Reduction of an Eight-State Mechanism of Cotransport to a Six-State Model Using a New Computer Program

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ABSTRACT A computer program was developed to allow easy derivation of steady-state velocity and binding equations for multireactant mechanisms including or without rapid equilibrium segments. Its usefulness is illustrated by deriving the rate equation of the most general sequential iso ordered ter ter mechanism of cotransport in which two Na^+ ions bind first to the carrier and mirror symmetry is assumed. It is demonstrated that this mechanism cannot be easily reduced to a previously proposed six-state model of Na^+-D-glucose cotransport, which also includes a number of implicit assumptions. In fact, the latter model may only be valid over a restricted range of Na^+ concentrations or when assuming very strong positive cooperativity for Na^+ binding to the glucose symporter within a rapid equilibrium segment. We thus propose an equivalent eight-state model in which the concept of positive cooperativity is best explained within the framework of a polymeric structure of the transport protein involving a minimum number of two transport-competent and identical subunits. This model also includes an obligatory slow isomerization step between the Na^+ and glucose-binding sequences, the nature of which might reflect the presence of functionally asymmetrical subunits.

INTRODUCTION

The high-affinity, Na^+-D-glucose cotransport system (SGLT1) has been considered throughout the years as representative of a larger class of membrane transport proteins that use electrochemical gradients for ions to accumulate organic molecules in a variety of prokaryote and eukaryote cells (Schultz and Curran, 1970; Crane, 1977; Semenza et al., 1984; Kimmich, 1990; Wright, 1993). The structural basis underlying the specific and efficient coupling of glucose transport to Na^+ flux is currently unknown, so most of our understanding of the coupling process per se relies heavily on a number of kinetic studies performed in brush-border membrane vesicles (Hopfer and Groseclose, 1980; Kessler and Semenza, 1983; Moran et al., 1988; Koepsell et al., 1990; Chenu and Berteloot, 1993), intact cells (Restrepo and Kimmich, 1985a,b, 1986; Kimmich and Randles, 1988), and SGLT1 cRNA-injected oocytes (Parent et al., 1992a; Loo et al., 1993; Hazama et al., 1997).

Most contemporary approaches to Na^+/glucose cotransport kinetics involved the mobile carrier concept, which mainly states that the activator (Na^+, N) and substrate (glucose, S) binding sites are only accessible from one side of the membrane at a time through isomerization of the unloaded transporter (Schultz and Curran, 1970; Heinz et al., 1972; Jacquez, 1972; Crane, 1977; Stein, 1981; Turner, 1981, 1983, 1985; Sanders et al., 1984; Läuger and Jauch, 1986). In this concept, coupling of the N and S fluxes results from the formation of an N-S carrier complex that undergoes another conformational change, accounting for the differences in compartmentalization of the two molecules during the transport cycle. With the demonstration that SGLT1 couples the transport of two Na^+ ions to one glucose molecule (Kimmich and Randles, 1980; Moran et al., 1988; Parent et al., 1992a; Chen et al., 1995), and because ordered substrate addition is usually assumed (Kimmich, 1990; Wright, 1993), it turns out that the simplest models of cotransport mostly considered in recent kinetic studies all belong to the family of terreactant systems, which is composed of the three possible eight-state sequential iso ordered ter ter mechanisms, i.e., the so-called S:N:N, N:S:N, and N:N:S models (Restrepo and Kimmich, 1985a). The first possibility seems to be ruled out by those kinetic studies, showing that Na^+ is mandatory for both glucose cotransport and phlorizin (a specific inhibitor of SGLT1) binding to the carrier (Restrepo and Kimmich, 1985b, 1986; Chenu and Berteloot, 1993; Oulianova and Berteloot, 1996). The second possibility is advocated by the Kimmich group (Kimmich, 1990), and its value as a likely kinetic scheme of Na^+/glucose cotransport relies on the validity of the assumption previously dismissed by Hopfer and Groseclose (1980) that N and S binding to the carrier occurs under rapid equilibrium conditions. Accordingly, these two kinetic schemes will not be considered any further in the present studies, although we acknowledge the fact that the N:S:N model has been used to satisfactorily describe a number of experimental results (Smith-Maxwell et al., 1990; Bennett and Kimmich, 1996). The last possibility is currently supported by a vast body of literature, mostly published by the Wright group (Wright, 1993) and involving steady-state and presteady-state kinetic studies in SGLT1 cRNA-injected oocytes (Parent et al., 1992a; Loo et al., 1993; Hazama et

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al., 1997). The characteristic feature of the kinetic scheme proposed by this group is that the expected eight-state N:N:S mechanism of cotransport was reduced to a six-state model in which the binding of the two Na^+ ions to the carrier is described as a single reaction step (Parent et al., 1992b). However, no direct proof of the two hypotheses originally claimed by Parent et al. (1992b) has ever been provided by the authors to justify such a simplification procedure.

So far, the major deterrent to the consideration of general ter ter mechanisms of cotransport has been the forbiddingly tedious nature of the derivation of steady-state velocity equations therefrom. Indeed, the number of valid King-Altman patterns would increase from 15 to 56 when going from the model of Parent et al. (1992b) to its eight-state counterpart, and a number of simplifications other than the reduction proposed by these authors have been consistently introduced in the past to minimize the complexity of the derivation process. Note that the latter usually involved various assumptions about the rate-limiting steps in the transport cycle (rapid equilibrium assumption) (Turner, 1981, 1983, 1985; Kimmich, 1990), the symmetry of binding events at the two membrane faces (Jacquez, 1972; Kimmich, 1990; Parent et al., 1992b), the symmetry of translocation rate constants (Schultz and Curran, 1970), and the lack of mobility of partially loaded forms of the carrier (Kimmich, 1990). To our knowledge, the effect of these assumptions on the mathematical expression of the rate law characterizing the most general model has never been evaluated.

From a theoretical point of view, it would appear that the derivation of a cotransport model should be as general as possible. However, to decrease the number of significant parameters that can be determined at the experimental level, it is usually recommended to use the simplest model predicting an equivalent rate law (Stein, 1981). Similarly, if the general form of a velocity equation relative to its dependence on a specific substrate concentration has been established experimentally, the rate law predicted by the most general model might prove more complex than necessary to explain the behavior of the real system (more terms than needed would appear in the theoretical velocity equation). For practical purposes, then, a number of variations in the design of a basic model often need to be evaluated, and with complex mechanisms at least, such a systematic approach is necessarily tedious and prone to human errors. It would thus be most advantageous to resort to computer programs to derive velocity equations; however, none of those previously published in the field of enzyme kinetics (Rhoads and Pring, 1968; Hurst, 1969; Cornish-Bowden, 1977; Fromm, 1979; Herries, 1984; Ishikawa et al., 1988) appeared to us to be readily suitable for achieving this goal.

In the present studies, we first present a new computer program that should allow anyone to derive velocity and binding equations for complex kinetic mechanisms involving multireactant systems with or without rapid equilibrium segments. The usefulness of the program is next demonstrated by deriving the rate equation of the general eight-state N:N:S mechanism of Na^+/glucose cotransport and by testing a number of hypotheses that would justify its reduction to the six-state model of Parent et al. (1992b). It is finally shown that the unique solution to the latter problem involves high positive cooperativity for Na^+ binding within a rapid equilibrium segment before a slow isomerization step itself, followed by either steady-state or rapid equilibrium addition of glucose, a kinetic mechanism that is best rationalized when assuming a dimeric structure of the SGLT1 protein.

MATERIALS AND METHODS

Theory

General considerations

The problem of writing the rate equation for a reaction mechanism has been greatly simplified by the schematic method of King and Altman (1956), which was reformulated in purely algebraic terms by Cornish-Bowden (1977) for computational purposes. Because the justification of the King-Altman approach was adduced by an analysis showing that it was based on the determinant method for solving nonhomogeneous linear equations (King and Altman, 1956), it was also proposed that a systematic solution of the simultaneous steady-state equations characterizing a kinetic mechanism removes the need to identify the King-Altman patterns, because terms that would have arisen from patterns containing closed loops automatically cancel (Hurst, 1967). As discussed by Cornish-Bowden (1977), the latter approach is slightly more general but may lead to longer execution times on a computer. From a practical point of view, however, its computerization does not require any understanding of the principles underlying full and efficient use of the rules to identify the valid King-Altman patterns (Hurst, 1969; Fromm, 1979; Herries, 1984; Ishikawa et al., 1988). Accordingly, the program to be presented below relies on the systematic expansion of determinants characterizing a matrix with dimension *n*, where *n* corresponds to the number of carrier species involved in a transport cycle.

Calculation of rate equations using the determinant method

The application of the determinant method (Hurst, 1967) to the analysis of complex transport mechanisms is illustrated by taking the general eight-state N:N:S mechanism of Na^+/glucose cotransport shown in Fig. 1 *A* as an example. This model is associated with the following set of differential equations:

$$\frac{dN_1}{dt} = -[k_{12} + k_{18}(Na_i)]N_1 + k_{21}N_2 + k_{81}N_8 \quad (1)$$

$$\frac{dN_2}{dt} = k_{12}N_1 - [k_{21} + k_{23}(Na_o)]N_2 + k_{32}N_3 \quad (2)$$

$$\frac{dN_3}{dt} = k_{23}(Na_o)N_2 - [k_{32} + k_{34}(Na_o) + k_{38}]N_3 + k_{43}N_4 + k_{83}N_8 \quad (3)$$

$$\frac{dN_4}{dt} = k_{34}(Na_o)N_3 - [k_{43} + k_{45}(S_o) + k_{47}]N_4 + k_{54}N_5 + k_{74}N_7 \quad (4)$$

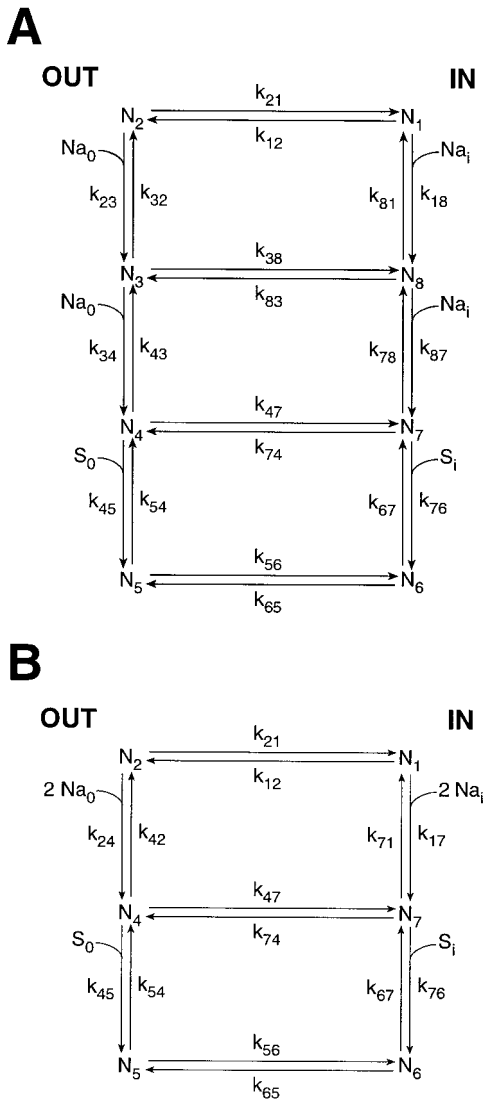

FIGURE 1 Kinetic models of Na^+-D-glucose cotransport. (*A*) Sequential iso ordered ter ter mechanism in which the two Na^+ ions bind first to the carrier and mirror symmetry is assumed. (*B*) Reduced six-state model proposed by Parent et al. (1992b), in which the two Na^+ ions add simultaneously to the carrier in a steady-state fashion. Note that the numbering of the different carrier species in *B* is different from that proposed originally by these authors and has been chosen to make easier a direct comparison with the model in *A*. In both *A* and *B*, Na_o and Na_i stand for external (OUT) and internal (IN) Na, and S_o and S_i represent external and internal glucose, respectively. Further details are given in the text.

$$\frac{dN_5}{dt} = k_{45}(S_o)N_4 - (k_{54} + k_{56})N_5 + k_{65}N_6 \tag{5}$$

$$\frac{dN_6}{dt} = k_{56}N_5 - (k_{65} + k_{67})N_6 + k_{76}(S_i)N_7 \tag{6}$$

$$\frac{dN_7}{dt} = k_{47}N_4 + k_{67}N_6 - [k_{74} + k_{76}(S_i) + k_{78}]N_7 + k_{87}(Na_i)N_8 \tag{7}$$

$$\frac{dN_8}{dt} = k_{18}(Na_i)N_1 + k_{38}N_3 + k_{78}N_7 - [k_{81} + k_{83} + k_{87}(Na_i)]N_8 \tag{8}$$

which express the time dependence of the N_i carrier forms. Under steady-state conditions, where all $dN_i/dt = 0$, and because the N_i carrier forms are linked through the conservation equation,

$$N_T = \sum_{i=1}^{8} N_i \tag{9}$$

in which N_T is constant and represents the total amount of transport protein, the concentrations of the N_i carrier forms can be found by solving the matrix equation

$$\begin{pmatrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ A_{21} & A_{22} & A_{23} & 0 & 0 & 0 & 0 & 0 \\ 0 & A_{32} & A_{33} & A_{34} & 0 & 0 & 0 & A_{38} \\ 0 & 0 & A_{43} & A_{44} & A_{45} & 0 & A_{47} & 0 \\ 0 & 0 & 0 & A_{54} & A_{55} & A_{56} & 0 & 0 \\ 0 & 0 & 0 & 0 & A_{65} & A_{66} & A_{67} & 0 \\ 0 & 0 & 0 & A_{74} & 0 & A_{76} & A_{77} & A_{78} \\ A_{81} & 0 & A_{83} & 0 & 0 & 0 & A_{87} & A_{88} \end{pmatrix} \cdot \begin{pmatrix} N_1 \\ N_2 \\ N_3 \\ N_4 \\ N_5 \\ N_6 \\ N_7 \\ N_8 \end{pmatrix} = \begin{pmatrix} N_T \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix} \tag{10}$$

In Eq. 10, the first line expresses the fact that Eq. 9 has been substituted for Eq. 1, and the algebraic expressions of the A_{ji} elements in the main matrix on the left-hand side correspond to the coefficients of the N_i carrier forms to be found in the system of j equations (j = 2–8) defined above. The steady-state rate can then be calculated from the known steady-state concentrations of those N_i carrier forms that appear in the definition of the velocity equations

$$v^S = \frac{d(S_i)}{dt} = k_{67}N_6 - k_{76}(S_i)N_7 \tag{11}$$

$$v^{Na} = \frac{d(Na_i)}{dt} = k_{78}N_7 + k_{81}N_8 - k_{18}(Na_i)N_1 - k_{87}(Na_i)N_8 \tag{12}$$

relative to substrate (v^S) or Na^+ (v^{Na}) transport, respectively.

Application to transport models involving rapid equilibrium segments

The simplest hypothesis that may be involved in reducing the complexity of a kinetic mechanism is based on the time-scale separation hypothesis, which states that not all transitions between the elementary reactions leading to substrate flux proceed at the same rate, so that only the slowest ones can be seen in realistic experiments. Accordingly, some steps along the transport pathway may be assumed to be very rapid when compared to others, so as to have reached near-equilibrium during the steady-state phase of the reaction. Most computer programs developed in the past cannot derive rate equations when the mechanism involves rapid equilibrium

segments (Rhoads and Pring, 1968; Hurst, 1969; Fromm, 1979; Herries, 1984). Moreover, the Cornish-Bowden program (1977) requires some preparation of the data for presentation to the computer, and the software of Ishikawa et al. (1988) does not take advantage of the possibility of reducing the matrix size when applying Cha's rules (Cha, 1968). The latter is illustrated in the following, using the model shown in Fig. 2 *A*, which formally states that the binding of the first Na^+ ion to the inside- and outside-facing carrier sites occurs under rapid equilibrium. Note that this mechanism illustrates one of the hypotheses formulated by Parent et al. (1992b) to validate the reduction of the general eight-states model of cotransport shown in Fig. 1 *A* to their six-state model presented in Fig. 1 *B*.

Following Cha's rules (Cha, 1968), each group of carrier forms at equilibrium with each other can be treated as a single block (named **x** and **y** in Fig. 2 *A*), and all rate constants leading away from these blocks should be weighted according to the reactive fraction f_i of those carrier species involved in each block. From the definition of the dissociation constants given by Eqs. 13–14,

$$K_{18} = \frac{N_1(\mathrm{Na_i})}{N_8} = \frac{k_{81}}{k_{18}} \tag{13}$$

$$K_{23} = \frac{N_2(\mathrm{Na_o})}{N_3} = \frac{k_{32}}{k_{23}} \tag{14}$$

the composite carrier species N_x and N_y, constitutive of the two blocks **x** and **y**, can be expressed by Eqs. 15 and 16, respectively:

$$N_x = N_2 + N_3 = N_2\left[1 + \frac{(\mathrm{Na_o})}{K_{23}}\right] \tag{15}$$

$$N_y = N_1 + N_8 = N_1\left[1 + \frac{(\mathrm{Na_i})}{K_{18}}\right] \tag{16}$$

from which the f_i fractions can be calculated as shown in Eqs. 17–20:

$$f_1 = \frac{N_1}{N_y} = \frac{K_{18}}{K_{18} + (\mathrm{Na_i})} \tag{17}$$

$$f_2 = \frac{N_2}{N_x} = \frac{K_{23}}{K_{23} + (\mathrm{Na_o})} \tag{18}$$

$$f_3 = \frac{N_3}{N_x} = \frac{(\mathrm{Na_o})}{K_{23} + (\mathrm{Na_o})} \tag{19}$$

$$f_8 = \frac{N_8}{N_y} = \frac{(\mathrm{Na_i})}{K_{18} + (\mathrm{Na_i})} \tag{20}$$

The six-state mechanism shown in Fig. 2 *B* is thus formally equivalent to its eight-state counterpart in Fig. 2 *A*, so that six differential equations are now sufficient to fully describe the proposed kinetic scheme with concomitant reduction of the matrix dimensions in Eq. 10. When the new set of differential equations is established, the conservation equation (Eq. 9) should be modified to account for Eqs. 15 and 16, and a number of apparent rate constants now need to be used with algebraic expressions, as indicated in Fig. 2 *B*. Note that the complex expressions connecting the N_x and N_y carrier species result from the rule of additivity of parallel pathways proposed by Volkenstein and Goldstein (1966). Note also that the definition of v^{Na} given in Eq. 12 has to be modified as shown in Eq. 21:

$$v^{Na} = 2[k_{78}N_7 - k_{87}(\mathrm{Na_i})N_8] \tag{21}$$

where $N_8 = f_8 N_y$ according to Eq. 20. The reduced kinetic scheme thus appears equivalent to a cotransport mechanism leading to the steady-state release of two Na^+ ions in a single step, as proposed in the model of Parent et al. (1992b).

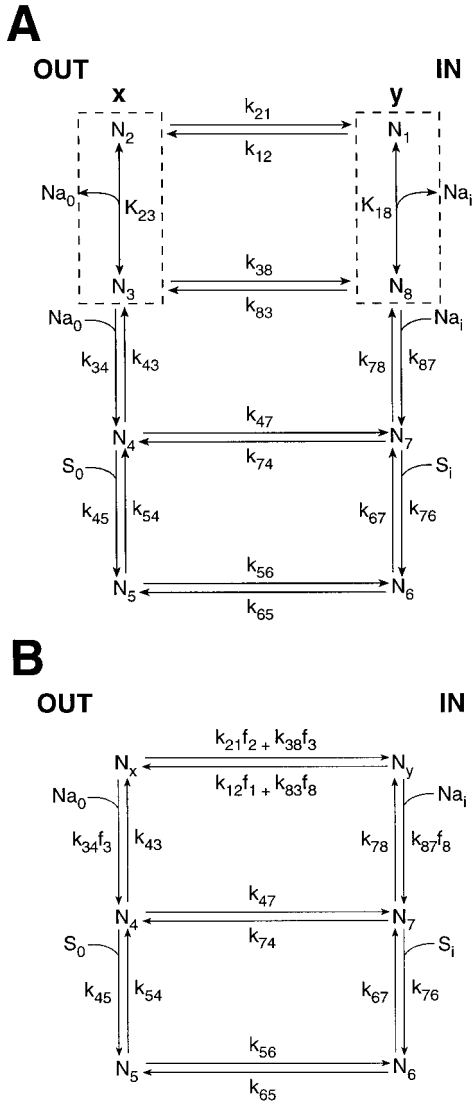

FIGURE 2 Simplified models of Na^+-D-glucose cotransport. (*A*) It is assumed, as proposed by Parent et al. (1992b), that rapid equilibrium binding of the first Na^+ ion is followed by steady-state addition of the second Na^+ ion. **x** and **y** represent the two blocks within the transport cycle (*marked in dashed lines*), where the carrier species are linked through a rapid equilibrium reaction. (*B*) Equivalent six-state model reduced according to Cha's rules (Cha, 1968). N_X and N_Y represent the summation of those carrier species that are linked through a rapid equilibrium sequence in the transport cycle. In both *A* and *B*, the numbering of the carrier species is similar to that indicated in the legend to Fig. 1. Further details are given in the text.

Computerization of the determinant method

The main features of the program are presented in Fig. 3 as a flowchart. The program was written in Mathematica language, which is well suited for mathematical applications like linear algebra, analytical calculations, and algebraic manipulations (Wolfram, 1993). Moreover, in contrast to traditional programming languages such as Fortran or BASIC, which handle only numerical computations, Mathematica also performs symbolic and graphical computations (Wolfram, 1993).

The data input method is illustrated in the Appendix, using the kinetic mechanism of cotransport shown in Fig. 2 *A*. The process is interactive, includes several tests to avoid human errors, and can be checked as a whole

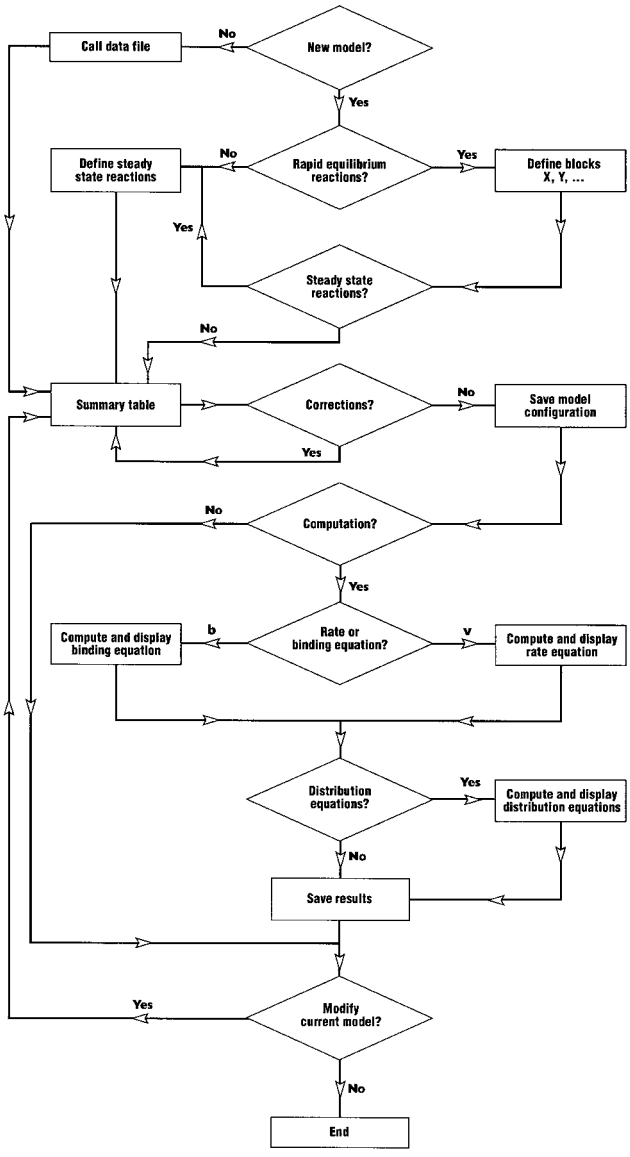

FIGURE 3 Flowchart of the computer program developed in the present studies

from the summary table presented at the end of the input session. The latter can be used further as a guide to performing necessary corrections in the current model or to introduce modifications in a preedited model file. Note that the program self-establishes, for any particular model, its characteristic matrix Eq. 10 and, if required by the model, the algebraic expression of the dissociation constants involved in the rapid equilibrium reactions according to their definitions, as in Eqs. 13 and 14.

The program output may then provide the user with the algebraic expressions of (see Appendix) 1) the fractional concentration factors f_i calculated according to their definitions, as in Eqs. 17–20, if any; 2) the velocity or binding equation relative to the release of a particular product or fixation to the transporter of any molecule included in the basic model (substrate, inhibitor, etc.); and 3) the steady-state distribution equations relevant to all carrier species involved in a particular model. Note that the velocity/binding equation is displayed in one run under its generic form, which is the most useful one for experimental applications, and that the algebraic expressions of the constant and substrate coefficient terms can be listed upon request.

Implementation

The program was written for a text-based interface configuration of Mathematica and was implemented on the mainframe computer at the University of Montreal (UNIX system). It has since been run with similar efficiency on a Pentium PC Pro (200 MHz, 32 Mbytes RAM) with a notebook configuration of Mathematica. In its present form, the program has been devised without any limitation to the number of either carrier species or elementary reactions involved, but these may be dictated by the memory space available on specific computers.

RESULTS

Solution of the eight-state model of cotransport (Fig. 1 *A*)

For the purpose of the present studies, zero-trans initial rates of transport only will be considered, because such velocity equations have been used by Parent et al. (1992b) to analyze their experimental data. Relative to glucose (v_i^S) and Na^+ (v_i^{Na}) transport, their generic forms resulting from computer calculation are shown in Eqs. 22 and 23:

$$\frac{v_i^S}{N_T} = \frac{a_0(Na_o)^2(S_o)}{b_0 + b_1(Na_o) + b_2(Na_o)^2 + [c_0 + c_1(Na_o) + c_2(Na_o)^2](S_o)} \quad (22)$$

$$\frac{v_i^{Na}}{N_T} = \frac{[a_1 + a_2(S_o)](Na_o) + [a_3 + a_4(S_o)](Na_o)^2}{b_0 + c_0(S_o) + [b_1 + c_1(S_o)](Na_o) + [b_2 + c_2(S_o)](Na_o)^2} \quad (23)$$

respectively, with constant and substrate coefficient terms (macro constants) as listed in Table 1. Although presented differently to emphasize the fact that S (Eq. 22) or Na^+ (Eq. 23) can be taken as the variable substrate, the denominator expressions of Eqs. 22–23 are, in fact, identical, as conditioned by the conservation Eq. 9. However, their numerator expressions are quite different and reflect the fact that glucose and Na^+ are released through alternative pathways on the *trans* side. Note, then, that the use of Eq. 22 assumes that glucose transport is measured by a radio tracer technique, whereas Eq. 23 may apply to either $^{22}Na^+$ uptake or the Na^+ current flowing through the Na^+ and glucose transport pathways. It follows from these considerations that the measure of the v_i^{Na}/v_i^S ratio (Lee et al., 1994) may not be the best method for determining the coupling stoichiometry of cotransport, because noninteger numbers are generally expected from this approach. Indeed, it can easily be verified from Table 1 that a true coupling stoichiometry of 2.0 may only be observed in the total absence of Na^+ leak pathways (i.e., when $k_{38} = k_{47} = k_{74} = k_{83} = 0$), in which case $a_1 = a_2 = a_3 = 0$ and $a_4 = 2a_0$. Note, however, that the introduction of the latter modifications into the basic model does not affect the general form of the denominator expression of Eqs. 22–23, i.e., none of the constant or substrate coefficient terms can be eliminated by this assumption.

TABLE 1 Derivation of the general eight-state mechanism of cotransport

Macro constants	Algebraic expressions
a_0	$k_{12}k_{23}k_{34}k_{45}k_{56}k_{67}(k_{74} + k_{78})(k_{81} + k_{83})$
a_1	$k_{12}k_{23}k_{38}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{43}k_{74} + k_{43}k_{78} + k_{47}k_{78})k_{81}$
a_2	$k_{12}k_{23}k_{38}k_{45}k_{56}k_{67}k_{78}k_{81}$
a_3	$k_{12}k_{23}k_{34}k_{47}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})k_{78}(2k_{81} + k_{83})$
a_4	$k_{12}k_{23}k_{34}k_{45}k_{56}k_{67}k_{78}(2k_{81} + k_{83})$
b_0	$(k_{12} + k_{21})(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{43}k_{74} + k_{43}k_{78} + k_{47}k_{78})(k_{32}k_{81} + k_{38}k_{81} + k_{32}k_{83})$
b_1	$(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{12}k_{23}k_{38}k_{43}k_{74} + k_{12}k_{23}k_{38}k_{43}k_{78} + k_{12}k_{23}k_{38}k_{47}k_{78} + k_{12}k_{23}k_{43}k_{74}k_{81} + k_{23}k_{38}k_{43}k_{74}k_{81} + k_{12}k_{23}k_{43}k_{78}k_{81} + k_{23}k_{38}k_{43}k_{78}k_{81} + k_{12}k_{23}k_{47}k_{78}k_{81} + k_{12}k_{34}k_{47}k_{78}k_{81} + k_{21}k_{34}k_{47}k_{78}k_{81} + k_{23}k_{38}k_{47}k_{78}k_{81} + k_{12}k_{23}k_{43}k_{74}k_{83} + k_{12}k_{23}k_{43}k_{78}k_{83} + k_{12}k_{23}k_{47}k_{78}k_{83})$
b_2	$k_{23}k_{34}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{12}k_{47}k_{78} + k_{12}k_{47}k_{81} + k_{12}k_{74}k_{81} + k_{12}k_{78}k_{81} + k_{47}k_{78}k_{81} + k_{12}k_{47}k_{83} + k_{12}k_{74}k_{83} + k_{12}k_{78}k_{83})$
c_0	$(k_{12} + k_{21})k_{45}k_{56}k_{67}k_{78}(k_{32}k_{81} + k_{38}k_{81} + k_{32}k_{83})$
c_1	$k_{45}k_{56}k_{67}k_{78}(k_{12}k_{23}k_{38} + k_{12}k_{23}k_{81} + k_{12}k_{34}k_{81} + k_{21}k_{34}k_{81} + k_{23}k_{38}k_{81} + k_{12}k_{23}k_{83})$
c_2	$k_{23}k_{34}k_{45}(k_{12}k_{56}k_{67}k_{78} + k_{12}k_{56}k_{67}k_{81} + k_{12}k_{56}k_{74}k_{81} + k_{12}k_{65}k_{74}k_{81} + k_{12}k_{67}k_{74}k_{81} + k_{12}k_{56}k_{78}k_{81} + k_{12}k_{65}k_{78}k_{81} + k_{12}k_{67}k_{78}k_{81} + k_{56}k_{67}k_{78}k_{81} + k_{12}k_{56}k_{67}k_{83} + k_{12}k_{56}k_{74}k_{83} + k_{12}k_{65}k_{74}k_{83} + k_{12}k_{67}k_{74}k_{83} + k_{12}k_{56}k_{78}k_{83} + k_{12}k_{65}k_{78}k_{83} + k_{12}k_{67}k_{78}k_{83})$

The macro constants shown correspond to the constant and substrate coefficient terms appearing in Eqs. 22 and 23 given in the text, which characterize the velocity equations derived for the kinetic mechanism of cotransport depicted in Fig. 1 *A*.

Solution of the six-state model of Parent et al. (1992b) (Fig. 1 *B*)

In our studies, the numbering of the n carrier species was chosen in such a way that N_1 and N_2 always represent the unloaded forms of the transporter independently of the n value. Moreover, the notation used in Fig. 1 *B* emphasizes the similarities and the differences between the six- and eight-state models. The initial velocity equations were thus recalculated to fit this new formalism as compared to the original work of Parent et al. (1992b); their generic forms are given by

$$\frac{v_i^S}{N_T} = \frac{a_{0P}(Na_o)^2(S_o)}{b_{0P} + b_{2P}(Na_o)^2 + [c_{0P} + c_{2P}(Na_o)^2](S_o)} \quad (24)$$

$$\frac{v_i^{Na}}{N_T} = \frac{[a_{3P} + a_{4P}(S_o)](Na_o)^2}{b_{0P} + c_{0P}(S_o) + [b_{2P} + c_{2P}(S_o)](Na_o)^2} \quad (25)$$

for glucose and Na^+ transport, respectively. The algebraic expressions of the different macro constants are listed in Table 2 and can be compared directly to their counterparts lacking the P indices in Table 1.

A comparison of Eqs. 22–23 with Eqs. 24–25 clearly indicates that the absence of (Na_o) and $(Na_o)(S_o)$ coefficient terms in the denominator expressions of the latter is a major difference between the characteristic velocity equations predicted by the two models shown in Fig. 1, *A* and *B*, respectively. Moreover, whereas the numerator expression of $v^S{}_i$ is similar in form for the two models, that for v_i^{Na} also lacks the (Na_o) and $(Na_o)(S_o)$ coefficient terms when derived for the six-state model. Because the macro constants a_1 and a_2 in the numerator of Eq. 23 account for a Na^+ leak pathway through the one Na^+-loaded carrier complex in the eight-state model (N_3 to N_8 transitions in Fig. 1 *A*), it can be concluded that an implicit hypothesis of the six-state model of Parent et al. (1992b) is that such a leak does not exist. Indeed, setting $k_{38} = k_{83} = 0$ cancels out the (Na_o) and $(Na_o)(S_o)$ coefficient terms in the numerator of Eq. 23 and emphasizes the 2 factor in the algebraic expressions of the macro constants a_3 and a_4 (Table 1).

Note that the macro constants a_{3P} and a_{4P} in Eq. 25 characterize the Na^+ fluxes through the leak and cotransport pathways, respectively. It is readily apparent, then, that these two fluxes will be equal, provided that Eq. 26 is satisfied:

$$(S_o) = \frac{a_{3P}}{a_{4P}} = \frac{k_{47}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})}{k_{45}k_{56}k_{67}} \quad (26)$$

The algebraic expression on the right side of Eq. 26 is equivalent, when accounting for the differences in notations, to that previously derived through a different ap-

TABLE 2 Derivation of the six-state model of cotransport (Parent et al., 1992b)

Macro constants	Algebraic expressions
a_{0P}	$k_{12}k_{24}k_{45}k_{56}k_{67}(k_{71} + k_{74})$
a_{3P}	$2k_{12}k_{24}k_{47}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})k_{71}$
a_{4P}	$2k_{12}k_{24}k_{45}k_{56}k_{67}k_{71}$
b_{0P}	$(k_{12} + k_{21})(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{42}k_{71} + k_{47}k_{71} + k_{42}k_{74})$
b_{2P}	$k_{24}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{12}k_{47} + k_{12}k_{71} + k_{47}k_{71} + k_{12}k_{74})$
c_{0P}	$(k_{12} + k_{21})k_{45}k_{56}k_{67}k_{71}$
c_{2P}	$k_{24}k_{45}(k_{12}k_{56}k_{67} + k_{12}k_{56}k_{71} + k_{12}k_{65}k_{71} + k_{12}k_{67}k_{71} + k_{56}k_{67}k_{71} + k_{12}k_{56}k_{74} + k_{12}k_{65}k_{74} + k_{12}k_{67}k_{74}$

The macro constants shown correspond to the constant and substrate coefficient terms appearing in Eqs. 24 and 25 given in the text, which characterize the velocity equations derived for the kinetic mechanism of cotransport depicted in Fig. 1 *B*.

proach by Chen et al. (1995) for the kinetic constant K_c, which was defined as the extracellular sugar concentration giving equal Na^+ currents through the inward Na^+ leak and the coupled Na^+-sugar influx.

The steady-state current flowing through the carrier under zero-trans conditions (I) can be determined from Eq. 25 and is given by Eq. 27:

$$I = FN_T v_i^{Na} = 2FN_T\left[\frac{[\varphi + \epsilon(S_o)](Na_o)^2}{\alpha + \beta(S_o) + [\chi + (S_o)](Na_o)^2}\right] \quad (27)$$

in which F is the Faraday constant, and the right side of the last equality corresponds to the expression previously derived by Parent et al. (1992b). Taking into account the differences in formalism, it was verified that the algebraic expressions of the macro constants relative to v_i^{Na} as defined by Eqs. 25 and 27 satisfy the equalities shown in Eq. 28:

$$a_{3P} = 2\lambda\varphi; \quad a_{4P} = 2\lambda\epsilon; \quad b_{0P} = \lambda\alpha; \quad c_{0P} = \lambda\beta; \quad {}_{2P} = \lambda\chi; \quad c_{2P} = \lambda \quad (28)$$

Still, it should be noted that the 2 factor showing up in the complete form of Eq. 25 (see Table 1) results from our definition of the initial rate, given by

$$v_i^{Na} = 2k_{71}N_7 \quad (29)$$

which accounts for the inside release of two Na^+ ions. In contrast, it clearly appears that the concept that led Parent et al. (1992b) to the correct Eq. 27 is ill defined, because, as shown by

$$I = -z_c F(k_{21}N_2 - k_{12}N_1) \quad (30)$$

(adapted from the original publication to fit the notations shown in Fig. 1 *B*), the 2 coefficient results from the assumption that the valence of the ion-binding site on the empty carrier (z_c) is equal to -2 (Parent et al., 1992b). Indeed, because the definition of the steady state assumes that there is no net flow of carrier forms from one side of the membrane to the other, a corollary implication is that there should be no net flow through the membrane of those charges that might be involved at the level of the different carrier species. Accordingly, steady-state currents can only measure the charges associated with ion fluxes. This fact has been correctly appreciated in similar studies by Chen et al. (1995), in which the steady-state current was defined as

$$I = nF(k_{21}N_2 - k_{12}N_1) \quad (31)$$

(adapted from the original publication to fit the notations shown in Fig. 1 *B*), where n accounts for the 2:1 stoichiometry of SGLT1. Still, a word of caution should be given regarding the definition of the steady-state velocity equation relative to the free carrier recycling step (N_1 to N_2 transitions) as in Eqs. 30 and 31, rather than to the Na-releasing step as in Eq. 29. Indeed, applying the former concept to the eight-state model (Fig. 1 *A*) failed to give the correct steady-state equation in the presence of an extra Na^+ leak pathway represented by the N_3 to N_8 transitions.

First attempts to reduce the general eight-state model of cotransport to the six-states model of Parent et al. (1992b)

Our first attempts to justify a reduction of the general eight-state model of cotransport (Fig. 1 *A*) to the six-state model shown in Fig. 1 *B* aimed at testing the two hypotheses proposed by Parent et al. (1992b). The first hypothesis, which assumed that there are two equivalent binding sites for Na^+ ions on the carrier protein, can easily be introduced into the model of Fig. 1 *A* by setting $k_{23} = k_{34}$, $k_{43} = k_{32}$, and $k_{78} = k_{81}$ (note that we also assumed that $k_{38} = k_{83} = 0$ as implicit in the model of Parent et al. (1992b); see above). It can easily be verified from Table 1 that these equalities did not allow us to cancel any of the (Na_o) and $(Na_o)(S_o)$ coefficient terms in the denominator of Eqs. 22–23. This result is not unexpected, however, when considering the rule stating that consecutive steps are not additive (Volkenstein and Goldstein, 1966). The second hypothesis, which assumed that binding of the first Na^+ ion to the carrier is in rapid equilibrium and that binding of the second Na^+ ion to the carrier is rate limiting, can be introduced into the model of Fig. 1 *A* as shown in Fig. 2. The computer analysis of this model (see Appendix) led to a v_i^{Na} equation similar in form to Eq. 23. with macro constants as listed in

TABLE 3 Derivation of a simplified eight-state mechanism of cotransport

Macro constants	Algebraic expressions
a_1	0
a_2	0
a_3	$2k_{12}k_{34}k_{47}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})k_{78}$
a_4	$2k_{12}k_{34}k_{45}k_{56}k_{67}k_{78}$
b_0	$K_{23}(k_{12} + k_{21})(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{43}k_{74} + k_{43}k_{78} + k_{47}k_{78})$
b_1	$k_{12}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{43}k_{74} + k_{43}k_{78} + k_{47}k_{78})$
b_2	$k_{34}(k_{54}k_{65} + k_{54}k_{67} + k_{56}k_{67})(k_{12}k_{47} + k_{12}k_{74} + k_{12}k_{78} + k_{47}k_{78})$
c_0	$K_{23}(k_{12} + k_{21})k_{45}k_{56}k_{67}k_{78}$
c_1	$k_{12}k_{45}k_{56}k_{67}k_{78}$
c_2	$k_{34}k_{45}(k_{12}k_{56}k_{67} + k_{12}k_{56}k_{74} + k_{12}k_{65}k_{74} + k_{12}k_{67}k_{74} + k_{12}k_{56}k_{78} + k_{12}k_{65}k_{78} + k_{12}k_{67}k_{78} + k_{56}k_{67}k_{78})$

The macro constants shown correspond to the constant and substrate coefficient terms appearing in Eq. 23 given in the text, which characterizes the velocity equation derived for the kinetic mechanism of cotransport depicted in Fig. 2 *A*.

Table 3 (note that $a_1 = a_2 = 0$, because of the additional constraint that $k_{38} = k_{83} = 0$ in these calculations). Accordingly, the two hypotheses proposed by Parent et al. (1992b) must be rejected.

Our further attempts to reduce the eight-state model of cotransport involved the analysis of models lacking the N_3-N_8 transition in Fig. 1 *A* and differing in the number and position of the rapid equilibrium steps that can be introduced into the transport cycle. Their characteristic velocity equations were established using our computer program, and the results showed that v_i^{Na} was similar in form to Eq. 23 (with $a_1 = a_2 = 0$), with a number of modifications in some of the substrate coefficient terms in the denominator as listed in Table 4. Note that 1) it does not matter to the general form of Eq. 23 whether S binds to the carrier in a steady-state or rapid equilibrium reaction, a conclusion that was verified to apply to the model of Parent et al. (1992b), so that Eqs. 23 and 25 do not represent a unique solution to the full steady-state models shown in Fig. 1, *A* and 1 *B*, respectively; 2) all models in which $c_0 = 0$, including those leading to the disappearance of the $(Na_o)(S_o)$ coefficient term c_1 in the denominator of Eq. 23, must be rejected because the rate law characterizing the model of Parent et al. (1992b) contains a finite c_{0P} constant (see Eq. 25); and 3) in no case was it possible to cancel out both of the macro constants b_1 and c_1 defining, respectively, the (Na_o) and $(Na_o)(S_o)$ coefficient terms in the denominator of Eq. 23. The latter point is not unexpected, because the macro constant b_1 expresses the fact that one of the two Na^+ ions must precede further Na^+ and glucose binding to the transporter, as clearly indicated in Table 4 from the analysis of the simplified model, in which all reactants add to the transporter under rapid equilibrium conditions.

Conditions of application of the model of Parent et al. (1992b)

The above studies demonstrate that there is no kinetic basis so far that could justify the reduction of the general eight-state model of cotransport (Fig. 1 *A*) to the six-state model shown in Fig. 1 *B*. It can be observed, however, that Eq. 25 is equivalent to the Hill equation,

$$v_i^{Na} = \frac{V_{maxP}^{Na}(Na_o)^{n_H}}{(K_{mP}^{Na})^{n_H} + (Na_o)^{n_H}} \tag{32}$$

in which the parameters V_{maxP}^{Na} and K_{mP}^{Na} would represent the apparent V_{max} and K_m of Na^+ transport estimated at a fixed concentration of glucose (their algebraic expressions can be easily determined from Eq. 25 after dividing both its numerator and denominator by the coefficient of the $(Na_o)^2$ term in the denominator) and the Hill number $n_H = 2$. In agreement with this view and, thus, attesting at the experimental level to the validity of Eq. 32, Parent et al. (1992a) reported n_H values of 1.9–2.1 for Na^+ activation of the steady-state currents through SGLT1. One may then question the conditions under which Eq. 23 (with $a_1 = a_2 = 0$, see justifications above) could be approximated by Eq. 32.

To answer this question and to allow easier comparison of Eqs. 32 and 23, it is most convenient to analyze the latter under its equivalent form:

$$v_i^{Na} = \frac{V_{max}^{Na}(Na_o)^2}{K_{m1}^{Na}K_{m2}^{Na} + K_{m2}^{Na}(Na_o) + (Na_o)^2} \tag{33}$$

which is composed entirely of kinetic constants, defined as

$$V_{max}^{Na} = \frac{a_3 + a_4(S_o)}{b_2 + c_2(S_o)} N_T \tag{34}$$

$$K_{m1}^{Na} = \frac{b_0 + c_0(S_o)}{b_1 + c_1(S_o)} \tag{35}$$

$$K_{m2}^{Na} = \frac{b_1 + c_1(S_o)}{b_2 + c_2(S_o)} \tag{36}$$

Note that Eqs. 35 and 36 would characterize the apparent affinities of Na^+ binding to the first and second carrier sites, respectively. Because the denominator of Eq. 33 can be factorized under the two forms shown in Eq. 37,

$$\begin{gathered} K_{m2}^{Na}[K_{m1}^{Na} + (Na_o)] + (Na_o)^2; \\ K_{m1}^{Na}K_{m2}^{Na} + \left[\frac{K_{m2}^{Na}}{(Na_o)} + 1\right](Na_o)^2 \end{gathered} \tag{37}$$

it will degenerate to the denominator form of Eq. 32 when $K_{m2}^{Na} \ll (Na_o) \ll K_{m1}^{Na}$. Accordingly, the model of Parent et al. (1992b) may approximate the general eight-state mechanism of cotransport over a restricted range of Na^+ concentrations, provided that the apparent affinity for binding of the second Na^+ ion is much higher than that of the first.

Alternatively, it is possible to estimate the apparent n_H value that one may expect from a Hill plot analysis of Eq. 33. Because n_H is given in this approach by the slope of the $Ln[v/(V_{max} - v)]$ versus Ln(S) plot at $v = V_{max}/2$ (Segel,

TABLE 4 Attempts to reduce the general eight-state mechanism of cotransport using the rapid equilibrium assumption

Rapid equilibrium segments	Effect on denominator expressions
$N_2 - N_3$	None
$N_3 - N_4$	$c_0 = 0$
$N_4 - N_5$	None
$N_2 - N_4$	$c_0 = c_1 = 0$
$N_2 - N_3$ and $N_4 - N_5$	None
$N_3 - N_5$	$c_0 = 0$
$N_2 - N_5$	$c_0 = c_1 = 0$

The rapid equilibrium segments shown have been introduced into the kinetic mechanism of cotransport depicted in Fig. 1 *A*. The effect of these modifications from the basic model was tested by computer derivation of the initial velocity equations characterizing each of the simplified schemes and subsequent comparison of their denominator expressions with those of Eqs. 22 and 23 given in the text.

1975), its value can be evaluated from

$$n_H = \left[\frac{\mathrm{d\ Ln} \dfrac{v_i^{Na}}{V_{max}^{Na} - v_i^{Na}}}{\mathrm{d\ Ln\ (Na_o)}} \right]_{(Na_o)_{0.5}} \quad (38)$$

which simply translates this fact into mathematical terms. Equation 38 can be transformed to its equivalent form,

$$n_H = \left[\frac{V_{max}^{Na}(Na_o)}{v_i^{Na}(V_{max}^{Na} - v_i^{Na})} \cdot \frac{\mathrm{d}\, v_i^{Na}}{\mathrm{d\ (Na_o)}} \right]_{(Na_o)_{0.5}} \quad (39)$$

after using the mathematical rules governing the derivation of logarithmic functions. When applied to Eq. 33, formal development of the terms in brackets in Eq. 39 and further rearrangements lead to

$$n_H = \frac{2K_{m1}^{Na} + (Na_o)_{0.5}}{K_{m1}^{Na} + (Na_o)_{0.5}} = 1 + \frac{K_{m1}^{Na}}{K_{m1}^{Na} + (Na_o)_{0.5}} \quad (40)$$

from which it is readily apparent that $1 \leq n_H \leq 2$ and $n_H = 2$, provided that $(Na_o)_{0.5} \ll K_{m1}^{Na}$. The $(Na_o)_{0.5}$ expression can be found by solving Eq. 33 for $(Na)_o$ after setting $v_i^{Na} = V_{max}^{Na}/2$, and the result is given by

$$(Na_o)_{0.5} = \frac{K_{m2}^{Na} + (K_{m2}^2 + 4K_{m1}^{Na}K_{m2}^{Na})^{1/2}}{2} \quad (41)$$

so that $n_H = 2$, provided that $2K_{m2}^{Na} \ll K_{m1}^{Na}$. Accordingly, then, the model of Parent et al. (1992b) may also approximate the general eight-state mechanism of cotransport in the case of very strong positive cooperativity for Na^+ binding to the transporter.

High positive cooperativity for Na^+ binding as a likely physical basis of the kinetic features of the six-state model of Parent et al. (1992b)

It should be acknowledged that the concept of positive cooperativity for Na^+ binding was coined by Parent et al. (1992b) in their original paper. However, it was neither demonstrated as such nor discussed further by these authors. Indeed, its formal development into a realistic kinetic scheme may have to address first a number of issues that have been overlooked so far, in part to keep the analysis of the general eight-state mechanism of cotransport as simple as possible, but also because most of these questions are not readily apparent from the model of Parent et al. (1992b). First, with a transporter known to possess two Na^+ binding sites, it is very unlikely to have a purely ordered mechanism of Na^+ binding unless there is extensive asymmetry in the configuration of the two sites. The demonstration by Levitzki (1978) that the latter situation is able to account for noncooperativity and negative cooperativity, but never for positive cooperativity, would suggest that a random addition of the two Na^+ ions represents the most relevant mechanism to be considered in the case of Na^+-D-glucose cotransport. Second, given the assumption of steady-state reactions for Na^+ binding in a random mechanism, our computer calculations showed that the resulting velocity equation contains $(Na_o)^3$ coefficient terms that do not exist in Eqs. 23 and 25. There is, thus, a rational basis for assuming that the random addition of the two Na^+ ions should occur within a rapid equilibrium segment of the transport cycle. Last, because the concept of a purely kinetic nature of positive cooperativity would appear to be incompatible with the rapid equilibrium assumption above (Segel, 1975) and with the observation that equilibrium binding curves of the nontransported inhibitor phlorizin are sigmoidal relative to Na^+ concentrations (Restrepo and Kimmich, 1986; Moran et al., 1988), we assume in the following that the Na^+ sites are interactive, i.e., the first occupancy of one of the two sites modifies the apparent affinity for Na^+ binding to the unoccupied site.

According to these considerations, the binding sequence of the two Na^+ ions can be depicted as shown in Fig. 4 *A* and corresponds to a block **x** in the transport cycle, verifying Eq. 42:

$$X = N_2 \left[1 + \frac{2(Na_o)}{K_{Na}} + \frac{(Na_o)^2}{\alpha K_{Na}^2} \right] \quad (42)$$

in which K_{Na}, the intrinsic dissociation constant for binding of the first Na^+ ion, is modified by a factor α accounting for the increased affinity of Na^+ binding to the second site ($\alpha < 1$ for positive cooperativity). The fractional concentrations of the N_i carrier forms within the block **x** can thus be expressed by

$$f_2 = \frac{N_2}{X} = \frac{\alpha K_{Na}^2}{\alpha K_{Na}^2 + 2\alpha K_{Na}(Na_o) + (Na_o)^2} \quad (43)$$

$$f_3 = \frac{N_3^* + N_3^{**}}{X} = \frac{2\alpha K_{Na}(Na_o)}{\alpha K_{Na}^2 + 2\alpha K_{Na}(Na_o) + (Na_o)^2} \quad (44)$$

$$f_4 = \frac{N_4}{X} = \frac{(Na_o)^2}{\alpha K_{Na}^2 + 2\alpha K_{Na}(Na_o) + (Na_o)^2} \quad (45)$$

from which it can be concluded that $f_3 \ll f_2$ and f_4, if $2\alpha K_{Na} \ll (Na_o) \ll K_{Na}/2$. Thus $f_3 \approx 0$ when $\alpha \ll ¼$, and this situation is equivalent to the simultaneous addition of two Na^+ ions with an apparent dissociation constant of αK_{Na}^2. Note that, under these conditions, the low concentration of the N_3 carrier species would also justify the approximation that $k_{38} = 0$, as implicitly assumed by Parent et al. (1992b) (see above). Still, as shown in Table 4, a general eight-state mechanism of cotransport in which the two Na^+ binding steps occur under rapid equilibrium conditions cannot pretend to mimic the behavior of the model of Parent et al. (1992b). Nonetheless, if we further assume the existence of a slow isomerization step between the Na^+ and glucose binding sequences, as depicted in Fig. 4 *B*, the characteristic velocity equation calculated by our computer program is similar in form to Eq. 25, with algebraic expressions of the macro constants as given in Table 5.

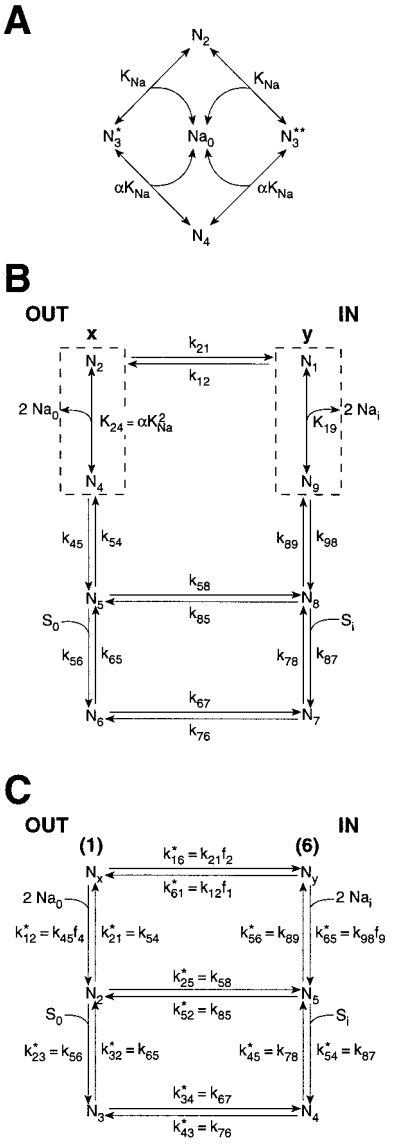

FIGURE 4 Positive cooperativity in Na^+ binding to the cotransport protein. (*A*) It is assumed that the conformations of the two Na^+ sites present on the free carrier facing toward the outside medium are equivalent (the free carrier is symmetrical), so that binding of the first Na^+ ion may occur at either site with similar affinity (dissociation constant K_{Na}). It is also assumed that the first Na^+ ion to bind induces a conformational change that results in increased affinity of Na^+ binding to the second site (dissociation constant αK_{Na}, where $\alpha \ll 1$ for high positive cooperativity). (*B*) Proposed eight-state model of Na^+-D-glucose cotransport that includes the concept of high positive cooperativity developed in *A*. Note that a slow isomerization step has been included between the Na^+ and glucose-binding sequences for reasons discussed in the text. (*C*) Equivalent six-state model of the mechanism shown in *B* after reduction according to Cha's rules (Cha, 1968). N_X and N_Y represent the summation of those carrier species that are linked through a rapid equilibrium sequence in the transport cycle. In both *A* and *B*, the numbering of the carrier species is similar to that shown in Fig. 1. In *C*, however, it is similar to that proposed originally by Parent et al. (1992b), to underscore the similarity of the two models (k^* corresponds to their definition in the original publication by these authors, whereas k is equivalent to those shown in *B*). Further details are given in the text.

DISCUSSION

A new versatile computer program aimed at resolving complex kinetic models

A very natural approach to the understanding of complex reaction mechanisms is through kinetic analysis. Still, steady-state kinetics can only be used efficiently to eliminate those mechanisms that failed to confirm model predictions at the experimental level and, conversely, to develop kinetic models from experimental data. It is thus necessary that a sufficiently general set of reaction mechanisms be considered if maximum benefit is to be gained from this kinetic approach. Yet, in the case of multisubstrate systems like complex cotransport mechanisms, the value of a systematic analysis is restricted by the intricacies of the algebraic manipulations involved in the derivation of their steady-state velocity equations. This difficulty in no way invalidates the possibility that such mechanisms do exist, and the availability of fully automatic techniques should prove invaluable to their analysis.

Cornish-Bowden (1977) has given a useful review of the many computer programs designed to derive enzyme kinetic rate equations, but none of these appear as simple to use, nor is any as versatile as the one proposed in the present studies. First, our program expands determinants in a systematic manner and, hence, avoids the complications linked to the determination of valid King-Altman patterns. Second, our approach introduces a very simple format of data presentation for rapid equilibrium, steady-state, or combined steady-state plus rapid equilibrium mechanisms with its automatic processing to form the determinant needed for solving the steady-state concentrations of the various carrier species involved. Accordingly, the use of the program does not require a detailed understanding of the mathematics involved in the calculations, and limits to a minimum the possibility of human errors associated with an extensive preparation of the data before their presentation to the computer. Note that the program 1) also handles irreversible steps in a mechanism and, hence, that it can be used from the start to derive velocity equations under zero-trans conditions, and 2) may be used to handle pre-steady-state kinetic studies according to the formalism developed by Wierzbicki et al. (1990), in which a number of carrier-state distributions need to be calculated at specified times. Third, the program output gives an easy-to-read form of the velocity or binding equations and, upon request, of the numerator expressions characterizing the carrier-state distribution equations. Finally, note that the current version of the program has been intensively tested and that it proved successful at calculating the velocity equations characterizing the most typical enzyme mechanisms analyzed by Segel (1975).

Application to the modelization of Na^+-D-glucose cotransport

In recent publications aimed at characterizing both the steady-state and pre-steady-state kinetics of SGLT1, it was

TABLE 5 Derivation of the allosteric eight-state mechanism of cotransport

Macro constants	Algebraic expressions
a_1	0
a_2	0
a_3	$2k_{12}k_{45}k_{58}(k_{65}k_{76} + k_{65}k_{78} + k_{67}k_{78})k_{89}$
a_4	$2k_{12}k_{45}k_{56}k_{67}k_{78}k_{89}$
b_0	$K_{24}(k_{12} + k_{21})(k_{65}k_{76} + k_{65}k_{78} + k_{67}k_{78})(k_{54}k_{85} + k_{54}k_{89} + k_{58}k_{89})$
b_1	0
b_2	$(k_{65}k_{76} + k_{65}k_{78} + k_{67}k_{78})(k_{12}k_{45}k_{58} + k_{12}k_{45}k_{85} + k_{12}k_{54}k_{85} + k_{12}k_{45}k_{89} + k_{12}k_{54}k_{89} + k_{12}k_{58}k_{89} + k_{45}k_{58}k_{89})$
c_0	$K_{24}(k_{12} + k_{21})k_{56}k_{67}k_{78}k_{89}$
c_1	0
c_2	$k_{56}(k_{12}k_{45}k_{67}k_{78} + k_{12}k_{45}k_{67}k_{85} + k_{12}k_{45}k_{76}k_{85} + k_{12}k_{45}k_{78}k_{85} + k_{12}k_{45}k_{67}k_{89} + k_{12}k_{45}k_{76}k_{89} + k_{12}k_{45}k_{78}k_{89} + k_{12}k_{67}k_{78}k_{89} + k_{45}k_{67}k_{78}k_{89})$

The macro constants shown correspond to the constant and substrate coefficient terms appearing in Eq. 23 given in the text, which characterizes the velocity equation derived for the kinetic mechanism of cotransport depicted in Fig. 4 *B*.

concluded that a six-state mechanism of cotransport could adequately describe the electrical properties of this transporter in cRNA-injected oocytes (Parent et al., 1992a,b; Loo et al., 1993; Hazama et al., 1997). It was originally stated that the proposed model would be equivalent to its more complete eight-state counterpart when assuming either identical Na^+ binding sites or fast binding of the first Na^+ ion as compared to the second (Parent et al., 1992b). In the absence of a formal demonstration regarding the validity of these claims, we first tried to evaluate their relevance to the proposed mechanisms, and it is demonstrated in the present studies that neither of the two assumptions made by Parent et al. (1992b) can, in fact, justify the suggested model reduction. It is further shown that the six-state model of cotransport 1) makes the implicit assumption that the Na^+-loaded carrier complex does not contribute in any way to the Na^+ leak pathway; 2) cannot be justified from the consideration alone that some steps in the transport cycle may occur at rates faster than those of others (Table 4); 3) does not provide a unique solution to the steady-state velocity equation derived by Parent et al. (1992b), because a number of rapid equilibrium segments could be introduced into the original model without modifying the generic form of the rate law, 4) ignores the fact that a random rather than a ordered Na^+ addition to the transporter is the more logical sequence to consider when two binding sites are involved and positive cooperativity is assumed, and 5) can be justified only by considering a restricted range of Na^+ concentrations or by assuming that there is strong positive cooperativity for Na^+ binding to the transport protein.

These considerations led us to propose an eight-state mechanism of cotransport that appears to be fully equivalent to the six-state model of Parent et al. (1992b) (Fig. 4) and presents the following characteristic features: 1) random binding of the two Na^+ ions within a rapid equilibrium segment in which strong positive cooperativity due to site-site interactions results in the apparent addition of the two molecules in a discrete reaction; 2) a slow isomerization step separating the Na^+ and glucose binding sequences; and 3) either steady-state or rapid equilibrium addition of glucose to the transporter, because these two possibilities gave an equivalent form of the characteristic velocity equation. According to point 3) above, an equivalent four-state model might thus prove sufficient to fully describe the proposed mechanism.

Because our studies suggest a kinetic basis that would justify the use of the six-state model of Parent et al. (1992b), which proved otherwise consistent with an impressive body of data to be found in the literature (Wright, 1993), it can be provisionally assumed that the eight-state mechanism shown in Fig. 4 *B* provides a coherent description of the kinetics of Na^+-D-glucose cotransport at the experimental level. A corollary implication, then, is that the model of Parent et al. (1992b) may contain two pseudo rate constants under zero-*trans* conditions. As indicated in Eqs. 46 and 47,

$$k_{12}^* = k_{45}\frac{(Na_o)^2}{\alpha K_{Na}^2 + (Na_o)^2} \tag{46}$$

$$k_{16}^* = k_{21}\frac{\alpha K_{Na}^2}{\alpha K_{Na}^2 + (Na_o)^2} \tag{47}$$

the algebraic reexpressions of the microscopic rate constants k_{12}^* and k_{16}^* originally defined in this model would demonstrate, in its equivalent version proposed in the present studies (Fig. 4 *C*), a clear dependence on Na^+ concentrations. Moreover, because the two rate constants above characterize putative membrane potential-dependent steps, the conclusions drawn by Parent et al. (1992b) about the fractional dielectric distance over which the carrier ion binding site moves on one hand, and the fraction of the membrane potential field between Na^+ ions and the Na^+ binding sites on the other hand, may need to be reevaluated. In any case, a unique set of numerical values should not be assumed under different experimental conditions, and, in agreement with this view, it can be noted that the model of Parent et al. (1992b) failed to perfectly describe their experimental data gathered at both 10 and 100 mM Na^+, and failed to give a quantitative description of some of the experimental data published in later studies from this group (Loo et al., 1993; Hazama et al., 1997).

Further comments on positive cooperativity and slow isomerization of a Na^+-loaded carrier complex

A correct interpretation of the kinetic features characterizing the eight-state mechanism of cotransport proposed in the present studies may have to await further characterization of the transporter at the structural level. Still, over the last 15 years, a number of inactivation radiation (Turner and Kempner, 1982; Takahashi et al., 1985; Béliveau et al., 1988; Stevens et al., 1990) and kinetic (Blank et al., 1989; Koepsell et al., 1990; Chenu and Berteloot, 1993; Gerardi-Laffin et al., 1993; Koepsell and Spangenberg, 1994) studies have suggested that SGLT1 may function as a polymeric protein composed of at least two similar subunits. The eight-state mechanism of cotransport proposed in Fig. 4 *B* is fully compatible with this concept and with the dimeric scheme of cotransport reported earlier by our group (Chenu and Berteloot, 1993). Indeed, as shown in Fig. 5, its formal development within the framework of a simple sequential model of Koshland et al. (1966) would provide both a structural and a physical basis for the hypothesis of strong positive cooperativity introduced in the present studies on different grounds. According to this theory (Koshland et al., 1966), the free carrier would be a symmetrical dimer (with subunit conformation A, circle), Na^+ binding to either one of the two subunits would induce a conformational change (square conformation B), and binding of a second Na^+ ion to the other subunit would reestablish a symmetrical dimer configuration. High positive cooperativity for Na^+ binding would thus result from the consideration that interaction between an A and a B subunit is far less favorable than interaction between two A and two B subunits, in which case $K_{AB}^2/K_{BB} = \alpha \ll 1$ (note that the interaction factor α has the same meaning as in Eq. 42, and K_{AB} and K_{BB} are defined in the legend to Fig. 5, with values relative to K_{AA} taken as unity).

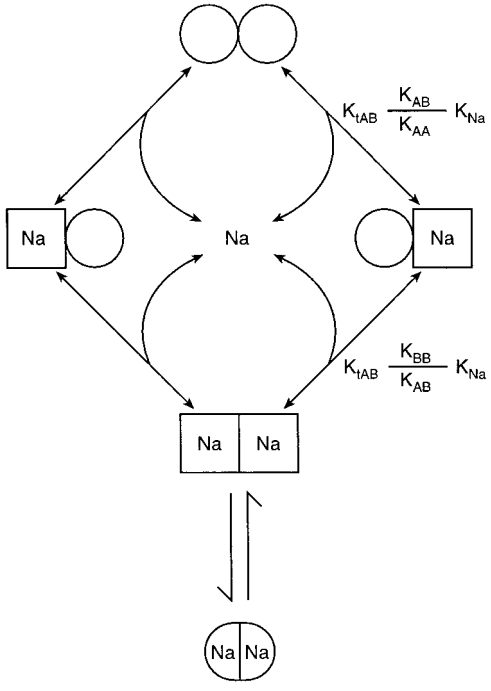

FIGURE 5 Proposed dimeric model of Na^+-D-glucose cotransport. It is assumed that the free carrier is a symmetrical dimer (with subunit conformation A, circle) onto which Na^+ binding to either one of the two subunits induces a conformational change (square conformation B). Binding of a second Na^+ ion to the other subunit then reestablishes a symmetrical dimer configuration. K_{Na} is the intrinsic affinity constant of Na^+ binding to the B conformation; K_{tAB} is an equilibrium constant characterizing the isomerization from the A to B configurations; and K_{AA}, K_{AB}, and K_{BB} are equilibrium constants that account for subunit interactions under the A-A, A-B, and B-B configurations, respectively. Note that the equilibrium constants are defined as association constants in this particular case to simplify the notations, as is usually done for allosteric kinetics (Segel, 1975). A slow isomerization step is included after the Na^+ addition sequence and is accompanied by a concerted conformational change to the hemicircle configuration. Further details are given in the text.

The proposal of a slow isomerization step separating the Na^+ and S binding sequences is compatible with previous data of Peerce and Wright (1984), showing that Na^+ binding to the glucose symporter induced a conformation change in the transporter which increases its affinity for glucose. The significance of the isomerization step is more difficult to grasp in the context of a monomeric or an oligomeric structure of SGLT1 and requires additional information or hypotheses to be fully evaluated. Nonetheless, as depicted in Fig. 4 *B*, this step can be viewed as an essential feature of the transport mechanism itself, in which a conformational change would lead to partial or complete occlusion of the two Na^+ ions within a channel-like structure (and, hence, to Na^+ transport in the absence of glucose that would account for the Na^+ leak pathway) with concomitant exposure of the glucose binding site and/or opening of a glucose channel that would finally result in Na^+ and glucose cotransport in the presence of the latter substrate. This most general view would fit nicely within the dynamic concept of cotransport proposed in the so-called rail hypothesis, in which Na^+ and glucose transport follows interacting routes (Semenza et al., 1985), possibly through two interacting channels (Ugolev and Metel'skii, 1990). A putative dimeric structure of SGLT1 would further raise the question of whether these two transport routes/channels are formed by the association of two subunits or are constitutive structures of each subunit. Note that either one of these two possibilities could be compatible with the previous evidence of one Na^+ and one glucose binding site per polypeptide chain of the transporter (Peerce and Wright, 1985, 1986), in which case one may also suspect the binding of more than one glucose molecule after Na^+ activation of glucose transport, as discussed in more detail in previous studies from our group (Chenu and Berteloot, 1993).

A multisubstrate, single-file model for ion-coupled transporters has recently been proposed by Su et al. (1996), which was found to account quantitatively for a number of kinetic characteristics associated with various cotransport systems. Because our results were obtained using the more classical alternating access mechanism of cotransport

(Schultz and Curran, 1970; Heinz et al., 1972; Jacquez, 1972; Crane, 1977; Stein, 1981; Turner, 1981, 1983, 1985; Sanders et al., 1984; Läuger and Jauch, 1986), one may argue that their significance is restricted to this class of models. The simplest way to introduce channel-like properties into the model shown in Fig. 4 *B* would be, as previously demonstrated by Stein (1981) for a four-state mechanism of facilitated diffusion, to eliminate the free-carrier recycling step (N_1 to N_2 transitions) from model consideration. Because this modification from the basic model did not change the generic form of Eq. 25 when the rate law was established under zero-*trans* conditions, it can be concluded that the results of the present studies also encompass those expected from pure channel-like kinetics. Moreover, as acknowledged by Su et al. (1996), the multi-substrate single-file channel is unable, in its present form, to deal with the phenomena of exchange and counterflow, both of which have been demonstrated to occur with the glucose symporter (Kessler et al., 1983; Dorando and Crane, 1984; Semenza et al., 1984). Indeed, as considered previously by Stein (1981), the presence of transport sites within the channel that are accessible to glucose from either side of the membrane would lead to zero inward substrate fluxes under infinite *trans* conditions. This problem could be overcome by assuming the existence of an asymmetrical molecule made of (at least) two identical and functional subunits in which concerted interactions between monomers upon site occupancy from the inside and/or the outside would determine the extent of substrate fluxes in both directions (Chenu and Berteloot, 1993). Note that similar views have already been proposed for the Na^+/H^+ (Otsu et al., 1989, 1992) and Cl^-/HCO_3^- (Salhany, 1992) exchangers.

APPENDIX: AN EXAMPLE OF COMPUTER-ASSISTED DERIVATION OF KINETIC MECHANISMS

The kinetic mechanism of cotransport shown in Fig. 2 *A* is taken as an example to illustrate the use of the computer program discussed under Materials and Methods when one wants to determine the velocity equation relative to Na^+ transport, which is given by Eq. 21 in the main text. The following dialogue is transcribed as it appears on the screen of the monitor when the computer program is run. Note that the bold characters correspond to the answers keyed in by the user and that the input dialogue is shown for typical reactions only.

Do you want to create a new model (y or n)? **y**
Do you want to create a model with rapid-equilibrium reactions (y or n)? **y**
Give the name block of the rapid equilibrium reactions: **X**
Print the rapid equilibrium reaction: **{2,3,Nao, K23}**
Block X: 2 ↔ 3 Substrate: Nao Dissociation constant: K23
Do you want to keep this reaction (y or n)? **y**
Do you want to add another reaction to the block X (y or n)? **n**
Do you want to add a rapid equilibrium reaction to another block (y or n)? **y**
Give the name of the block: **Y**
Print the rapid equilibrium reaction: **{1,8,Nai, K18}**
Block Y: 1 ↔ 8 Substrate: Nai Dissociation constant: K18
Do you want to keep this reaction (y or n)? **y**
Do you want to add another reaction to the block Y (y or n)? **n**
Do you want to add a rapid equilibrium reaction to another block (y or n) **n**
Do you want to introduce steady-state reactions (y or n)? **y**

The steady-state reactions: input data

Print the steady-state reaction: **{1,2,without, k12}**
The reaction:1→2 Substrate:without Rate constant:k12
Do you want to keep this reaction (y or n)? **y**
Do you want to add another reaction (y or n)? **y**
Print the steady-state reaction: **{2,1,without, k21}**
The reaction:2→1 Substrate:without Rate constant: k21
Do you want to keep this reaction (y or n)? **y**
Do you want to add another reaction (y or n)? **y**
Print the steady-state reaction: **{3,4,Nao, k34}**
The reaction:3→4 Substrate:Nao Rate constant:k34
Do you want to keep this reaction (y or n)? **y**
Do you want to add another reaction (y or n)? **y**
Print the steady-state reaction: **{4,3,without, k43}**
The reaction:4→3 Substrate:without Rate constant:k43
Do you want to keep this reaction (y or n)? **y**
Do you want to add another reaction (y or n)? **y**
do you want to add another reaction (y or n)? **n**

Summary table

1) 1→ 2 Substrate:without Rate constant:k12
2) 2→ 1 Substrate:without Rate constant:k21
3) 3→ 4 Substrate:Nao Rate constant:k34
4) 4→ 3 Substrate:without Rate constant:k43
5) 3→ 8 Substrate:without Rate constant:k38
6) 8→ 3 Substrate:without Rate constant:k83
7) 4→ 7 Substrate:without Rate constant:k47
8) 7→ 4 Substrate:without Rate constant:k74
9) 4→ 5 Substrate:So Rate constant:k45
10) 5→ 4 Substrate:without Rate constant:k54
11) 5→ 6 Substrate:without Rate constant:k56
12) 6→ 5 Substrate:without Rate constant:k65
13) 6→ 7 Substrate:without Rate constant:k67
14) 7→ 6 Substrate:Si Rate constant:k76
15) 7→ 8 Substrate:without Rate constant:k78
16) 8→ 7 Substrate:Nai Rate constant:k87
Do you want to add a steady-state reaction (y or n)? **n**
Do you want to clear a steady-state reaction (y or n)? **n**
Block X
1) 2↔3 Substrate:Nao Dissociation constant:K23
Block Y
1) 1 ↔ 8 substrate: Nai dissociation constant:K18
Do you want to add a rapid-equilibrium reaction (y or n)? **n**
Do you want to clear a rapid-equilibrium reaction (y or n)? **n**
Do you want to modify the model again (y or n)? **n**
Time required to construct the data: 0.16 s
Do you want to save this model (y or n)? **y**
Give it a name: **fig2a**
Do you want to start the computation (y or n)? **y**

Computation in progress. . .

Fractional distributions

$$X2: \quad \frac{K23}{K23 + Nao}$$

$$X3: \quad \frac{Nao}{K23 + Nao}$$

$$Y1: \quad \frac{K18}{K18 + Nai}$$

$$Y8: \quad \frac{Nai}{K18 + Nai}$$

Do you want to input a rate or binding equation (*v* or *b*)? **v**
Define the rate or binding equation: **2(k78 n[7] − k87 Nai n[8])**
Time required to calculate numerator and denominator: 32.84 s
Time required to simplify numerator and denominator: 3.85 s
The rate equation:

$$v/N_T = (C7\ Nai^2 + C3\ Nai^2\ Nao + C8\ Nao^2 + C5\ Nai\ Nao^2 + C4\ Nai^2\ Si + C1\ Nai^2\ Nao\ Si + C6\ Nao^2\ So + C2\ Nai\ Nao^2\ So)/(C40 + C36\ Nai + C29\ Nai^2 + C37\ Nao + C30\ Nai\ Nao + C21\ Nai^2\ Nao + C33\ Nao^2 + C24\ Nai\ Nao^2 + C14\ Nai^2\ Nao^2 + C38\ Si + C31\ Nai\ Si + C22\ Nai^2\ Si + C34\ Nao\ Si + C25\ Nai\ Nao\ Si + C15\ Nai^2\ Nao\ Si + C27\ Nao^2\ Si + C18\ Nai\ Nao^2\ Si + C10\ Nai^2\ Nao^2\ Si + C39\ So + C32\ Nai\ So + C23\ Nai^2\ So + C35\ Nao\ So + C26\ Nai\ Nao\ So + C16\ Nai^2\ Nao\ So + C28\ Nao^2\ So + C19\ Nai\ Nao^2\ So + C11\ Nai^2\ Nao^2\ So + C17\ Nai^2\ Si\ So + C12\ Nai^2\ Nao\ Si\ So + C20\ Nao^2\ Si\ So + C13\ Nai\ Nao^2\ Si\ So + C9\ Nai^2\ Nao^2\ Si\ So)$$

Constant number or *q* (*q*: quit)? **8**
Nao^2

$$2k12\ K18\ k34\ k47\ (k54\ k65 + k54\ k67 + k56\ k67)\ k78$$

Constant number or q (q: quit)? **6**
Nao^2 So

$$2\ k12\ K18\ k34\ k45\ k56\ k67\ k78$$

Constant number or q (q: quit)? q
Do you want to see the distribution equations (y or n)? **y**
Distribution equations:

$$n[1]/Nt = (C18 + C15\ Nao + C12\ Nao^2 + C16\ Si + C13\ Nao\ Si + C17\ So + C14\ Nao\ So + C11\ Nao^2\ So)/d$$

$$C11 = K18\ k34\ k45\ k56\ k67\ k78$$

$$C12 = K18\ k34\ k47(k54\ k65 + k54\ k67 + k56\ k67)k78$$

$$n[2]/Nt = (C28 + C25\ Nai + C22\ Nai^2 + C26\ Si + C23\ Nai\ Si + C21\ Nai^2\ Si + C27\ Si + C24\ Nai\ So)/d$$

$$C21 = K23\ k43\ k54\ k65\ k76\ k87$$

$$C22 = K23\ k43(k54\ k65 + k54\ k67 + k56\ k67)k74\ k87$$

Do you want to save this model (y or n)? **n**
Do you want to save this result (y or n)? **y**
Give it a name: **eqfig2a**
Do you want to pursue the work session with the current model (y or n)? **n**

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REFERENCES

Béliveau, R., M. Demeule, K. H. Ibnoul, M. Bergeron, G. Beauregard, and M. Potier. 1988. Radiation-inactivation studies on brush-border membrane vesicles. General considerations and application to the glucose and phosphate carriers. *Biochem. J.* 252:807–813.

Bennett, E., and G. A. Kimmich. 1996. The molecular mechanism and potential dependence of the Na^+/glucose cotransporter. *Biophys. J.* 70:1676–1688.

Blank, M. E., F. Bode, K. Bauman, and D. F. Diedrich. 1989. Computer analysis reveals changes in renal Na^+-glucose cotransporter in diabetic rats. *Am. J. Physiol.* 257:C385–C396.

Cha, S. 1968. A simple method for derivation of rate equations for enzyme-catalyzed reactions under the rapid equilibrium assumption or combined assumptions of equilibrium and steady state. *J. Biol. Chem.* 243:820–825.

Chen, X.-Z., M. J. Coady, F. Jackson, A. Berteloot, and J.-Y. Lapointe. 1995. Thermodynamic determination of the Na^+:glucose coupling ratio for the human SGLT1 cotransporter. *Biophys. J.* 69:2405–2414.

Chenu, C., and A. Berteloot. 1993. Allosterism and Na^+-D-glucose cotransport kinetics in rabbit jejunal vesicles: compatibility with mixed positive and negative cooperativities in a homo-dimeric or tetrameric structure and experimental evidence for only one transport protein involved. *J. Membr. Biol.* 132:95–113.

Cornish-Bowden, A. 1977. An automatic method for deriving steady-state rate equations. *Biochem. J.* 165:55–59.

Crane, R. K. 1977. The gradient hypothesis and other models of carrier-mediated active transport. *Rev. Physiol. Biochem. Pharmacol.* 78: 99–159.

Dorando, F. C., and R. K. Crane. 1984. Studies on the kinetics of Na^+ gradient coupled transport as found in brush-border membrane vesicles from rabbit jejunum. *Biochim. Biophys. Acta.* 772:273–287.

Fromm, H. J. 1979. Computer-assisted derivation of steady-state rate equations. *Methods Enzymol.* 63:84–103.

Gerardi-Laffin, C., P. Delque-Bayer, P. Sudaka, and J.-C. Poirée. 1993. Oligomeric structure of the sodium-dependent phlorizin binding protein from kidney brush-border membranes. *Biochim. Biophys. Acta.* 1151: 99–104.

Hazama, A., D. D. F. Loo, and E. M. Wright. 1997. Presteady-state currents of the Na^+/glucose cotransporter (SGLT1). *J. Membr. Biol.* 155:175–186.

Heinz, E., P. Geck, and W. Wilbrandt. 1972. Coupling in secondary active transport. Activation of transport by cotransport and/or countertransport with the fluxes of other solutes. *Biochim. Biophys. Acta.* 255:442–461.

Herries, G. 1984. The computerized derivation of steady-state rate equations for enzyme kinetics. *Biochem. J.* 223:551–553.

Hopfer, U., and R. Groseclose. 1980. The mechanism of Na^+-dependent D-glucose transport. *J. Biol. Chem.* 255:4453–4462.

Hurst, R. O. 1967. A simplified approach to the use of determinants in the calculation of the rate equation for a complex enzyme system. *Can. J. Biochem.* 45:2015–2039.

Hurst, R. O. 1969. A computer program for writing the steady-state rate equation for a multisubstrate enzymic reaction. *Can. J. Biochem.* 47: 941–944.

Ishikawa, H., T. Maeda, H. Hikita, and K. Miyatake. 1988. The computerized derivation of rate equations for enzyme reactions on the basis of the pseudo-steady-state assumption and the rapid-equilibrium assumption. *Biochem. J.* 251:175–181.

Jacquez, J. A. 1972. Models of ion and substrate cotransport and the effect of membrane potential. *Math. Biosci.* 13:71–93.

Kessler, M., and G. Semenza. 1983. The small-intestinal Na^+, D-glucose cotransporter: an asymmetric gated channel (or pore) responsive to $\Delta\psi$. *J. Membr. Biol.* 76:27–56.

Kimmich, G. A. 1990. Membrane potentials and the mechanism of intestinal Na^+-dependent sugar transport. *J. Membr. Biol.* 114:1–27.

Kimmich, G. A., and J. Randles. 1980. Evidence for an intestinal Na^+: sugar transport coupling stoichiometry of 2.0. *Biochim. Biophys. Acta.* 596:439–444.

Kimmich, G. A., and J. Randles. 1988. Na^+-coupled sugar transport: membrane potential-dependent K_m and K_i for Na^+. *Am. J. Physiol.* 255:C486–C494.

King, E. L., and C. Altman. 1956. A schematic method of deriving the rate laws for enzyme-catalyzed reactions. *J. Phys. Chem.* 60:1375–1378.

Koepsell, H., G. Fritzsch, K. Korn, and A. Madrala. 1990. Two substrate sites in the renal Na^+-D-glucose cotransporter studied by model analysis of phlorizin binding and D-glucose transport measurements. *J. Membr. Biol.* 114:113–132.

Koepsell, H., and J. Spangenberg. 1994. Function and presumed molecular structure of Na^+-D-glucose cotransport systems. *J. Membr. Biol.* 138: 1–11.

Koshland, D. E., Jr., G. Nemethy, and D. Filmer. 1966. Comparison of experimental binding data and theoretical models in protein containing subunits. *Biochemistry.* 5:365–385.

Läuger, P., and P. Jauch. 1986. Microscopic description of voltage effects on ion-driven cotransport systems. *J. Membr. Biol.* 91:275–284.

Lee, W. S., Y. Kanai, R. G. Wells, and M. A. Hediger. 1994. The high affinity Na^+/glucose cotransporter. *J. Biol. Chem.* 269:12032–12039.

Levitzki, A. 1978. Quantitative aspects of allosteric mechanisms. *Mol. Biol. Biochem. Biophys.* 28:1–104.

Loo, D. D. F., A. Hazama, S. Supplisson, E. Turk, and E. M. Wright. 1993. Relaxation kinetics of the Na^+/glucose cotransporter. *Proc. Natl. Acad. Sci. USA.* 90:5767–5771.

Moran, A., L. J. Davis, and R. J. Turner. 1988. High affinity phlorizin binding to the LLC-PK_1 cells exhibits a sodium:phlorizin stoichiometry of 2:1. *J. Biol. Chem.* 263:187–192.

Otsu, K., J. L. Kinsella, E. Koh, and J. P. Froehlich. 1992. Proton dependence of the partial reactions of the sodium-proton exchanger in renal brush border membranes. *J. Biol. Chem.* 267:8089–8096.

Otsu, K., J. L. Kinsella, B. S. Sacktor, and J. P. Froehlich. 1989. Transient state kinetic evidence for an oligomer in the mechanism of Na^+-H^+ exchange. *Proc. Natl. Acad. Sci. USA.* 86:4818–4822.

Oulianova, N., and A. Berteloot. 1996. Sugar transport heterogeneity in the kidney: two independent transporters or different transport modes through an oligomeric protein? 1. Glucose transport studies. *J. Membr. Biol.* 153:181–194.

Parent, L., S. Supplisson, D. D. F. Loo, and E. M. Wright. 1992a. Electrogenic properties of the cloned Na^+/glucose cotransporter. I. Voltage-clamp studies. *J. Membr. Biol.* 125:49–62.

Parent, L., S. Supplisson, D. D. F. Loo, and E. M. Wright. 1992b. Electrogenic properties of the cloned Na^+/glucose cotransporter. II. A transport model under nonrapid equilibrium conditions. *J. Membr. Biol.* 125:63–79.

Peerce, B. E., and E. M. Wright. 1984. Sodium-induced conformational changes in the glucose transporter of intestinal brush borders. *J. Biol. Chem.* 259:14105–14112.

Peerce, B. E., and E. M. Wright. 1985. Evidence for tyrosyl residues at the Na^+ site on the intestinal Na^+/glucose cotransporter. *J. Biol. Chem.* 260:6026–6031.

Peerce, B. E., and E. M. Wright. 1986. Distance between substrate sites on the Na-glucose cotransporter by fluorescence energy transfer. *Proc. Natl. Acad. Sci. USA.* 83:8092–8096.

Restrepo, D., and G. A. Kimmich. 1985a. Kinetic analysis of mechanism of intestinal Na^+-dependent sugar transport. *Am. J. Physiol.* 248: C498–C509.

Restrepo, D., and G. A. Kimmich. 1985b. The mechanistic nature of the membrane potential dependence of sodium-sugar cotransport in small intestine. *J. Membr. Biol.* 87:159–172.

Restrepo, D., and G. A. Kimmich. 1986. Phlorizin binding to isolated enterocytes: membrane potential and sodium dependence. *J. Membr. Biol.* 89:269–280.

Rhoads, D. G., and M. Pring. 1968. The simulation and analysis by digital computer of biochemical systems in terms of kinetic models. *J. Theor. Biol.* 20:297–313.

Salhany, J. M. 1992. Band 3 quaternary states and allosteric control of function. *Prog. Cell Res.* 2:191–205.

Sanders, D., U.-P. Hansen, D. Gradmann, and C. L. Slayman. 1984. Generalized kinetic analysis of ion-driven cotransport systems: a unified interpretation of selective ionic effects on Michaelis parameters. *J. Membr. Biol.* 77:123–152.

Schultz, S. G., and P. F. Curran. 1970. Coupled transport of sodium and organic solutes. *Physiol. Rev.* 50:637–718.

Segel, I. W. 1975. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. Wiley Interscience, New York.

Semenza, G., M. Kessler, M. Hosang, J. Weber, and U. Schmidt. 1984. Biochemistry of the Na^+, D-glucose cotransporter of the small-intestinal brush border membrane. The state of the art in 1984. *Biochim. Biophys. Acta.* 779:343–379.

Semenza, G., M. Kessler, U. Schmidt, J. C. Venter, and C. M. Fraser. 1985. The small-intestinal sodium-glucose cotransporter(s). *Ann. N.Y. Acad. Sci.* 456:83–96.

Smith-Maxwell, C., E. Bennett, J. Randles, and G. A. Kimmich. 1990. Whole cell recording of sugar-induced currents in LLC-PK_1 cells. *Am. J. Physiol.* 258:C234–C242.

Stein, W. D. 1981. Concepts of mediated transport. *In* Membrane Transport. S. L. Bonting and J. J. H. H. M. de Pont, editors. Elsevier/North Holland, Amsterdam. 123–157.

Stevens, B. R., A. Fernandez, B. Hirayama, E. M. Wright, and E. S. Kempner. 1990. Intestinal brush border membrane Na^+/glucose cotransporter functions in situ as a homotetramer. *Proc. Natl. Acad. Sci. USA.* 87:1456–1460.

Su, A., S. Mager, S. L. Mayo, and H. A. Lester. 1996. A multi-substrate single-file model for ion-coupled transporters. *Biophys. J.* 70:762–777.

Takahashi, M., P. Malathi, H. Preiser, and C. Y. Jung. 1985. Radiation inactivation studies on the rabbit kidney sodium-dependent glucose transporter. *J. Biol. Chem.* 260:10551–10556.

Turner, J. 1981. Kinetic analysis of a family of cotransport models. *Biochim. Biophys. Acta.* 649:269–280.

Turner, J. 1983. Quantitative studies of cotransport systems: models and vesicles. *J. Membr. Biol.* 76:1–15.

Turner, R. J. 1985. Solution of carrier-type transport models: general solution for an arbitrarily complex rapid equilibrium model. *J. Membr. Biol.* 88:77–83.

Turner, R. J., and E. S. Kempner. 1982. Radiation inactivation studies of the renal brush-border membrane phlorizin-binding protein. *J. Biol. Chem.* 257:10794–10797.

Ugolev, A. M., and S. T. Metel'skii. 1990. Two-channel transporter versus a single-channel Na^+-dependent transporter for glucose and amino acids in rat and turtle. *Biomed. Sci.* 1:578–584.

Volkenstein, M. V., and B. N. Goldstein. 1966. A new method for solving the problems of the stationary kinetics of enzymological reactions. *Biochim. Biophys. Acta.* 115:471–477.

Wierzbicki, W., A. Berteloot, and G. Roy. 1990. Presteady-state kinetics and carrier-mediated transport: a theoretical analysis. *J. Membr. Biol.* 117:11–27.

Wolfram, S. 1993. Mathematica: A System for Doing Mathematics by Computer. Addison-Wesley, New York.

Wright, E. M. 1993. The intestinal Na^+/glucose ion-coupled cotransporter. *Annu. Rev. Physiol.* 55:575–589.