Use of experimental isotope-exchange fluxes in reversible enzyme and membrane transport models, assessed by simultaneous computer simulation of unidirectional and net chemical rates

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Steady-state rate equations for unidirectional (isotope-exchange) rates can become so complex, even for rather simple (reversible) enzyme or membrane transport models, that they are useless for detailed data analysis. In this paper a procedure is described for simultaneous simulation of net (chemical) and isotope-exchange rates. The method employs an expanded version of the basic model to monitor explicitly the fate of the label in an experiment. The procedure is quite general, and can be used for steady-state as well as transient kinetic situations, or it can be used in conjunction with existing interactive computer programs for steady-state model analysis. Three numerical examples are presented. First, it is shown, using the conventional (Post-Albers) model for Na⁺/K⁺-ATPase, that the change in concentration of a labelled intermediate after a change in experimental conditions does not in general reflect the change in the total concentration of that intermediate, and thus labelled intermediate concentrations may be misleading. Second, using a standard co-transport model and a prototype active-transport model (equivalent to a ligand-ATPase), it is shown that the ratio of tracer transport fluxes at steady state yields transport stoichiometries which depend on the experimental conditions, are different from the net apparent stoichiometries, and whose changes with conditions are also different from that of the net stoichiometries. It follows that conclusions drawn on the basis of experimentally determined tracer fluxes should be viewed with some caution. Specifically, a measured influx stoichiometry ligand/ATP (in the ATPase case) of higher than 1:1 does not necessarily imply the existence of more than one site for either ligand on the enzyme.

INTRODUCTION

The goal in studies of the kinetics of enzyme-catalysed reactions, in solution or in a membrane transport system, is to arrive at a kinetic model capable of describing the experimental observations in the sense that the behaviour of the measured rates under a variety of experimental conditions should be satisfactorily reproduced by the rate equation derived from the model.

The rate of change of the individual kinetic-state concentrations (state probabilities) E_i in a model are described by differential equations of the form

$$\frac{dE_i}{dt} = \sum_j (k_{ji} E_j - k_{ij} E_i) \quad (1)$$

where the k_{ij} are rate constants from state i to state j and the summation is over all states directly connected to the state E_i . For a kinetic mechanism with n states, the steady-state solution for the state probabilities is obtained by solving the algebraic equations

$$\frac{dE_i}{dt} = 0, \quad i = 1 \quad \text{to} \quad n-1 \quad (2)$$

$$\sum_{i=1}^n E_i = 1 \quad (3)$$

and the steady-state rate $v = d[\text{prod}]/dt$ is then obtained from an equation of the form

$$v = k_{i,i+1} E_i - k_{i+1,i} E_{i+1} \quad (4)$$

As is well known, diagram procedures directly yielding the solutions of the steady-state equations are available [1,2].

In many cases rate measurements are carried out using isotopically labelled substrates, and the rates are thus unidirectional. If such measurements are initial (steady-state) rates, i.e.

performed in the absence of products of the reaction, no problems arise with the interpretation of the results. The measured rates are equal to the net 'chemical' rates, and hence the above procedures for deriving the relevant rate equation for the model can be used.

In the presence of products, however, this is no longer true. This is the general situation in membrane transport systems, where the transported ligand(s) are both substrate(s) (on one side of the membrane) and product(s) (on the other). It should be emphasized that neither of the terms in eqn. (4) represents a unidirectional flux (as measured by the use of tracers). For that, one needs to know what fraction of the steady-state concentration of the E_i stems from the point in the mechanism at which a labelled compound is added.

Diagram methods have been developed to obtain these fractional concentrations and rate equations for isotope exchange [3,4]. However, even for relatively simple models, these equations in terms of the rate constants of the model become very complex. For example, in the exchange rate equation for P_i -ATP exchange for the model in Fig. 1(a) used in the following section, the denominator will contain 315 terms, each a product of several rate constants. Of course, such an expression can be formally simplified by collecting terms, and subsequently used for non-linear regression procedures, but the physical significance of the parameters obtained is then obscured.

This paper presents a simple procedure for computation of the fractional concentrations of intermediates stemming from the addition of substrate and product respectively, and simultaneously for the total steady-state concentrations of the intermediates. This then permits a comparison of net chemical rates and unidirectional rates. In numerical examples it is then shown that, in general: (i) no conclusions regarding the total steady-state concentrations of intermediates can be drawn on the basis of measurements of labelled intermediate concentrations during

exchange; (ii) transport stoichiometries calculated from ratios of unidirectional fluxes in membrane co-transport models, as well as in a prototype active-transport model, may be very different from the actual net stoichiometry of the model; furthermore, such stoichiometries depend on the concentrations of the transported ligands in a particular run, as well as on their gradients across the membrane, in ways that may be different from those of stoichiometries calculated from the net fluxes, and they are thus not characteristic of the model (with associated rate constants), as are the stoichiometries calculated from ratios of the net fluxes. Nor can the number of binding sites per enzyme unit be easily inferred from such measurements.

The question of exchange-flux stoichiometries and their possible difference from net-flux stoichiometries was addressed a few years ago by Heinz [5]. He considered a rather special case of an ordered co-transport system without slippage and derived for this model, expanded as here to include explicitly the labelled states, a formal expression for the ratio of the exchange fluxes of the transported ligands. However, the possible magnitude of the deviation from the model stoichiometry could not be assessed from his work, and it is in fact stated that the error in the stoichiometry calculated in this way may be expected to be small. In contrast, it is shown in this paper that the deviations may be quite large, and that uncritical use of exchange-flux stoichiometries may lead to erroneous conclusions regarding the properties of the underlying mechanism.

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THEORY AND METHODS

The procedure for simulation of unidirectional rates at steady state in an enzyme kinetic mechanism is best described by consideration of a specific model. The method is quite general and can easily be implemented for any model of enzyme action, including isotope exchange at equilibrium, and calculation of pre-steady-state rates for isotope exchange.

The model shown in Fig. 1(a) is a simplified version of the Post-Albers scheme [6,7] for the action of the membrane-bound transport enzyme Na^+/K^+ -ATPase. This is the enzyme responsible for the active transport of Na^+ and K^+ against their electrochemical gradients through mammalian cell membranes, utilizing free energy derived from the concomitant hydrolysis of ATP to ADP and inorganic phosphate:

When the enzyme is studied under conditions where no net transport of ions occurs, such as in broken membrane preparations, the only reaction that can be monitored is the hydrolysis reaction (5). The activity of the enzyme is strongly influenced by Na^+ and K^+ , but in Fig. 1(a) only the action of the latter is explicitly shown.

To obtain a numerical steady-state solution for the model in Fig. 1(a), the rates of change of the kinetic intermediates are set equal to zero, and the five independent linear algebraic equations, together with the enzyme conservation equation, are solved by computer. In matrix form, the equations may be written

$$AX = B \quad (6)$$

where A is the coefficient matrix, consisting of linear combinations of rate constants, X is the vector of steady-state concentrations of the kinetic intermediates, and B is a vector of constants, the elements of which are all zero except one, which is unity, if the system is normalized so that all enzyme species concentrations are fractions of the total enzyme concentration.

Fig. 1. (a) Essential features of the Post-Albers model for Na^+/K^+ -ATPase and (b) expanded model used in the exchange simulation

(a) In the absence of K^+ , the enzyme hydrolyses ATP using the cycle B, in the clockwise direction; in the presence of K^+ , the overall cycle $C = A + B$ is presumed to dominate. The action of Na^+ is not explicitly shown. Also, ADP is presumed in the calculations to inhibit competitively by binding to the empty enzyme forms. T and P denote ATP and phosphate respectively. E_1 and E_2 denote different conformational states of the Na^+/K^+ -ATPase. The species enclosed in boxes are presumed to be in rapid equilibrium. Several cycle relations among rate constants result from small cycles between forms 1 and 5, as well as between states 1 and 2. In (b) the asterisk in the left part of the Figure denotes a labelled species. Note that the state E_1 is shown twice in the diagram for purely graphical reasons.

Several computer programs, such as KINSIM [8], are available for interactive processing of such equations.

The solutions are the (fractional) steady-state concentrations of the intermediates, from which the net rate in any step may be calculated. For the model in Fig. 1(a), the net hydrolysis rate is obtained from:

$$v_{\text{net}} = k_{51}[\text{E}_2\text{P}] - k_{15}[\text{E}_2] \quad (7)$$

To obtain numerical values of the unidirectional rate, which is the quantity being measured with isotopically labelled ATP, say, it is necessary to keep track of the part of a particular species stemming from addition of the labelled compound. The ATP- P_i exchange rate for this model is

$$v_{\text{hyd}}^* = k_{51}[\text{E}_2\text{P}^*] \quad (8)$$

where $[\text{E}_2\text{P}^*]$ is the concentration of labelled E_2P . (In this paper the term 'ATP- P_i exchange rate' is defined as the hydrolysis rate measured using labelled ATP, in the presence or absence of unlabelled products. Likewise, P_i -ATP exchange is the unidirectional synthesis rate of ATP, using labelled P_i and unlabelled ATP and ADP.) It is clear from the work of Cleland [4] for the calculation of exchange rates that the labelled species concentration $[\text{E}_2\text{P}^*]$ will always be given by an expression of the form

$$[\text{E}_2\text{P}^*] = [\text{ATP}^*]f(k_i, \text{ATP}, \text{ADP}, \text{P}_i) \quad (9)$$

where $[\text{ATP}^*]$ is the concentration of labelled substrate, and f is a function of rate constants and ligand concentrations. Denoting by α the fraction of the total ATP concentration that is labelled,

it follows that the concentration of E_2P stemming solely from the addition of ATP to the enzyme is

$$[E_2P]_{ATP} = \frac{[E_2P^*]}{\alpha} \quad (10)$$

since $[ATP^*] = \alpha[ATP]$. We thus need to compute $[E_2P^*]$ (or corresponding quantities for different exchange processes) for a given value of α .

To achieve this we first expand the model in Fig. 1(a) such that those enzyme states that will become labelled during measurement of a particular exchange rate are explicitly present in the model and distinguishable from the corresponding unlabelled states. If we wish to study the rates for $ATP \rightarrow P_i$ and $P_i \rightarrow ATP$ exchange for the model in Fig. 1(a), the expansion of the model results in the scheme shown in Fig. 1(b), where the states becoming labelled during the exchange have been marked with an asterisk. (Note that for graphical reasons the state E_1 is represented twice in the diagram.) The rate constants connecting the labelled species are the same as those connecting the corresponding unlabelled ones.

In order to calculate the steady-state concentrations of all species in this enlarged model, all second-order rate constants corresponding to ligand addition have to be multiplied by the appropriate ligand concentrations. For the calculation of the unidirectional rate for $P_i \rightarrow ATP$ exchange, we let the fraction of the added P_i that contains the label be $\alpha < 1$. Neither ATP nor ADP contains labelled phosphate under these conditions. We therefore take for the value of the phosphate concentration, $[P^*]$, to multiply the rate constant connecting E_2 and E_2P^* in Fig. 1(b):

$$[P^*] = \alpha[P]$$

where $[P]$ is the total concentration of P_i . The P_i concentration to be used for the corresponding unlabelled addition is then $(1-\alpha)[P]$. Analogously, for simulation of this experiment, the concentration of labelled ATP is set to zero, so that there is no back-reaction in the steps at which labelled synthesized ATP is released from the enzyme. The steady-state equations, having the same form as eqn. (6), but with enlarged coefficient matrix A and vectors X and B corresponding to the enlarged model, are now solved. If α is small enough, the resulting steady-state concentrations of the labelled species will be small enough that the concentrations of the unlabelled species are not noticeably perturbed relative to the values obtained for the smaller model in Fig. 1(a) (see below). This ensures that the correct steady-state concentrations of the unlabelled enzyme species are available to the label. From the solutions for the labelled enzyme species, the exchange rate $v_{syn.}$ for this case is then calculated from

$$v_{syn.} = \frac{k_{96}[E_1 T^*] + k_{10,1}[E_2 T^*]}{\alpha} \quad (11)$$

Similarly, for the exchange rate in the opposite direction, the steady-state equations are again solved, but now the labelled substrate concentration is $\alpha[ATP]$, that of the unlabelled substrate $(1-\alpha)[ATP]$, and that of the labelled phosphate is set to zero. The exchange rate for $ATP \rightarrow P_i$ is subsequently calculated from

$$v_{hyd.} = \frac{k_{71}[E_2 P^*]}{\alpha} \quad (12)$$

The fraction of a species concentration stemming from a particular ligand addition will in general vary from species to species at steady state. This in turn implies that one cannot, in general, use the amount of label in a particular enzyme form to represent the actual steady-state concentration of that form (see below).

NUMERICAL RESULTS

General

To achieve the high accuracy needed in solving the equations, the solutions of which may differ by several orders of magnitude, double precision is used throughout in the computations, which were performed on a VAX 6210 computer. The equations are solved by Gaussian elimination, using Crout's algorithm with partial pivoting (the so-called LU-decomposition method) and subsequent iterated improvement of the solution (see ref. [9] for details). To avoid possible problems with matrix elements $A_{ij} = 0$, all such elements were set to 10^{-4} in the computations. In the present case, this procedure yields an accuracy (round-off error) of about 10^{-13} of the steady-state concentrations.

Rate constants

The rate constants in the model are not all independent; they must satisfy detailed balance at equilibrium. Thus, in any closed cycle in the model, the product of rate constants in the clockwise direction (including, where appropriate, the concentration of ligand added) must equal the product of rate constants in the counterclockwise direction. This ensures that the net flux in any cycle, and the associated entropy production, are zero at equilibrium, as they must be according to the second law of thermodynamics. For example, for cycle A in Fig. 1(a), the result of which is merely the addition of ATP and its subsequent release from the enzyme, the concentration terms cancel, and the condition of detailed balance yields the relation:

$$\frac{\prod_{\text{forward}} k_i}{\prod_{\text{backward}} k_j} = 1 \quad (13)$$

On the other hand, for cycle b, the result of which is the complete conversion of substrate into products, no such cancellation of (equilibrium) concentrations occurs, and the resulting condition for the rate constants is

$$\frac{\prod_{\text{forward}} k_i}{\prod_{\text{backward}} k_j} = \frac{[ADP]_e [P_i]_e}{[ATP]_e} = K_{eq.} \quad (14)$$

where $K_{eq.}$ is the equilibrium constant for reaction (1). Thus the rate constants are related to the equilibrium constant of the catalysed reaction, as they must be, since the presence of a catalyst cannot change the position of the equilibrium nor the value of the equilibrium constant. A relation similar to eqn. (14) applies to the larger cycle C (= cycle A + cycle B), but this condition follows from eqns. (13) and (14) and thus is not a new independent relation between rate constants. A new relation will be obtained, however, in models in which two distinct non-overlapping pathways for conversion of substrates into products are present. The appropriate relations are automatically satisfied in the program used here by submitting as data all rate constants except one in each elementary cycle; the missing rate constant is then calculated in the program using the relations above.

Value of α

The population obtained in a computation of the labelled states, and hence the extent of perturbation of the steady-state values of the unlabelled concentrations, will depend on the value of α . Thus a small value of α is indicated. On the other hand, if α is too small, the concentrations of the labelled species approach the round-off error, yielding unreliable values for these species. Also, the results for the unidirectional rates, i.e. for the concentrations of the part of a particular species that stems from the

Fig. 2. Steady-state concentration of E_1P stemming from the point of addition of the labelled ligand (i.e. $[E_1P^*]/\alpha$ (—), see the text) and that of the total concentration E_1ATP (----) for various values of α

(a) $ATP \rightarrow P_i$ exchange (i.e. hydrolysis) and (b) $P_i \rightarrow ATP$ exchange (i.e. synthesis) simulations.

point of addition of the labelled compound, E_1^*/α , must be independent of α . We thus expect a range of α -values satisfying these criteria.

In Fig. 2(a) is shown the variation of the labelled species concentrations, divided by α , as a function of α for a particular choice of rate constants and ligand concentrations. Also shown is the variation of the steady-state concentration of the unlabelled state E_1ATP in Fig. 1(b) (as an indicator of the steady-state concentrations of unlabelled intermediates; right-hand ordinate). These results pertain to the calculation of the unidirectional hydrolytic rate of ATP ($ATP \rightarrow P_i$ exchange). Similarly, Fig. 2(b) shows the results for the calculation of the $P_i \rightarrow ATP$ exchange rate. It can be seen that, for the rate of synthesis, values of α in the range $5 \times 10^{-6} < \alpha < 10^{-1}$ will satisfy the criteria above, whereas, for the rate of hydrolysis, the corresponding values must be in the range $5 \times 10^{-7} < \alpha < 3 \times 10^{-3}$. It is clear that the precise value for α is irrelevant for the results, provided that it is in the proper range.

It should be pointed out here that the reason for the change in the unlabelled E_1ATP concentration for values of α close to 1 is really that the calculation is then performed at a different ATP or P_i concentration, namely $(1 - \alpha)$ times the total concentration. Since the concentrations of the unlabelled species are not given by equations analogous to eqn. (9), one cannot 'normalize' the unlabelled concentrations merely by dividing by $(1 - \alpha)$.

A knowledge of the steady-state concentrations of unlabelled intermediates can be used to provide a check for consistency of the calculated unidirectional rates. For the model in Fig. 1, the net rate of hydrolysis may be found for example from eqn. (7),

and this rate is related to the unidirectional rates for hydrolysis and synthesis by the equation:

$$v_{\text{net}} = v_{\text{hyd.}} - v_{\text{syn.}} \quad (15)$$

Does the measurement of the concentration of a labelled intermediate reflect the true steady-state concentration of that species?

An example to illustrate this point is shown in Fig. 3, containing some results from simulations of the rates of synthesis ($P_i \rightarrow ATP$ exchange) and hydrolysis ($ATP \rightarrow P_i$ exchange) for the model in Fig. 1 with and without K^+ (5 mM) in the medium. (See the Appendix for more details of the model and the rate constants.) The conventional procedure used to measure the amount of phosphoenzyme, EP, ($= E_1P + E_2P + E_2PK$ in the model) is to precipitate the phosphoenzyme with acid, filtrate and count the label incorporated. If it is believed that the values so obtained truly reflect the steady-state concentrations, the results of this procedure would then indicate, using first the synthesis results (second set of bars in Fig. 3), that the concentration of phosphoenzyme on addition of K^+ had increased by a factor of about 10, whereas in fact the steady-state (total, first set of bars) concentration of phosphoenzyme has actually decreased. A similar calculation, corresponding to an experiment with labelled ATP (third set of bars), shows that the labelled phosphoenzyme concentration does decrease in the presence of K^+ , but to a larger extent than the actual steady-state concentration. In neither case, then, is the value of the labelled phosphoenzyme concentration a good indicator of the steady-state concentration, and may be misleading, as in the case of the synthesis results.

The magnitude of the possible discrepancies between steady-state concentrations calculated from exchange experiments and those obtained using 'chemical' procedures (if they exist) will depend not only on the rate constants, but also on the particular model. Thus one cannot make general statements as to which rate constants are 'critical' in a model. The data presented in Fig. 3 merely illustrate that, for quite realistic values in the model used here, such discrepancies can be quite large.

The physical reasons for these discrepancies may be understood

Fig. 3. Simulation of the results of a determination of the phosphoenzyme concentration: total EP concentration ('no label'), in $P_i \rightarrow ATP$ exchange experiments and in $ATP \rightarrow P_i$ exchange

Open bars, no K^+ ; hatched bars, with 5 mM- K^+ .

as follows. Consider any unlabelled state, say E_1P , and suppose we measure ATP- P_i exchange. There are positive contributions to E_1P from transitions $E_1ATP \rightarrow E_1P$, and a part of these are due to the addition of ATP to E_1 . Positive contributions to E_1P are also obtained from the transitions $E_2P \rightarrow E_1P$ which are, in part, due to P_i adding to E_2 and E_2K . To obtain the net one-way flux through the model in the positive direction, i.e. the direction of hydrolysis of ATP, we need to consider only those contributions (in addition to the negative contributions from transitions $E_1P \rightarrow E_1ATP$ or $E_1P \rightarrow E_2P$) that are a result of the addition of ATP to E_1 . This is achieved by introducing the cycle with the labelled states, since, in a particular experiment, there will be input in this cycle only from the substrate end or the product end, but not both. It is therefore not surprising that the distribution of labelled species differs from that of the unlabelled ones at steady state, and hence the specific activities of the kinetic states are not equal.

The results obtained with and without K^+ in this model can be qualitatively understood with reference to the rate constants and further details of the model shown in the Appendix. The addition of K^+ to the enzyme causes a new set of states for the enzyme to be available, and since the K^+ -bound empty enzyme has a lower affinity for ATP (see Table A1), the concentration of the K^+ -bound states are increased at the expense of the phosphoenzyme, leading to a decreased amount of EP. For the labelled EP, however, the situation is different. All things being equal, the concentration of a labelled species is larger the closer it is to the point in the mechanism at which a labelled compound is being added. In a synthesis experiment the addition of K^+ increases the rate constant for P_i addition to E_2K , as compared with that for E_2 (see Table A1), and even though the release rate constant is also increased, these features in combination with the changed K^+ affinity in the E_2 pool, relative to the E_2P pool (because of a detailed balance relation between the rate constants in the small cycle $E_2-E_2K-E_2KP-E_2P-E_2$), leads to a marked increase in the EP* concentration. In a hydrolysis experiment, on the other hand, the EPs are far away from the addition of label, and there is no effect of the increase of k_{on} for P_i since labelled P_i is not added. As the release of P_i is increased, however, the total labelled EP concentration decreases in the presence of K^+ , to a larger extent than does the unlabelled EP because that species receives a positive contribution from the addition of (unlabelled) P_i .

Stoichiometry of transport in a membrane co-transport model

The model is shown in Fig. 4(a). It transports the ligands N and L from outside to inside. N is the driving ligand, moving down its concentration gradient, and L, the driven ligand, is transported against its concentration gradient. To obtain a net inwards movement of the ligands, we must have $[L]_{in}/[L]_{out} < [N]_{out}/[N]_{in}$, i.e. the driving force (the gradient in the chemical potential of N) must be larger than the opposing force for L. This is, however, not a sufficient condition for net inwards transport of L, as will be detailed below. The model has some slippage: if the rate constants k and l in Fig. 4(a) are comparable with the other rate constants in the mechanism, some N may be transported without L, and the net transport stoichiometry L/N will be smaller than 1:1. On the other hand, if k and l are very small, there is effectively no slippage, and the net transport stoichiometry will always be 1:1. Both cases will be considered here.

The expanded model for the general case that both ligands are simultaneously labelled is shown in Fig. 4(b). An asterisk and a filled circle respectively are used to indicate the labelled forms of N and L. There are 14 states. The input data in the simulations are the concentrations of the ligands on both sides of the

Fig. 4. (a) A simple prototype co-transport model and (b) the expanded model

(a) Both ligands N and L are transported from out to in. The ligand N drives L against its concentration gradient. The enzyme states E_1 and E_2 designate accessibility to the outside and inside respectively. The rate constants are second or first order where appropriate. Their concentration unit is molar. The rate constants k and l have the values 0.1 and 1 respectively for the cases with slippage, and 10^{-8} and 10^{-7} for the no-slippage cases. In (b) an asterisk and a filled circle respectively denote labelled ligands N and L.

membrane and the rate constants in the basic model (Fig. 4a). The program then assigns rate constants to the appropriate transitions in Fig. 4(b). Whether a single- or double-label experiment is simulated is governed in the program by assigning zero or non-zero values to the appropriate 'specific activities' α of the ligands N and L. In these simulations $\alpha = 10^{-4}$ (for both ligands) was found to be suitable in all cases. A model without slippage is simulated by setting the rate constants k and l in Fig. 4(a) equal to 10^{-8} and 10^{-7} respectively, and for a model with slippage the rate constants are set to 0.1 and 1 respectively. For both cases the transport stoichiometries were calculated from the ratios of the appropriate fluxes, i.e. net (chemical) fluxes and one-way inward fluxes, as functions of (i) $[L]_{out}$, at constant $[L]_{in}$, $[N]_{out}$ and $[N]_{in}$; (ii) $[L]_{in}$, at constant $[L]_{out}$, $[N]_{out}$, $[N]_{in}$; (iii) $[N]_{out}$, at constant $[L]_{out}$, $[L]_{in}$ and $[N]_{in}$; (iv) $[L]_{in}$ and $[L]_{out}$ with constant $[L]_{in}/[L]_{out} = 2$, and constant $[N]_{out}$, $[N]_{in}$.

The results are shown in Tables 1–4. It is noted first that in Tables 1 and 2 the 'slippage' results may be negative, despite the fact that in all cases the condition $[N]_{out}/[N]_{in} > [L]_{in}/[L]_{out}$ is satisfied. Apparently this is not a sufficient condition for obtaining positive net flux inwards. Such a condition may be found by

Table 1. Variation of flux stoichiometry (from net and influxes) with $[L]_{out}$

$[L]_{in} = 20 \text{ mM}$; $[N]_{out} = 150 \text{ mM}$; $[N]_{in} = 5 \text{ mM}$. No slippage/slippage: $k = 10^{-8}/0.1$; $l = 10^{-7}/1$.

Varied species $[L]_{out}$ (mM)	No slippage		Slippage	
	$[L]/[N]$ (net)	$[L]/[N]$ (in)	$[L]/[N]$ (net)	$[L]/[N]$ (in)
2	1.0	1.16	-0.09	0.20
4	1.0	1.18	0.09	0.35
6	1.0	1.18	0.21	0.46
8	1.0	1.19	0.32	0.56
10	1.0	1.19	0.40	0.63
12	1.0	1.19	0.47	0.69
14	1.0	1.19	0.52	0.74
16	1.0	1.20	0.57	0.79
18	1.0	1.20	0.61	0.83
20	1.0	1.20	0.65	0.86

Table 2. Variation of flux stoichiometry with $[L]_{in}$

$[L]_{out} = 10 \text{ mM}$; $[N]_{out} = 150 \text{ mM}$; $[N]_{in} = 5 \text{ mM}$. No slippage/slippage: $k = 10^{-8}/0.1$; $l = 10^{-7}/1$.

Varied species $[L]_{in}$ (mM)	No slippage		Slippage	
	$[L]/[N]$ (net)	$[L]/[N]$ (in)	$[L]/[N]$ (net)	$[L]/[N]$ (in)
10	1.0	1.10	0.48	0.58
20	1.0	1.19	0.40	0.63
30	1.0	1.28	0.32	0.68
40	1.0	1.36	0.24	0.72
50	1.0	1.46	0.16	0.76
60	1.0	1.54	0.07	0.81
70	1.0	1.63	-0.02	0.85
80	1.0	1.70	-0.11	0.89
90	1.0	1.78	-0.21	0.93
100	1.0	1.86	-0.31	0.98

Table 3. Variation of flux stoichiometry with $[N]_{out}$

$[N]_{in} = 5 \text{ mM}$; $[L]_{out} = 10 \text{ mM}$; $[L]_{in} = 20 \text{ mM}$. No slippage/slippage: $k = 10^{-8}/0.1$; $l = 10^{-7}/1$.

Varied species $[N]_{out}$ (mM)	No slippage		Slippage	
	$[L]/[N]$ (net)	$[L]/[N]$ (in)	$[L]/[N]$ (net)	$[L]/[N]$ (in)
20	1.0	1.56	0.11	0.91
40	1.0	1.35	0.31	0.75
60	1.0	1.28	0.35	0.69
80	1.0	1.23	0.37	0.67
100	1.0	1.22	0.39	0.65
120	1.0	1.20	0.39	0.64
140	1.0	1.19	0.40	0.63
160	1.0	1.19	0.40	0.63
180	1.0	1.18	0.41	0.62
200	1.0	1.18	0.41	0.62

the use of the flux-diagram technique developed by Hill [2]. When there is a substantial flux through the step $E_1N \rightarrow E_2N$, the net fluxes of the ligands will receive contributions from one or more of the cycle fluxes in Fig. 4(a). There are three cycles, and

Table 4. Variation of flux stoichiometry with $[L]_{out}$ and $[L]_{in}$ with constant $[L]_{in}/[L]_{out} = 2$

$[N]_{out} = 150 \text{ mM}$; $[N]_{in} = 5 \text{ mM}$. No slippage/slippage: $k = 10^{-8}/0.1$; $l = 10^{-7}/1$.

Varied species $[L]_{in} = 2[L]_{out}$ (mM)	No slippage		Slippage	
	$[L]/[N]$ (net)	$[L]/[N]$ (in)	$[L]/[N]$ (net)	$[L]/[N]$ (in)
20	1.0	1.19	0.40	0.62
40	1.0	1.41	0.55	0.99
60	1.0	1.62	0.62	1.28
80	1.0	1.83	0.67	1.54
100	1.0	2.07	0.70	1.79
120	1.0	2.28	0.72	2.04
140	1.0	2.51	0.74	2.27
160	1.0	2.72	0.75	2.50
180	1.0	2.96	0.76	2.78
200	1.0	3.18	0.77	2.94

the net inwards flux of L will consist of fluxes associated with the lower elementary cycle and the overall cycle, one or both of which may become negative, leading to a negative total flux for the ligand. Using the rate constant values in Fig. 4(a), the flux-diagram method yields the following condition for zero net flux of L inwards:

$$Y_L = \frac{200[N]_{out} + 20 + 21 Y_N}{200[N]_{out} + 40 + Y_N} \quad (16)$$

where

$$Y_L = \frac{[L]_{in}}{[L]_{out}}; \quad Y_N = \frac{[N]_{out}}{[N]_{in}}$$

It is thus seen that the value of the gradient for L that yields zero net flux depends not only on the gradient for N, Y_N , but also on the concentration $[N]_{out}$. Using the values employed here ($[N]_{out} = 150 \text{ mM}$ and $Y_N = 30$), we obtain for Y_L (flux L = 0):

$$Y_L(0) = 6.8$$

and so the transport stoichiometry will change sign when $[L]_{in} = 68 \text{ mM}$ in Table 2 and for $[L]_{out} = 2.94 \text{ mM}$ in Table 1. For higher values of $[L]_{in}$ (Table 2) and lower values for $[L]_{out}$ (Table 1) the net flux of L is negative.

In Table 1, the gradient in N is constant ($[N]_{out}/[N]_{in} = 30$), whereas the gradient in L is varied by variation in $[L]_{out}$, keeping $[L]_{in}$ constant. The net and influx stoichiometries are not very different, and, in the slippage case, both increase with $[L]_{out}$. In Table 2, the gradient for N is again constant, and the gradient in L is varied by changing $[L]_{in}$. There are considerable differences between the net and influx stoichiometries: in the no-slippage case, it would depend on conditions whether the tracer stoichiometry is found to be 1 or 2 (within the range of the varied species concentrations). Also, in the slippage case the net stoichiometry decreases, and the tracer stoichiometry increases, as $[L]_{in}$ is increased, corresponding to a change in the ratio $[L]_{in}/[L]_{out}$ from 1 to 10. In Table 3, the gradient in L is constant and the ratio $[N]_{out}/[N]_{in}$ is varied from 4 to 40 by changing $[N]_{out}$. Again a difference in behaviour of the two stoichiometries is noted: the influx stoichiometry decreases whereas, in the slippage case, the net stoichiometry increases, as $[N]_{out}$ is increased. Finally, in Table 4, the gradients in the ligands are constant, but the concentrations of the ligand L on both sides of

the membrane are increased (in constant ratio). Irrespective of the presence or absence of slippage, the measured influx stoichiometries are between 1 and 3, depending on the particular choice of concentrations.

Actually, it is not surprising that a change in a ligand concentration should result in changes in the properties of the model. Since such a change results in a corresponding change in the appropriate pseudo-first-order constant, there will be a shift in kinetic importance of the various intermediates and therefore also in the magnitude of the fluxes. However, the order of magnitude and the properties of the flux ratios are not easy to evaluate on an intuitive basis.

A prototype ligand-ATPase model

The diagram in Fig. 4(a) can easily be made to correspond to an active-transport model, in which an enzyme (an ATPase) transports a ligand L from out to in against its concentration gradient. The necessary changes in the model are shown in Fig. 5. The rate constants have been changed to correspond to an ATPase whose ATP dissociation constant is 1 mM. Also, one of

Fig. 5. A simple prototype membrane active-transport model with the same geometry as the model in Fig. 4

The enzyme E is an ATPase which transports the ligand L from out to in against its gradient. T and D denote ATP and ADP respectively. The rate constants used in the computations are indicated. For the no-slippage case, the rate constants *k* and *l* are set to 10⁻³ and 10⁻⁵ respectively, and for the cases with slippage to 10 and 0.1. In the latter case, some ATP is hydrolysed without concomitant transport of L. The states E₁ and E₂ designate, as in Fig. 4, accessibility of the transport sites for L towards the outside and inside of the membrane respectively. ATP, ADP, and P_i are presumed to act only on inside sites. The expanded model for this model is identical with that in Fig. 4(b). The concentration unit implied in the values of the second-order rate constants is molar.

the detailed balance relations is changed, such that eqn. (14) is used (with *K*_{eq} = 10⁶ M). To mimic the case of a model with slippage, the rate constants *k* and *l* are set to 10 M⁻¹·s⁻¹ and 0.1 s⁻¹ respectively, and in the no-slippage case these values are multiplied by 10⁻⁴. The other rate constants used here are shown in Fig. 5. *α* was set to 10⁻⁴ in all cases. A few results from calculation of net and influx stoichiometries for transport, i.e. number of ligand molecules transported per molecule of ATP hydrolysed, are shown in Tables 5 and 6. The results are similar in nature to those discussed above: the tracer flux stoichiometries are, as expected, different from the net stoichiometries and do not yield information regarding the actual stoichiometry of transport (or coupling ratio), whether or not the model has slippage. Also, in Table 6 (last three columns) the behaviours of the two stoichiometries as a function of the varied species are different. Even under conditions where the two sides of the membrane have the same ligand concentrations (Table 6, first three columns), the influx coupling ratio will vary between 1 and 2. One cannot therefore draw definite conclusions on the basis of such measurements as to the precise transport properties or number of ligand binding sites per enzyme unit of the underlying model.

For a qualitative understanding of the discrepancies for this ligand-ATPase model, or the co-transport model considered above, consider the latter case, with no slippage for simplicity. There is only one cycle in the basic diagram, and hence the net flux ratio [L]/[N] will be 1. However, in the corresponding enlarged model there are 10 cycles which are used to different degrees of the two ligands depending on the rate constants. Some labelled L will be transported by unlabelled N, some unlabelled L by labelled N, and only in the cycle transporting only labelled (or only unlabelled) ligands will the transport stoichiometry be 1.

In summary, a procedure has been described allowing the simulation of experimental results from tracer flux measurements at steady state. Use is made of an expanded version of the basic model, in which labelled forms of the enzyme are explicitly present in the model. This procedure can be used together with existing computer programs to model experimental results interactively at steady state, and also for the study of transient kinetics (in which a set of simultaneous differential equations would have to be solved). Applying this method to three different models, it is shown that conclusions, based on tracer flux measurements or concentrations of labelled intermediates, regarding total steady-state concentrations of intermediates, as well as transport stoichiometries (coupling ratios), should be viewed with some caution. Also, from a measured influx stoichiometry of transport larger than 1:1, it cannot in general be

Table 5. Stoichiometry in active-transport model at constant gradients at different ligand concentrations

[ATP] = 3 mM. [ADP] = 1 mM. [P_i] = 10 mM. [L]_{in}/[L]_{out} = 10.

Varied species		No slippage		Slippage	
[L] _{out} (mM)	[L] _{in} (mM)	[L]/[ATP] (net)	[L]/[ATP] (in)	[L]/[ATP] (net)	[L]/[ATP] (in)
4	40	1.0	1.29	0.68	0.97
6	60	1.0	1.44	0.74	1.18
8	80	1.0	1.58	0.78	1.36
10	100	1.0	1.73	0.80	1.53
12	120	1.0	1.88	0.82	1.69
14	140	1.0	2.02	0.83	1.85
16	160	1.0	2.17	0.84	2.00
18	180	1.0	2.31	0.85	2.16
20	200	1.0	2.46	0.85	2.31
22	220	1.0	2.61	0.86	2.47

Table 6. Stoichiometry of transport in active-transport model with slippage at various ligand concentrations, with $[L]_{out} = [L]_{in}$ (cols. 1–3) and at various $[L]_{in}$ at constant $[L]_{out} = 10$ mM (cols. 4–6)

$[L]_{in} = [L]_{out}$ (mM)	$[L]/[ATP]$ (net)	$[L]/[ATP]$ (in)	$[L]_{in}$ (mM) ($[L]_{out} = 10$ mM)	$[L]/[ATP]$ (net)	$[L]/[ATP]$ (in)
10	0.88	0.95	10	0.88	0.95
20	0.94	1.08	20	0.87	1.02
30	0.96	1.17	30	0.86	1.08
40	0.97	1.26	40	0.85	1.15
50	0.97	1.34	50	0.84	1.21
60	0.98	1.42	60	0.84	1.28
70	0.98	1.49	70	0.83	1.34
80	0.98	1.57	80	0.82	1.40
90	0.99	1.64	90	0.81	1.47
100	0.99	1.72	100	0.80	1.53

concluded that more than one site for one or the other ligand exists.

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REFERENCES

- King, E. L. & Altman, C. (1956) *J. Phys. Chem.* **60**, 1375–1378
- Hill, T. L. (1977) *Free Energy Transduction in Biology. The Steady-State Kinetic and Thermodynamic Formalism*, Academic Press, New York
- Chen, Y. D. (1990) *Biophys. Chem.* **35**, 55–63
- Cleland, W. W. (1967) *Annu. Rev. Biochem.* **36**, 77–112
- Heinz, E. (1985) *Ann. N.Y. Acad. Sci.* **456**, 1–9
- Post, R. L., Kume, S., Tobin, T., Orcutt, B. & Sen, A. K. (1969) *J. Gen. Physiol.* **54**, 306s–326s
- Albers, R. W. (1967) *Annu. Rev. Biochem.* **36**, 727–756
- Barshop, B. A., Wrenn, R. F. & Frieden, C. F. (1983) *Anal. Biochem.* **130**, 134–145
- Press, W. H., Flannery, B. P., Teukolsky, S. A. & Vetterling, W. T. (1986) *Numerical Recipes*, Cambridge University Press, Cambridge

APPENDIX

The rate constants used for the simulations under ‘Does the measurement of the concentration of a labelled intermediate reflect the true steady-state concentration of that species?’ in the main paper are shown in Table A1. Some values were taken from previously published kinetic work [1] based on steady-state kinetic measurements. Several steps not explicitly shown in the model in Fig. 1 include the cycles $E_2-E_2T = E_2KT-E_2K = E_2$

and $E_2-E_2P = E_2KP-E_2K = E_2$ (species assumed in rapid equilibrium are connected by ‘=’) and the analogous cycles in the left hand part of Fig. 1(b). Several rate constants in these sequences were assigned by trial and error in an effort to fit kinetic results for ATP synthesis and hydrolysis by Plesner *et al.* [2]. The rate constants for the steps involving interconversion of the phosphoenzymes E_1P and E_2P , which cannot be obtained from

Table A1. Parameters used in the simulations for Na^+/K^+ -ATPase

T $\equiv$ ATP.

(a)

Step	Rate constant	Step	Rate constant
$E_2 \rightarrow E_2T$	$1.2 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$	$E_2P \rightarrow E_1P$	$3.4 \times 10^3 \text{ min}^{-1}\dagger$
$E_2T \rightarrow E_2$	$4.0 \times 10^2 \text{ min}^{-1}$	$E_2P \rightarrow E_2$	$5.0 \times 10^2 \text{ min}^{-1}$
$E_2K \rightarrow E_2KT$	$1.2 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$	$E_2 \rightarrow E_2P$	$1.1 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$
$E_2KT \rightarrow E_2K$	$2.0 \times 10^4 \text{ min}^{-1}$	$E_2K \rightarrow E_2KP$	$4.4 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$
$E_2T \rightarrow E_1T$	$1.0 \times 10^5 \text{ min}^{-1}$	$E_2KP \rightarrow E_2K$	$4.0 \times 10^4 \text{ min}^{-1}$
$E_1T \rightarrow E_2T$	$2.0 \times 10^{-2} \text{ min}^{-1*}$	$E_2 \rightarrow E_1$	$3.3 \times 10^2 \text{ min}^{-1}$
$E_1T \rightarrow E_1P$	$4.0 \times 10^4 \text{ min}^{-1}$	$E_1 \rightarrow E_2$	$3.0 \times 10^{-4} \text{ min}^{-1}$
$E_1P \rightarrow E_1T$	$6.0 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$	$E_1 \rightarrow E_1T$	$4.8 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$
$E_1P \rightarrow E_2P$	$3.6 \times 10^4 \text{ min}^{-1}\dagger$	$E_1T \rightarrow E_1$	$3.6 \times 10^2 \text{ min}^{-1}$

(b)

Step	Dissociation constant (M)	Step	Dissociation constant (M)
$ATP = ADP + P_i$	5×10^6	$E_2K = E_2 + K^+$	2.5×10^{-3}
$E_1ADP = E_1 + ADP$	1×10^{-5}	$E_2PK = E_2P + K^+$	$5 \times 10^{-3*}$
$E_2ADP = E_2 + ADP$	1×10^{-4}	$E_2KATP = E_2ATP + K^+$	0.125

* Calculated from detailed balance relation.

† Valid for $[Na^+] = 0.6$ M [3].

ordinary steady-state kinetic work, were computed by an 'iterative' procedure, using the kinetic effect of the inhibitor oligomycin on the rate of hydrolysis, as described by Plesner [3]. Some rate constants, marked in Table A1 by an asterisk, were computed by a detailed balance relation for the appropriate cycle. The dephosphorylation rate constants for E_2P and E_2KP were assigned so as to yield the correct rate of hydrolysis in the presence of both P_i and ADP (and in the presence and absence of K^+), as published [2]. In addition, the competitive action of ADP on the rate of ATP hydrolysis has been incorporated. The dissociation constants for ADP from the two forms of the enzyme are shown in Table A1.

The species in Fig. 1 enclosed in boxes are presumed to be in rapid equilibrium. Each box in the model is considered to be one kinetic state. As a result some rate constants between the kinetic states are ligand-concentration-dependent [4]. For example, the rate constant for the transition $E_2KT \rightarrow E_2K$ in Fig. 1(a) is

$$k = \frac{k_0[K^+]/K_2}{1 + [K^+]/K_2} \quad (A1)$$

where K_2 is the dissociation constant for the reaction

and k_0 is the intrinsic rate constant.

For those not familiar with matrix notation (eqn. 6 in the main paper), a brief explanation of the construction of the matrix A and vectors X and B is given here.

The model in Fig. 1(a) has six states. Denoting the (relative) concentrations of these states E_i , $i = 1-6$, i.e. $E_i = [\text{state } i]/E_0$, the steady-state equations are

$$\frac{dE_i}{dt} = 0; \quad i = 1-6 \quad (A2)$$

One such equation, for state 3, is:

$$\frac{dE_3}{dt} = k_{23}E_2 + k_{63}E_6 + k_{43}E_4 - (k_{32} + k_{36} + k_{34})E_3 = 0 \quad (A3)$$

However, only five of the six equations (A2) are independent because of enzyme conservation. So, as the sixth independent equation in the unknowns E_i we have

$$\sum_{i=1}^{i=6} E_i = 1 \quad (A4)$$

The matrix equation, eqn. (6), is merely a compact way of writing these six equations. The column vector X contains the six variables E_i , and the (column) vector B contains the right-hand sides of the six equations, i.e. five zeros and one element = 1. The recipe for the matrix A is

$$A_{rc} = k_{cr}, r \neq c$$

$$A_{rr} = - \sum_{i=1}^{i=6} k_{ri}$$

Note that $k_{rc} = 0$ if there is no transition $E_r \rightarrow E_c$. Thus the third row in A in our case will be

$$[0, k_{23}, -(k_{32} + k_{36} + k_{34}), k_{43}, 0, k_{63}] \quad (A5)$$

The use of the enlarged model, Fig. 1(b), of course does not change eqn. (A3). But since there are now ten kinetic states, the matrix A has 10 rows and 10 columns. The third row of this larger matrix consists of the elements in eqn. (A5) and, in addition, four zeros, since E_3 does not interact with any of the states E_7-E_{10} .

REFERENCES

1. Plesner, I. W. & Plesner, L. (1981) *Biochim. Biophys. Acta* **648**, 231-246
2. Plesner, L., Karlsmose, B. & Lüscher, M. E. (1990) *Biochim. Biophys. Acta* **1040**, 167-174
3. Plesner, I. W. (1991) in *The Sodium Pump: Recent Developments* (Kaplan, J. H. & de Weer, P., eds.), pp. 345-349, The Rockefeller University Press, New York
4. Cha, S. (1968) *J. Biol. Chem.* **243**, 820-825

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