Wright, 1986). In the "simultaneous" mechanism the ion N and the substrate S bind to the transporter C, forming a ternary complex NCS which alternates between states $NC'S$ and $NC''S$ with inward-facing and outward-facing binding sites. In the "consecutive" (or "ping-pong") mechanism the transporter in state C_1 binds N at the extracellular side ($C_1 + N_{ext} \rightarrow C_1N$), undergoes a conformational transition ($C_1N \rightarrow NC_2$) and releases the ion to the cytoplasm ($NC_2 \rightarrow C_2 + N_{cyt}$). When the transporter is in state C_2, the substrate S may be bound from the extracellular side and released to the cytoplasm after conformational rearrangement ($S_{ext} + C_2 \rightarrow SC_2 \rightarrow C_1S \rightarrow S_{cyt}$

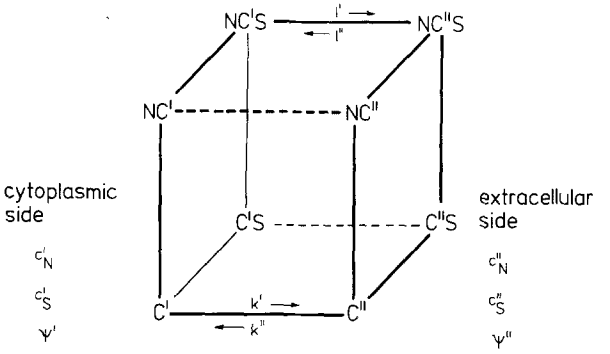


Fig. 1. "Simultaneous" mechanism for the transport of ion N and substrate S by the cotransporter C. The binding sites for N and S are alternately accessible from the cytoplasmic side (states C', NC', CS', $NC'S$) and from the extracellular side (states C'', NC'', $C''S$, $NC''S$). It is assumed that conformational transitions switching the binding sites from the inward-facing to the outward-facing configuration are possible only in the empty and fully occupied states (C and NCS). c'_N, c''_N, c'_S and c''_S are cytoplasmic and extracellular concentrations of N and S; ψ' and ψ'' are the electrical potentials on the cytoplasmic and extracellular side, respectively

$+ C_1$). In the following we discuss the kinetic properties of these two transport models for later comparison with experimental data.

"Simultaneous" Mechanism

This transport mechanism, which involves the formation of a ternary complex NCS between ion N, substrate S and transporter C, is schematically represented in Fig. 1. The binding sites for N and S are alternately accessible from the cytoplasmic side (states C', NC', $C'S$, $NC'S$) and from the extracellular side (states C'', NC'', $C''S$, $NC''S$).

In the following we assume that the conformational transitions moving the binding sites from an inward-facing to an outward-facing configuration are possible only in the empty ($C' \leftrightarrow C''$) and in the fully occupied state ($NC'S \leftrightarrow NC''S$). This assumption which implies complete coupling of the fluxes of N and S is consistent with the finding (Jauch et al., 1986) that the alanine-independent Na^+-current is only a small fraction of the total current which is measured at saturating alanine concentrations. We further assume that the rate constants for binding and release of N and S are large so that the association-dissociation reactions in the membrane-solution interface are always in equilibrium. This assumption, which is frequently used in the analysis of cotransport systems (Schultz, 1986) can be justified only *a posteriori* by comparison between theoretical predictions and experimental results.

Denoting the fraction of transporter molecules which are in state A by $x[A]$, the association-dissociation reactions at the cytoplasmic interface are described by equilibrium constants K'_N, K'_S, $K_N^{S'}$ and $K_S^{N'}$:

$$K'_N = \frac{x[C']c'_N}{x[NC']}; \qquad K_N^{S'} = \frac{x[C'S]c'_N}{x[NC'S]} \tag{1}$$

$$K'_S = \frac{x[C']c'_S}{x[C'S]}; \qquad K_S^{N'} = \frac{x[NC']c'_S}{x[NC'S]} \tag{2}$$

c'_N and c'_S are the cytoplasmic concentrations of Na^+ and S; $K_N^{S'}$ and $K_S^{N'}$ are the equilibrium constants for the dissociation of Na^+ and S, respectively, from the ternary complex $NC'S$. Analogous relations hold for the extracellular interface. The equilibrium constants K'_N, $K_N^{S'}$, etc., as well as the translocation rate constants k', k'', l' and l'' (Fig. 1) depend, in general, on membrane potential $V \equiv \psi' - \psi''$. According to the principle of microscopic reversibility, the kinetic constants are connected by

$$\frac{K'_N}{K_N^{S'}} = \frac{K'_S}{K_S^{N'}}; \qquad \frac{K''_N}{K_N^{S''}} = \frac{K''_S}{K_S^{N''}} \tag{3}$$

$$\frac{K''_N K_S^{N''}}{K'_N K_S^{N'}} \cdot \frac{k''l'}{k'l''} = \exp(u) \tag{4}$$

$$u \equiv \frac{\psi' - \psi''}{RT/F} = \frac{V}{RT/F}. \tag{5}$$

R is the gas constant, T the absolute temperature, and F the Faraday constant. Equation (3) follows directly from Eqs. (1) and (2); for a derivation of Eq. (4), *see* Läuger (1984).

If the membrane contains N transporter molecules per unit area, the electric current density I associated with sodium-coupled substrate transport is given by

$$I = e_o N(l'x[NC'S] - l''x[NC''S]) \tag{6}$$

where e_o is the elementary charge. Introducing the abbreviations

$$n \equiv \frac{c_N}{K_N}; \; s \equiv \frac{c_S}{K_S} \tag{7}$$

$$Q \equiv \frac{K_N}{K_N^S} = \frac{K_S}{K_S^N} \tag{8}$$

$$P \equiv 1 + n + s + nsQ \tag{9}$$

and using the notation $n' \equiv c'_N/K'_N$, $Q' \equiv K'_N/K_N^{S'}$, etc., the following relation is obtained for the current density I (Turner & Silverman, 1980):

$$I = \frac{e_o N}{\chi} \frac{k' l''}{K_N'' K_S^{N''}} [c_N' c_S' \exp(u) - c_N'' c_S''] \quad (10)$$

$$\chi \equiv P'(k'' + l'' n'' s'' Q'') + P''(k' + l' n' s' Q']. \quad (11)$$

For the experimental determination of kinetic parameters it is advantageous to measure the current at finite concentrations of Na^+ and S on one side and vanishing concentrations on the other ("zero-*trans*" experiment). Under this condition the expression for the current considerably simplifies. If the inward current $I'' \equiv I(c_N' = c_S' = 0)$ is measured for vanishing cytoplasmic concentrations of Na^+ and S, Eqs. (10) and (11) reduce to

$$I'' = I_{\infty,S}'' \frac{c_S''}{c_S'' + L_S''} \quad (12)$$

$$L_S'' \equiv K_S'' \frac{1 + k''/k' + c_N''/K_N''}{1 + c_N''(1 + l''/k')/K_N^{S''}} \quad (13)$$

$$I_{\infty,S}'' \equiv I_\infty'' \frac{c_N''}{c_N'' + K_N^{S''}/(1 + l''/k')} \quad (14)$$

$$I_\infty'' \equiv -e_o N(1/k' + 1/l'')^{-1}. \quad (15)$$

L_S'' is the half-saturation concentration of S, $I_{\infty,S}''$ the maximum current for $c_S'' \to \infty$ and I_∞'' the maximum current which is observed when both c_N'' and c_S'' are very large. Under the conditions $k'', l'' \ll k'$, $K_N'' \approx K_N^{S''}$, or $k'' \ll k'$, $c_N'' \to 0$, L_S'' becomes equal to the equilibrium dissociation constant K_S''. In general, however, L_S'' is a function of extracellular Na^+ concentration c_N''. When the inward current is measured as a function of c_N'', an equivalent form of Eq. (12) may be used:

$$I'' = I_{\infty,N}'' \frac{c_N''}{c_N'' + L_N''} \quad (16)$$

$$L_N'' \equiv K_N'' \frac{1 + k''/k' + c_S''/K_S''}{1 + c_S''(1 + l''/k')/K_S^{N''}} \quad (17)$$

$$I_{\infty,N}'' \equiv I_\infty'' \frac{c_S''}{c_S'' + K_S^{N''}/(1 + l''/k')}. \quad (18)$$

Analogous equations for the outward current $I' \equiv I(c_N'' = c_S'' = 0)$ are obtained by exchanging the superscripts ' and " in Eqs. (12)–(18).

"Consecutive" (or "Ping-Pong") Mechanism

The "consecutive" mechanism does not require formation of a ternary complex NCS; the conformational transitions occur when either N *or* S is bound. As depicted in Fig. 2, in conformation C_1 the transporter binds N from the extracellular side. Thereafter a transition to state C_2N can occur, followed by release of N to the cytoplasm. In state C_2 the substrate S is bound from the extracellular side and released to the cytoplasm after the conformational transition $SC_2 \to SC_1$ has taken place.

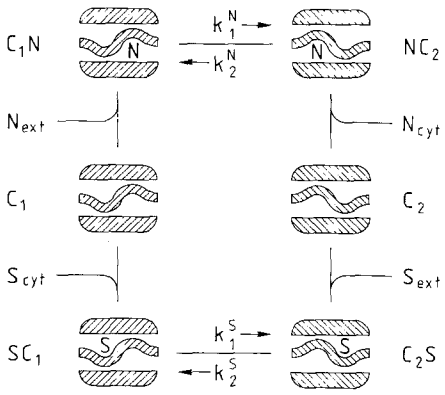


Fig. 2. "Consecutive" (or "ping-pong") mechanism for the coupled transport of ion N and substrate S. The cotransporter C can exist in two conformations C_1 and C_2. C_1 binds N from the extracellular side *or* S from the cytoplasm; C_2 binds N from the cytoplasm *or* S from the extracellular side. When the cotransporter goes through the cycle in clockwise direction starting in state C_1, N is bound from the extracellular side and released to the cytoplasm after the conformational transition $C_1N \to NC_2$ has taken place. In state C_2 a substrate molecule S is bound from the extracellular medium and released to the cytoplasm

We assume that direct transitions between C_1 and C_2 do not occur (Fig. 2), so that the fluxes of N and S are completely coupled. For a discussion of the more general case of arbitrary coupling ratio, *see* Ciani (1984) and Läuger (1985). We further assume (as before) that the binding/unbinding reactions at the interface are always in equilibrium. In analogy to Eqs. (1) and (2) equilibrium dissociation constants K_N', K_N'', K_S' and K_S'' may be introduced:

$$K_N' = \frac{x[C_2]c_N'}{x[C_2N]}; \qquad K_N'' = \frac{x[C_1]c_N''}{x[NC_1]} \quad (19)$$

$$K_S' = \frac{x[C_1]c_S'}{x[SC_1]}; \qquad K_S'' = \frac{x[C_2]c_S''}{x[C_2S]}. \quad (20)$$

(Note that in the "consecutive" mechanism N and S bind to different states (C_1 and C_2) of the transporter, whereas in the "simultaneous" mechanism K_N' and K_S' refer to the same state C'.)

In addition to the equilibrium dissociation constants, the model contains the rate constants k_1^N, k_1^S, k_2^N and k_2^S for transitions between conformations 1 and 2 (Fig. 2). According to the principle of microscopic reversibility, the equilibrium constants and rate constants are connected by

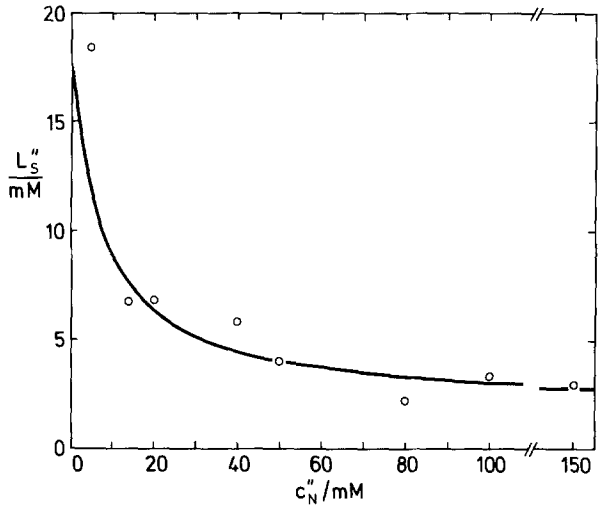


Fig. 3. Half-saturation concentration L_S'' of L-alanine at the extracellular site as a function of extracellular sodium concentration c_N'' [Eq. (12)]. The experimental points have been taken from Fig. 11 of the companion paper and from three additional experiments similar to those represented in that figure. The curve has been drawn according to Eq. (13) using the following parameter values: $K_S''(1 + k''/k') = 18$ mM, $K_S''/K_N'' = 0.28$, and $(1 + l''/k')/K_N^{S''} = 0.14$ mM^{-1}

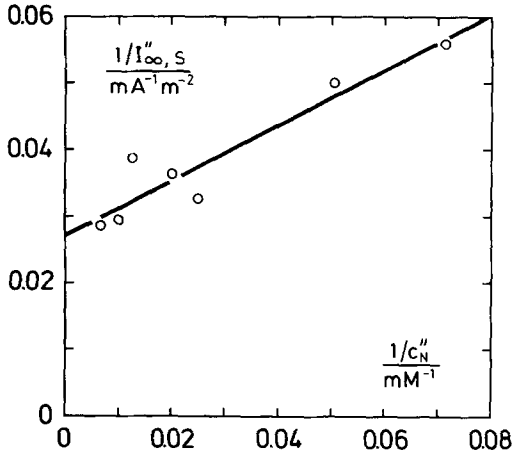


Fig. 4. Double-reciprocal plot of the maximum-inward current $I''_{\infty,S}$ at infinite extracellular alanine concentration ($c_S'' \to \infty$), as a function of extracellular sodium concentration c_N''. $I''_{\infty,S}$ is referred to unit area of the cell membrane. The experimental points have been taken from Fig. 11 of the companion paper. The straight line has been obtained by linear regression (using $I''_{\infty,S}$ as weighting factor) according to Eq. (14) with $I''_\infty = -37.0$ mA/m^2 and $K_N^{S''}/(1 + l''/k') = 15.1$ mM

$$\frac{K_N'' K_S''}{K_N' K_S'} \cdot \frac{k_2^N k_1^S}{k_1^N k_2^S} = \exp(u). \tag{21}$$

The current I may be calculated from the relation

$$I = e_o N(k_2^N x[NC_2] - k_1^N x[C_1 N]). \tag{22}$$

The result reads:

$$I = \frac{e_o N}{\theta} \frac{k_1^N k_2^S}{K_N'' K_S''} [c_N' c_S' \exp(u) - c_N'' c_S'']. \tag{23}$$

$$\theta \equiv (1 + n' + s'')(k_1^N n'' + k_1^S s') + (1 + n'' + s')(k_2^N n' + k_2^S s'') \tag{24}$$

where $n' \equiv c_N'/K_N'$, $s' \equiv c_S'/K_S'$, etc., as before.

In the zero-*trans* experiment, the inward current I'' is given again by Eq. (12), with

$$L_S'' \equiv K_S'' \frac{a''}{1 + a''} \cdot \frac{c_N''}{c_N'' + K_N''/(1 + a'')} \tag{25}$$

$$I''_{\infty,S} \equiv I''_\infty \frac{c_N''}{c_N'' + K_N''/(1 + a'')} \tag{26}$$

$$I''_\infty \equiv -e_o N(1/k_1^N + 1/k_2^S)^{-1} \tag{27}$$

where $a'' \equiv k_1^N/k_2^S$. By comparing Eqs. (25) and (26) with Eqs. (13) and (14) it is seen that in the "consecutive" model, L_S'' vanishes for small c_N'', whereas L_S'' remains finite for $c_N'' \to 0$ in the "simultaneous" model. Furthermore, the ratio $I''_{\infty,S}/L_S''$ is independent of sodium concentration c_N'' in the "consecutive" model, but is a function of c_N'' in the "simultaneous" model. From the general treatment of Ciani (1984) it may be shown that both conclusions (L_S'' proportional to c_N'' for small c_N'' and $I''_{\infty,S}/L_S''$ independent of c_N'') still hold for arbitrary rate constants of the association/dissociation reactions, provided that the rate of direct interconversion $C_1 \leftrightarrow C_2$ is vanishingly small.

Analysis of Inward Currents as a Function of c_N'' and c_S''

In the accompanying paper (Jauch et al., 1986) experiments have been described in which the inward current I'' has been measured under "zero-*trans* conditions, i.e., for vanishing intracellular concentrations of Na^+ and S ($c_N' = c_S' = 0$) and finite concentrations c_N'' and c_S'' in the external medium. The dependence of I'' on c_N'' and c_S'' could be represented by a Michaelis-Menten relation of the type of Eqs. (12) or (16). Equations (12) and (16) are equivalent theoretical representations of $I''(c_N'', c_S'')$; Eq. (12) contains the maximum current $I''_{\infty,S}$ and the half-saturation concentration L_S'' which are functions of c_N'', whereas Eq. (16) contains $I''_{\infty,S}$ and L_N'' which depend on c_S''. The functions $L_S''(c_N'')$, $I''_{\infty,S}(c_N'')$, $L_N''(c_S'')$ and $I''_{\infty,N}(c_S'')$ are represented in Figs. 3–6. L_S'' is found to decrease with increasing c_N'', approaching a finite value ($L_S'' \simeq 2$ mM) for $c_N'' \to \infty$ (Fig. 3). A similar concentration dependence is observed for the half-

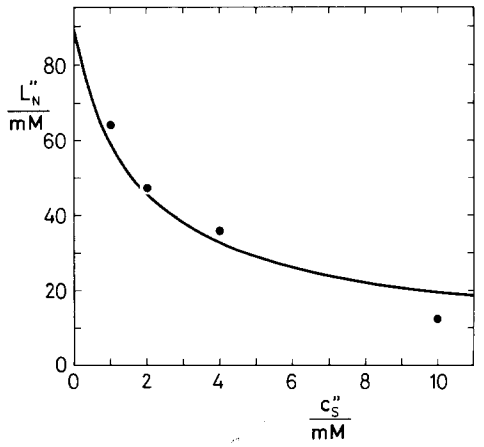


Fig. 5. Half-saturation concentration L''_N of Na^+ at the extracellular site as a function of extracellular alanine concentration c''_S [Eq. (16)]. The experimental points have been taken from Figs. 12 and 13 of the companion paper. The curve has been drawn according to Eq. (17) using the following parameter values: $K''_N(1 + k''/k') = 90$ mM, $K''_N/K''_S = 4.66$, and $(1 + l''/k')/K''^{N''}_S = 0.125$ mM^{-1}

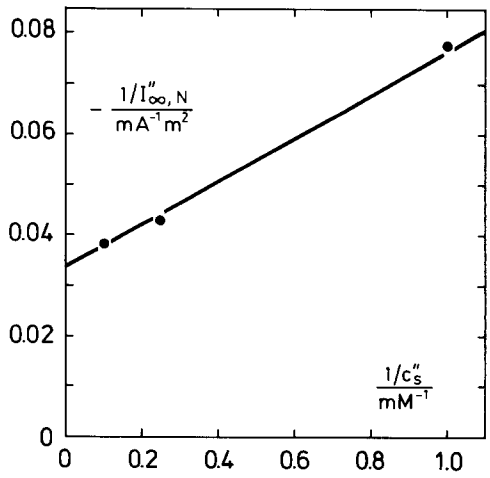


Fig. 6. Double-reciprocal plot of the maximum inward-current $I''_{\infty,N}$ at infinite extracellular Na^+ concentration ($c''_N \to \infty$), as a function of extracellular alanine concentration c''_S. $I''_{\infty,N}$ is referred to unit area of the cell membrane. The experimental points have been taken from Fig. 13 of the companion paper. The straight line has been drawn according to Eq. (18) with $I''_\infty = -30$ mA/m² and $K''^{N''}_S/(1 + l''/k') = 1.27$ mM

saturation concentration of sodium, L''_N (Fig. 5). Thus, binding of one substrate decreases the half-saturation concentration of the other substrate.

As seen from Figs. 4 and 5, $1/I''_{\infty,S}$ and $1/I''_{\infty,N}$ are linear functions of $1/c''_N$ and $1/c''_S$, respectively, as expected from Eqs. (14) and (18). In accordance with Eqs. (14) and (18), the extrapolated values $I''_{\infty,S}(c''_N \to \infty)$ and $I''_{\infty,N}(c''_S \to \infty)$ agree within the limits of experimental error (−37 and −30 mA/m²).

Experimental Discrimination between "Simultaneous" and "Consecutive" Models

The experimental results represented in Figs. 3 and 4 are essential for a distinction between "simultaneous" and "consecutive" models. According to Eq. (25) the half-saturation concentration L''_S is predicted to vanish in the limit of low external sodium concentration ($c''_N \to 0$) in the case of the "consecutive" (or "ping-pong") mechanism. This prediction is at variance with the experimentally observed sodium-concentration dependence of L''_S; as seen from Fig. 3, L''_S approaches a finite value in the limit $c''_N \to 0$. Furthermore, according to Eqs. (25) and (26), the ratio $L''_S/I''_{\infty,S}$ should be independent of c''_N, whereas the experimental values of $L''_S/I''_{\infty,S}$ clearly vary with extracellular sodium concentration. From these findings the model of Fig. 2 can be excluded as a possible mechanism of the Na^+, alanine cotransporter in pancreatic acinar cells. By similar arguments Kessler and Semenza (1983) and Restrepo and Kimmich (1985) came to the conclusion that the intestinal Na^+, sugar cotransporter does not function by a "consecutive" mechanism.

Evaluation of Kinetic Parameters of the "Simultaneous" Mechanism from Experimental Data

It is seen from Eqs. (13) and (14) that the following four independent parameters may be determined from the sodium-concentration dependence of L''_S and $I''_{\infty,S}$: $K''_N(1 + k''/k')$, K''_N/K''_S, $K''^{S''}_N/(1 + l''/k')$ and I''_∞. Since $L''_N(c''_S)$ and $I''_{\infty,N}(c''_S)$ contain exactly the same combinations of kinetic constants, no additional information is obtained from Eqs. (17) and (18). However, in order to minimize influences of measuring errors, it is preferable to use all experimental quantities (L''_S, L''_N, $I''_{\infty,S}$, $I''_{\infty,N}$) for numerical evaluation. By averaging the numerical values given in the legends of Figs. 3–6, the following estimates of kinetic parameters are obtained:

$$K''_N(1 + k''/k') \simeq 77 \text{ mM}$$
$$K''_N/K''_S = K''^{S''}_N/K''^{N''}_S \simeq 4.1$$
$$K''^{S''}_N/(1 + l''/k') \simeq 16 \text{ mM}$$
$$I''_\infty = -e_o N(1/k' + 1/l'')^{-1} \simeq -33 \text{ mA/m}^2.$$

From $K''_N(1 + k''/k')$, the equilibrium dissociation constant of sodium at the external site is found to be $K''_N < 77$ mM. This means that the external sodium site (with no amino-acid bound) is close to saturation at physiological Na^+ concentrations.

According to $K''_N/K''_S \simeq 4.1$ and $K''_N < 77$ mM, the dissociation constant K''_S of alanine must be smaller than 19 mM.

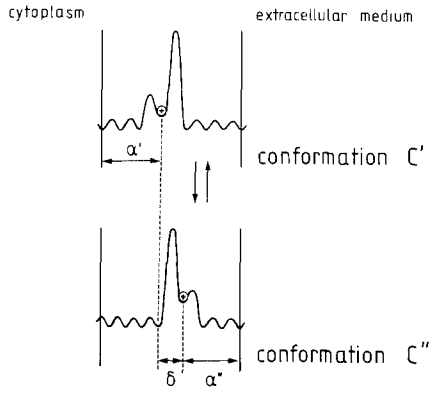


Fig. 7. Energy profile of the driving ion (Na^+) along the transport pathway. In conformation C' of the transporter, the ion-binding site is accessible from the cytoplasm, and in conformation C'' from the extracellular medium. α', α'' and δ are the relative dielectric distances along the pathway of the ion

For the dissociation constant of sodium from the ternary complex $NC''S$, a lower limit, $K_N^{S''} > 16$ mM, can be estimated from the value of $K_N^{S''}/(1 + l''/k')$ as given above. Since K_N''/K_S'' is about 4.1, this means, according to Eq. (3), that $K_S^{N''}$ must be larger than 16 mM/4.1 $\simeq$ 3.9 mM.

The limiting value of $I''_{\infty,S}$ for $c_N'' \to \infty$ (Fig. 4) represents the maximum possible inward current ($I''_\infty \simeq -30$ mA/m^2) which can be supplied by the cotransport system under the condition $c_N' = c_S' = 0$, c_N'', $c_S'' \to \infty$. According to Eq. (15), I''_∞ is proportional to the product of the number N of transporter molecules per unit area of the membrane times the maximum turnover rate for inward flux, $f'' \equiv (1/k' + 1/l'')^{-1}$. From steady-state current measurements alone it is not possible to evaluate N and f'' separately. Since in voltage-pulse experiments (Jauch et al., 1986) no alanine-dependent transient could be detected in the time-range above 10 msec, it is likely that the smallest rate constant of the overall transport reaction is larger than 100 sec^{-1}. With $I''_\infty \simeq -30$ mA/m^2, this would mean that N is smaller than 2000 μm^{-2}.

Sidedness of the Cotransporter

In the companion paper experiments have been described in which outward and inward currents have been recorded under mirror-symmetrical conditions, i.e., for equal but opposite concentration gradients. In the whole experimental range of c_N and c_S, the outward current I' (measured under the condition $c_N' = c_N$, $c_S' = c_S$, $c_N'' = c_S'' = 0$) was found to be equal within a factor of two to the inward current I'' (measured under the condition $c_N' = c_S' = 0$, $c_N'' = c_N$, $c_S'' = c_S$). If the relation $I' \simeq I''$ holds for all values of c_N and c_S, the coefficients of c_N and c_S in the expressions for I' and I'' [Eqs. (12)–(15)] must be approximately equal. This means that

$$K_N'(1 + k'/k'') \simeq K_N''(1 + k''/k')$$
$$K_N'/K_S' = K_N^{S'}/K_S^{N'} \simeq K_N''/K_S'' = K_N^{S''}/K_S^{N''}$$
$$K_N^{S'}/(1 + l''/k') \simeq K_N^{S''}/(1 + l'/k'')$$
$$1/k' + 1/l'' \simeq 1/k'' \simeq 1/l''.$$

These relations could be satisfied assuming that the cotransporter is symmetrical with respect to all kinetic parameters ($K_N' \simeq K_N''$, $k'' \simeq k''$, etc.). Such a detailed functional symmetry would be surprising, however, in view of the fact that a membrane-spanning molecule is likely to be structurally asymmetric.

Current-Voltage Characteristic

Since sodium-coupled substrate transport is associated with charge translocation, the transport rate must depend on membrane potential. By measuring the current-voltage characteristic of the cotransport system, information on the nature of the individual charge-translocation steps may be obtained (Geck & Heinz, 1976; Turner, 1981; Kessler & Semenza, 1983; Restrepo & Kimmich, 1985*b*; Läuger & Jauch, 1986). The interpretation of experimental current-voltage curves is complicated by the fact that, in principle, any single step in the transport cycle may be affected by the electric field. For a complete analysis of $I(V)$ curves the numerical values of some of the rate constants of the transport cycle must be known. As long as such detailed information is not available, a number of simplifying assumptions have to be introduced. In the following we assume, as before, that the binding reactions are at equilibrium and that the fluxes of Na^+ and S are completely coupled.

When the current-voltage characteristic is studied under the condition of vanishing intracellular concentrations of Na^+ and S ($c_N' = c_S' = 0$), only the external binding equilibria have to be considered. Since the substrate S is electrically neutral, the dissociation constants K_S'' and $K_S^{N''}$ may be assumed to be voltage-independent. The dependence of the dissociation constants K_N'' and $K_N^{S''}$ on voltage $V = (RT/F)u$ may be represented by (Läuger & Jauch, 1986):

$$K_N'' = \tilde{K}_N'' \exp(\alpha'' u) \quad (28)$$
$$K_N^{S''} = \tilde{K}_N^{S''} \exp(\alpha'' u). \quad (29)$$

$\tilde{K}_N''$ and $\tilde{K}_N^{S''}$ are the values of K_N'' and $K_N^{S''}$ at zero voltage. α'' is a phenomenological coefficient representing the relative dielectric distance between the sodium-binding site and the external membrane-so-

lution interface (Fig. 7); implicit in Eqs. (28) and (29) is the assumption that binding of S does not appreciably change the geometry of the sodium site. In a similar way, the voltage-dependence of the translocation rate constants k' and l' may be described by (Läuger & Jauch, 1986)

$$k' = \tilde{k}' \exp[(z_L\delta + \eta)u/2] \quad (30)$$

$$l' = \tilde{l}' \exp\{[(z_L + 1)\delta + \eta]u/2\}. \quad (31)$$

$z_L e_o$ is the electric charge of the empty binding site which is assumed to move over the same dielectric distance δ as the Na^+ ion in the transition $C' \leftrightarrow C''$ (Fig. 7). The parameter η describes contributions of additional charge translocations which may result, for instance, from the rotation of polar groups in the transporter molecule. The rate constants k'' and l'' for the reverse transitions are obtained from Eqs. (30) and (31) by changing the sign of the exponent.

The current-voltage measurements described in part I of this paper have been carried out at large extracellular alanine concentration ($c_S'' > K_S''$, $c_S'' > K_S^{N''}$). Under this condition Eqs. (17) and (18) simplify to $L_N'' \approx K_N^{S''}/(1 + l''/k')$ and $I_{\infty,N}'' \approx I_\infty''$. According to Eqs. (16) and (31)–(33), the voltage dependence of $I_{\infty,N}''$ and L_N'' is then given by

$$I_{\infty,N}'' \approx \frac{e_o N \tilde{k}' \exp(\varepsilon u/2)}{1 + (\tilde{k}'/\tilde{l}'') \exp[(\varepsilon + \delta/2)u]} \quad (32)$$

$$L_N'' \approx \frac{\tilde{K}_N^{S''} \exp(\alpha'' u)}{1 + (\tilde{l}''/\tilde{k}') \exp[-(\varepsilon + \delta/2)u]} \quad (33)$$

$$\varepsilon \equiv z_L\delta + \eta. \quad (34)$$

As shown in Fig. 14 of the companion paper (Jauch et al., 1986), the maximum current $I_{\infty,N}''$ was virtually independent of voltage, whereas the voltage dependence of L_N'' could be represented by the empirical relation $L_N'' = \tilde{L}_N'' \exp(0.65\ u)$. Assuming that intrinsic charge displacements in the transport (apart from movements of the binding site) are negligible ($\eta \approx 0$), the experimental findings can be explained by two different sets of assumptions:

a) z_L arbitrary, $\delta \approx 0$, $\alpha'' \approx 0.65$

b) $z_L = 0$, $\alpha'' + \delta/2 \approx 0.65$, $\tilde{k}' \ll \tilde{l}''$.

In case *a* in which the ion-binding site may be neutral ($z_L = 0$) or charged, the voltage-dependence of the current exclusively results from a voltage effect on the equilibrium constant of ion binding. This corresponds to an "ion-well" situation in which a fraction of transmembrane voltage drops between the ion-binding site and the external medium (Mitchell, 1969; Hopfer & Groseclose, 1980; Aronson, 1984; Restrepo & Kimmich, 1985*b*). In case *b* in which the ligand system has zero charge ($z_L = 0$), an arbitrary displacement of the ion-binding site may be associated with the conformational transition $NC'S \rightarrow NC''S$. As long as no information on the magnitude of $\tilde{k}'$ and $\tilde{l}''$ is available, the two possibilities cannot be distinguished. The possibility that the binding site of the sodium, sugar cotransporter of small intestine is negatively charged has been discussed by Kessler and Semenza (1983), and by Restrepo and Kimmich (1986).

Discussion

Two principally different mechanisms for ion-coupled substrate transport have been proposed so far (Semenza et al., 1984). In the "simultaneous" mechanism (Fig. 1) the cotransporter C forms a ternary complex NCS; coupled translocation of N and S results from conformational transitions between state $NC'S$ with the binding sites facing the cytoplasm and state $NC''S$ with the binding sites facing the extracellular medium. In the "consecutive" (or "ping-pong") mechanism (Fig. 2) formation of a ternary complex is not necessary; binding of N at the extracellular site in conformation C_1 is followed by translocation to the cytoplasmic side, leaving the transporter in conformation C_2 in which S can be bound from the extracellular side and translocated to the cytoplasm. One of the main results of this study is the demonstration that the experimentally observed dependence of half-saturation concentration L_S'' on sodium concentration c_N'' is incompatible with the "consecutive" model.

For the analysis of the experimental results in terms of the "simultaneous" mechanism we have assumed that the translocation steps are rate limiting so that the interfacial binding reactions always remain near equilibrium. This assumption is consistent with the fact that binding of substrates to enzymes is usually much faster than subsequent reaction steps, but in the absence of direct experimental evidence the assumption should be regarded as tentative. It is pertinent to mention that the observed Michaelis-Menten behavior of current, which agrees with the predictions of Eqs. (12)–(16), does not provide evidence for fast binding of Na^+ and S. It has been shown by Sanders et al. (1984) that the model of Fig. 1 with ordered binding but without the assumption of interfacial equilibrium exhibits the same general form of the ion- and substrate-concentration dependence of the current as indicated by Eqs. (12)–(18).

Coupling Mechanism

Accepting the equilibrium-binding model as a minimum model able to account for the experimental findings, certain kinetic parameters of the transport

system can be evaluated from the measurements. As discussed in a previous section, estimates for the dissociation constants K_N'', K_S'', $K_N^{S''}$ and $K_S^{N''}$ of sodium and alanine can be obtained from the experimental Michaelis-Menten parameters. The values of the equilibrium dissociation-constants are important for the understanding of the coupling mechanism. Coupling between the fluxes of Na^+ and S (Geck & Heinz, 1976) can be of the *velocity type* (rate constants of conformational transitions $NC' \leftrightarrow NC''$ and $C'S \leftrightarrow C''S$ vanishingly small) or of the *affinity type* (concentrations of NC and CS much lower than concentration of NCS), or may consist in a combination of both mechanisms. In a more formal way, these possibilities may be discussed by introducing the ratio ρ_N'' of substrate-coupled to uncoupled sodium influx, as well as the ratio ρ_S'' of sodium-coupled to uncoupled substrate-influx. Under the assumption of equilibrium binding, ρ_N'' and ρ_S'' are given by

$$\rho_N'' \equiv \frac{l''}{k_N''} \cdot \frac{x[NC''S]}{x[NC'']} = Q'' \frac{l''}{k_N''} \cdot \frac{c_S''}{K_S''} \tag{35}$$

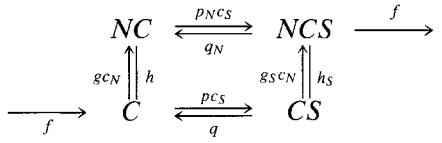
$$\rho_S'' \equiv \frac{l''}{k_S''} \cdot \frac{x[NC''S]}{x[C''S]} = Q'' \frac{l''}{k_S''} \cdot \frac{c_N''}{K_N''}. \tag{36}$$

k_N'' and k_S'' are the rate constants of the transitions $NC'' \rightarrow NC'$ and $C''S \rightarrow C'S$, respectively. "Affinity coupling" corresponds to $Q'' \equiv K_N''/K_N^{S''} = K_S''/K_S^{N''} \gg 1$ and "velocity coupling" to $k_N'', k_S'' \ll l''$. From the estimates $K_N'' < 77$ mM and $K_N^{S''} > 16$ mM derived above, it follows that Q'' cannot be larger than $77/16 \simeq 5$.

An experimental estimate for the degree of coupling can be obtained from the ratio $\rho_{I,\mathrm{exp}}'' \equiv I''(c_S'' > 0)/I''(c_S'' = 0)$ of alanine-dependent to alanine-independent inward current. As described in the companion paper, $\rho_{I,\mathrm{exp}}''$ is about 30 at low sodium and saturating alanine concentration. $\rho_{I,\mathrm{exp}}''$ contains contributions from unspecific leakage pathways (such as passive ionic channels) and therefore represents a lower limit for the intrinsic ratio ρ_I'' of the cotransporter. At low c_N'' and saturating c_S'', ρ_I'' is approximately given by $Q''l''/k_N''$, as discussed in the Appendix. With $\rho_I'' \simeq Q''l''/k_N'' > \rho_{I,\mathrm{exp}}'' \simeq 30$ and $Q'' < 5$ (*see above*) the ratio l''/k_N'' may be estimated to be >6. This indicates that coupling results from a velocity- or from a mixed (velocity- and affinity-) mechanism.

"Ordered" or "Random" Binding?

In the theory of enzymatic bisubstrate reactions a distinction is made between random and ordered binding (and release) of substrates (Segel, 1975). The possibility that ion and substrate bind in an ordered sequence to the cotransporter has been discussed, for instance, by Hopfer and Groseclose (1980), Turner (1981, 1983) and by Restrepo and Kimmich (1985*a*). "Ordered binding" may be defined by considering the stationary state of the transport system in which transitions $NC'S \rightarrow NC''S$ and $C'' \rightarrow C'$ occur at a constant net-rate f:



(37)

(Since this reaction scheme applies to both sides of the membrane, the superscripts ' and " have been omitted). Formation of the ternary complex may occur in two ways, via $C \rightarrow NC \rightarrow NCS$ (net rate f_N) or via $C \rightarrow CS \rightarrow NCS$ (net rate f_S):

$$f_N = g c_N x[C] - h x[NC] = p_N c_S x[NC] - q_N x[NCS] \tag{38}$$

$$f_S = p c_S x[C] - q x[CS] = g_S c_N x[CS] - h_S x[NCS]. \tag{39}$$

Binding is "ordered" if $f_N \gg f_S$ (N must bind before S can bind) or $f_N \ll f_S$ (S must bind before N can bind). The same definition applies to ordered release ($f = f_N + f_S < 0$). "Random" binding (or release) means that $f_N \simeq f_S$. Straightforward calculation yields

$$\frac{f_N}{f_S} = \frac{1/h_S + c_N/K_N^S q}{1/q_N + c_S/K_S^N h}. \tag{40}$$

Thus, ordered binding and release via $C \leftrightarrow NC \leftrightarrow NCS$ ($f_N \gg f_S$) occurs when one (or both) steps in the pathway $C \leftrightarrow CS \leftrightarrow NCS$ become very slow ($p,q \approx 0$ and/or $g_S, h_S \approx 0$).

Under the condition of interfacial equilibrium, ordered binding via $C \rightarrow NC \rightarrow NCS$ requires the concentration of the binary complex CS to be always negligible. This is the case for $K_S \rightarrow \infty$, $K_N^S \rightarrow 0$ (according to Eq. (3) the product $K_S K_N^S = K_N K_S^N$ must remain finite for $K_S \rightarrow \infty$). Under this condition Eqs. (8) and (14) yield

$$Q \rightarrow \infty; I_{\infty,S} \approx I_\infty. \tag{41}$$

This means that the saturating current at high alanine concentration should become independent of sodium concentration if the binding sequence is $C \rightarrow NC \rightarrow NCS$. Conversely, ordered binding via $C \rightarrow CS \rightarrow NCS$ occurs when $K_N \rightarrow \infty$, $K_S^N \rightarrow 0$, meaning that

$$Q \to \infty;\ I_{\infty,N} \approx I_{\infty}. \tag{42}$$

Figures (11) and (13) of the preceding experimental paper (Jauch et al., 1986) show that $I''_{\infty,S}$ depends on c''_N and that $I''_{\infty,N}$ depends on c''_S. Furthermore, Q'' has been estimated to be <5 (*see above*). These findings are at variance with the predictions of Eqs. (41) and (42), meaning that ordered binding is unlikely in the case of the sodium, alanine-cotransporter. It is pertinent to note that a mechanism involving "ordered binding" ($f_N \gg f_S$ or $f_N \ll f_S$) is merely a limiting case of the general mechanism of Fig. 1, and that in real cotransport systems the ratio f_N/f_S is always finite.

Voltage-Dependence of Transport Rates

In the accompanying experimental paper (Jauch et al., 1986) it has been shown that the alanine-driven inward current I'' decreases with increasing inside-positive membrane potential V, the shape of the $I''(V)$ curve depending on extracellular sodium concentration c''_N. The function $I''(c''_N,V)$ could be represented by a Michaelis-Menten equation with a voltage-dependent half-saturation concentration of sodium and a nearly voltage-independent maximum current. A possible interpretation of this finding consists in the assumption that a sodium ion entering the binding site from the extracellular medium has to traverse part of the transmembrane voltage. In addition to such a "sodium-well" effect, translocation of the sodium binding site within the membrane dielectric may also contribute to the observed voltage dependence of transport rate.

Voltage effects on sodium-driven cotransport systems have been demonstrated previously in experiments with renal or intestinal brushborder vesicles. An inside-negative membrane potential was found to enhance Na^+-coupled influx of D-glucose (Murer & Hopfer, 1974; Hilden & Sacktor, 1982; Kessler & Semenza, 1983; Kaunitz & Wright, 1984). Similar results were obtained in studies of Na^+-driven amino-acid transport in brush-border vesicles (Burckhardt, Kinne, Stange & Murer, 1980; Ganapathy & Leibach, 1983) and of Na^+-driven sugar transport in epithelial cells (Carter-Su & Kimmich, 1980; Restrepo & Kimmich, 1985*a*). In combination with other kinetic experiments quantitative studies of voltage-effects on cotransport systems are likely to yield valuable mechanistic information in the future.

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Appendix

Fluxes of Na^+ and S and Reversal Potential in the Case of Incomplete Coupling

We consider a generalized version of the reaction scheme of Fig. 1 in which the transporter C is able to bind ν Na^+ ions (N) and σ substrate molecules (S). We assume that transitions between configurations with inward- and outward-facing binding sites may occur not only in the fully-loaded state $N_\nu C S_\sigma$ but also in states $N_\nu C$ and CS_σ:

$$N_\nu C' S_\sigma \underset{l''}{\overset{l'}{\rightleftarrows}} N_\nu C'' S_\sigma \tag{A1}$$

$$N_\nu C' \underset{k_N''}{\overset{k_N'}{\rightleftarrows}} N_\nu C'' \tag{A2}$$

$$C' S_\sigma \underset{k_S''}{\overset{k_S'}{\rightleftarrows}} C'' S_\sigma \tag{A3}$$

Under this condition the ratio of the fluxes of N and S is no longer equal to the stoichiometric ratio ν/σ (the stoichiometric ratio is defined as the ratio of the number of sodium to the number of amino-acid binding sites involved in coupled transport). We assume, as before, that the binding reactions in the membrane-solution interface are in equilibrium. We further assume that the values of the equilibrium constants are such that the concentrations of intermediate forms $N_x C$ and CS_y with $1 \le x < \nu$ and $1 \le y < \sigma$ are always negligible. The equilibrium dissociation constants K_N and K_S (and, in an analogous way, K_N^S and K_S^N) are now defined by

$$K_N = \frac{x[C]c_N^\nu}{x[N_\nu C]}; \qquad K_S = \frac{x[C]c_S^\sigma}{x[CS_\sigma]}. \tag{A4}$$

In addition to Eqs. (3) and (4) the following relations hold:

$$\frac{K_S''}{K_S'} \cdot \frac{k_S' k''}{k_S'' k'} = \frac{K_S^{N''}}{K_S^{N'}} \cdot \frac{l' k_N''}{l'' k_N'} = 1. \tag{A5}$$

In analogy to Eq. (6) the flux Φ_N of Na^+ (which is referred to a single transporter molecule) is given by

$$\Phi_N/\nu = k_N' x[N_\nu C'] - k_N'' x[N_\nu C''] + l' x[N_\nu C' S_\sigma] - l'' x[N_\nu C'' S_\sigma]. \tag{A6}$$

An analogous relation holds for the flux Φ_S of S. The result reads

$$\Phi_N = \frac{\nu}{\chi}[A_N(c_N'^\nu e^{\nu u} - c_N''^\nu) + B_N(c_N'^\nu c_S''^\sigma e^{\nu u} - c_N''^\nu c_S'^\sigma) + C(c_N'^\nu c_S'^\sigma e^{\nu u} - c_N''^\nu c_S''^\sigma)] \tag{A7}$$

$$\Phi_S = \frac{\sigma}{\chi}[A_S(c_S'^\sigma - c_S''^\sigma) + B_S(c_S'^\sigma c_N''^\nu e^{-\nu u} - c_S''^\sigma c_N'^\nu) + C(c_S'^\sigma c_N'^\nu e^{\nu u} - c_S''^\sigma c_N''^\nu)] \tag{A8}$$

$$\chi \equiv P'H'' + P''H' \tag{A9}$$

$$H \equiv k + nslQ + k_N n + k_S s \tag{A10}$$

$$n \equiv c_N^\nu/K_N; \qquad s \equiv c_S^\sigma/K_S \tag{A11}$$

$$A_N \equiv \frac{k_N'' k'}{K_N''} + \frac{l'' k_S'}{K_N^{S''}} s' s'' \tag{A12}$$

$$B_N \equiv \frac{k_N'' k_S'}{K_N'' K_S'}; \; C \equiv \frac{l'' k'}{K_N'' K_S^{N''}} = \frac{l'' k'}{K_S'' K_N^{S''}}. \tag{A13}$$

A_S and B_S are obtained from A_N and B_N by exchanging the sub- and superscripts N and S. P and Q are defined by Eq. (9) and (8).

The reversal potential V_r for which Φ_N vanishes is obtained from Eq. (A7) in the form

$$V_r = \frac{RT}{\nu F} \ln \frac{c_N''^\nu c_S''^\sigma + D c_N''^\nu c_S'^\sigma + E c_N''^\nu}{c_N'^\nu c_S'^\sigma + D c_N'^\nu c_S''^\sigma + E c_N'^\nu} \tag{A14}$$

$$D \equiv B_N/C; \qquad E \equiv A_N/C. \tag{A15}$$

Since D and E depend, in general, on voltage, Eq. (A14) represents an implicit equation for V_r. In the limit $k'_N, k''_N, k'_S, k''_S \to 0$ (complete coupling), Eq. (A14) reduces to the thermodynamic relation

$$V_r = \frac{RT}{\nu F}\left(\nu \ln \frac{c''_N}{c'_N} + \sigma \ln \frac{c''_S}{c'_S}\right). \tag{A16}$$

Equation (A14) is important for the analysis of experiments in which the reversal potential was measured under the condition $c'_N = c''_N$, $c''_S > c'_S$ (Jauch et al., 1986). In these experiments V_r was found to be close to $(RT/F)\ln(c''_S/c'_S)$, which is the predicted value for complete coupling and $\sigma/\nu = 1$ [Eq. (A16)]. According to

$$V_r = \frac{RT}{\nu F} \ln \frac{c''^{\sigma}_S + Dc'^{\sigma}_S + E}{c'^{\sigma}_S + Dc''^{\sigma}_S + E} \qquad (c'_N = c''_N) \tag{A14a}$$

incomplete coupling ($D,E > 0$) always tends to *diminish* the absolute magnitude of V_r, meaning that σ/ν cannot be smaller than unity. The results of the experiments described in the companion paper are thus incompatible with a sodium-alanine stoichiometry of 2 : 1.

Using Eq. (A7), the ratio $\rho''_I \equiv I''(c''_S > 0)/I''(c''_S = 0)$ of substrate-independent (sodium-driven) inward current can be calculated. Under the condition $c'_N = c'_S = 0$, $\nu = \sigma = 1$, the result is obtained as

$$\rho''_I = \left[1 + \frac{c''_S}{K''^{N''}_S} \cdot \frac{l''}{k''_N}\right] \Big/ \left[1 + \frac{c''_S}{K''^{N''}_S} \cdot \frac{K''^{S''}_N(k' + k''_S) + c''_N(k' + l'')}{K''_N(k' + k'') + c''_N(k' + k''_N)}\right]. \tag{A17}$$

At low sodium and saturating substrate concentration, Eq. (A17) assumes the form

$$\rho''_I \approx \frac{K''_N}{K''^{S''}_N} \cdot \frac{l''}{k''_N} \cdot \frac{k' + k''}{k' + k''_S}. \tag{A18}$$

In the case of a symmetric transporter ($k' \approx k''$) with $k''_S \leq k'$, Eq. (A18) further reduces to

$$\rho''_I \simeq \frac{K''_N}{K''^{S''}_N} \cdot \frac{l''}{k''_N} = Q'' \frac{l''}{k''_N}. \tag{A19}$$