Use of progress curves to estimate the co-substrate-to-substrate flow ratio of a symport mechanism

Application to the isoleucine–Na⁺ symport of mouse ascites-tumour cells and to the lactose–proton symport

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The model envisages two components in the process, whereby H' equivalents of co-substrate and S' equivalents of substrate accumulate in the cellular compartment in time t . The first is the flow through the symport, n equivalents of co-substrate entering or leaving with each substrate equivalent. The second is the basal flow of co-substrate outside the symport. In certain specific circumstances n can be derived by plotting H'/t against S'/t . The principal requirement is that, whereas the ratio of the component flows must change in the interval t , the magnitude of the basal flow must either be zero or constant. The procedure is applied to published observations [West & Mitchell (1973) *Biochem. J.* 132, 587–592] on the lactose–proton symport of *Escherichia coli* [$n = 1.075 \pm 0.064(7)$] and to new observations on the isoleucine–Na⁺ symport of mouse ascites-tumour cells [$n = 1.136 \pm 0.120(18)$].

INTRODUCTION

Symport mechanisms concentrate substrates by coupling their flow across a cellular membrane to the spontaneous flow of a co-substrate ion, such as Na⁺ or H⁺, in the same direction (Harold, 1986). The flow ratio (n) is defined as the ratio of the respective flows of co-substrate and substrate that pass through the coupling mechanism (West, 1980; Eddy, 1982, 1987; Sanders, 1986). Thermodynamic arguments point to two possibilities: (1) the two flows are tightly coupled, n is a constant parameter and the extent to which the substrate is concentrated increases with n ; (2) in contrast, imperfect coupling of the two flows is characterized by values of n that vary with the concentration of either ligand. Whether n for a given system is fixed or variable is a matter of current debate (Eddy, 1982; Harold, 1986; Wright *et al.*, 1986; Sanders, 1986). One factor contributing to uncertainty about the exact magnitude of n is that the co-substrate or substrate may cross the cell membrane through parallel pathways outside the symport (Scheme 1). Furthermore, the operation of the symport tends to retard uptake of the co-substrate through these other basal pathways. That is because: (1) it depolarizes the cell membrane and (2) the co-substrate accumulates intracellularly (Philo & Eddy, 1978; Ballerín-Denti *et al.*, 1984; Kimmich & Randles, 1984). The magnitude of such effects being difficult to predict, the magnitude of the basal co-substrate flow (B') in the presence of substrate is correspondingly uncertain. In particular it may differ from the value exhibited in controls lacking the substrate in question (Eddy, 1977; Sanders *et al.*, 1983). These general considerations led us to examine whether both n and B' (Scheme 1) can be deduced by studying the time course of the uptake of both substrate

and co-substrate. We have applied our equations to (1) published observations on the lactose–proton symport of *Escherichia coli* (West & Mitchell, 1973) as well as to (2) new observations we have made on the isoleucine–Na⁺ symport of mouse ascites-tumour cells studied by Hacking & Eddy (1981).

MATERIALS AND METHODS

The mouse ascites-tumour cells were grown, collected and washed as described by Johnson & Eddy (1985). The standard Ringer solution, pH 7.2, contained 155 mM-Na⁺, 8 mM-K⁺, 131 mM-Cl[−], 16 mM-orthophosphate and 1.2 mM-MgSO₄. Preliminary work showed that, whereas 40 mM-isoleucine accelerated the uptake of ²²Na⁺ from solutions containing 8 mM-K⁺, 32 mM-Na⁺ and 123 mM-choline chloride, observations made at larger concentrations of Na⁺ were difficult to reproduce.

Assay of L-[¹⁴C]isoleucine uptake and ²²Na⁺ uptake

These were conducted in parallel cell suspensions of identical composition, except that one or other radioisotope was present in a given suspension. The tumour cells (2 ml) were first incubated at 37 °C for 30 min in the standard Ringer solution (50 ml). They were collected by centrifugation (1000 g, 2 min) and washed with 10 ml of a solution containing 32 mM-Na⁺, 8 mM-K⁺, 123 mM-choline chloride, 16 mM-orthophosphate and 1.2 mM-MgSO₄ (solution A). The tumour cells were collected again, washed twice further in a similar manner and suspended in 4.5 ml of solution A. The cell suspension (about 50 mg dry wt./ml) was kept at 0 °C. Portions (1 ml) were mixed with solution A (3 ml) containing ouabain at a final concentration of 0.3 mM, valinomycin (50 µg) and ²²Na⁺ (2.0 µCi). The cell suspension was kept

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Scheme 1. Simplified model of the net passive flows of substrate (S) and co-substrate (H^+) as represented in eqns. (2) and (4)

The system is assumed to be completely deficient in energy metabolism, so that active proton pumping has ceased, charge compensation occurring by outflow of K^+ . Exactly n equivalents of H^+ move with each equivalent of substrate (S) in either direction through the symport. The observed uptake of substrate at time t is S' ; that of co-substrate (H^+) is H' . The basal flow of co-substrate occurs outside the symport at a rate B' , the cumulative flow being represented by the appropriate integral. We consider in the text the case where B' is constant, a condition requiring that (1) the basal permeability to protons and (2) the electrochemical gradient driving their uptake are both constant. The instantaneous basal flow of substrate is represented as a first-order rate process governed by the product of the prevailing transmembrane solute gradient and a first-order rate constant. The cumulative basal flow of substrate (S') is derived by integration with respect to t . $[S]_0$ and $[S]_i$ are outer and inner substrate concentrations respectively.

at 37 °C for 8 min exactly. Parallel cell suspensions lacked $^{22}Na^+$. The purpose of this initial operation was to bring cellular Na^+ into approximate isotopic equilibrium with extracellular Na^+ , preliminary work having suggested that 8 min was sufficient for that purpose. Uptake of isoleucine was initiated by addition of 1 ml of 200 mM-isoleucine, in solution A, containing 1 μCi of ^{14}C as required. Samples (0.2 ml) were removed at intervals, each being added to 0.8 ml of modified Ringer solution (solution B) at 0 °C, layered over 1-bromododecane (0.5 ml). Solution B above contained 32 mM- Na^+ , 131 mM- K^+ , 16 mM-orthophosphate and 1.2 mM- $MgSO_4$. The tumour cells were next separated in an Eppendorf Microfuge at 10000 g for 2 min. The supernatant solution and the cell pellet were treated as described by Johnson & Eddy (1985). Each was assayed for ^{14}C or ^{22}Na as appropriate. We studied isoleucine uptake, during 30 min at 37 °C, from a 40 mM solution containing 40 mM- K^+ , 123 mM-choline chloride, 16 mM-orthophosphate, 1.2 mM- $MgSO_4$, 0.3 mM-ouabain and valinomycin (10 $\mu g/ml$). The purpose was to characterize the basal (Na^+ -independent) amino acid flow. The observations were used to determine a first-order rate constant (k) as described by Hacking & Eddy (1981).

Computations

The basal flow of amino acid in the presence of Na^+ was inferred from a knowledge of (1) k , the first-order rate constant for entry and exit of the amino acid (see above) and (2) the difference (D') between the respective values of cellular and extracellular [isoleucine] as a function of time (t). Nine assays of intracellular [isoleucine] were made in the interval up to 30 min (Fig. 2, below). The different values of D' were expressed as a third-order polynomial in t . The integral $k \cdot \int_0^t D' dt$ was then evaluated by using a program based on Simpson's Rule. The results are expressed in Fig. 2 (below) as the computed cellular isoleucine content that had entered through the Na^+ -independent pathway by time t .

Chemicals

L-[U- ^{14}C]isoleucine was obtained from Amersham International, Amersham, Bucks., U.K. Valinomycin and ouabain were obtained from Sigma, Poole, Dorset, U.K.

RESULTS

Description of the flows

(a) Let the substrate only enter or leave through the symport and in association with n protons (or Na^+) as co-substrate. In the model illustrated in Scheme 1 the two algebraic components of proton uptake are (1) n multiplied by the net substrate flow through the symport at time t and (2) B' , the basal rate of proton uptake at time t . The experimentally accessible quantities are H' and S' , the respective amounts of protons and substrate absorbed during the time interval t . Thus:

$$H' = n S' + \int_0^t B' dt \quad (1)$$

Let the basal proton flow proceed at a constant rate, B , during the interval t (see the Discussion section). Eqn. (1) then becomes:

$$\frac{H'}{t} = n \left(\frac{S'}{t} \right) + B \quad (2)$$

or

$$\frac{H'}{S'} = B \left(\frac{t}{S'} \right) + n \quad (3)$$

(b) The substrate may also enter or leave through a second parallel pathway characterized by the first-order rate constant, k . After time t , the net uptake of substrate through this route (S') is represented by the integral defined in Scheme 1. Eqn. (2) then becomes:

$$\frac{H'}{t} = n \cdot \frac{S' - S'}{t} + B \quad (4)$$

Properties of eqn. (2)

Lactose uptake into *Escherichia coli*, strain ML 308-225 (i^- , z^- , y^+), is assumed to occur only through the lactose-proton symport. Its time course into bacterial preparations deficient in energy metabolism was studied by West & Mitchell (1973), who reported seven mean values of the lactose uptake, as a function of time, in the period 15–80 s after the addition of lactose. As a hypothetical example, Fig. 1(a) (○) shows the time course of proton uptake that would occur if $n = 1$ and $B = 0$. □ and △ in Fig. 1(a) represent the corresponding

Fig. 1. Graphs illustrating the properties of eqn. (2)

The time course of the uptake of substrate, in units of nmol/ml of bacterial suspension, was modelled on the basis of the observations in Fig. 3(b) of West & Mitchell (1973). In (a), $\circ$ depicts the time course of the uptake of protons that would occur, in the period up to 80 s, if $n = 1$ and the basal flow of protons, $B = 0$. $\square$ and $\triangle$ show the corresponding proton uptake when, respectively, $B = +0.2$ units or -0.2 units. (b) Illustrates the transformation of the same observations in terms of eqn. (2). The slope of the line representing the variation of H^t/t with S^t/t defines the value of n . The intercept on the ordinate represents the parameter B .

behaviour when $B = 0.2 \text{ nmol} \cdot \text{s}^{-1}$ and $-0.2 \text{ nmol} \cdot \text{s}^{-1}$ respectively. Fig. 1(b) represents the same data in terms of eqn. (2), the values of n and B originally selected now defining the respective slope and intercept of the linear relationships. The same procedure was used to deduce the values of n and B that describe the magnitudes of the actual proton uptake (H^t) and lactose uptake (L^t) observed by West & Mitchell (1973). We obtained, using eqn. (2):

$$\frac{H^t}{t} = (1.075 \pm 0.064) \left(\frac{L^t}{t} \right) - 0.167 \pm 0.025 \quad (5)$$

with a linear correlation coefficient of $r = 0.991$. By using eqn. (3) the result was:

$$\frac{H^t}{L^t} = -(0.151 \pm 0.021) \left(\frac{t}{L^t} \right) + 1.032 \pm 0.059 \quad (6)$$

with a linear correlation coefficient of 0.955.

These two estimates of n , namely 1.075 ± 0.064 (7) and 1.032 ± 0.059 , are consistent with the approximate value of 1 that West & Mitchell (1973) deduced by extrapolating the ratio H^t/L^t to zero time to compensate for the postulated backflow of protons. We conclude that eqn. (2) represents a useful alternative way of combining the observations.

Application of eqn. (4) to the isoleucine- Na^+ symport

Hacking & Eddy (1981) showed that: (1) mouse ascites-tumour cells concentrated isoleucine poorly from

Fig. 2. Application of eqn. (4) to the time course of the uptake of isoleucine and of Na^+ in the presence of isoleucine

(a) The assays were carried out as described in the Materials and methods section. Uptake of isoleucine occurred from a 40 mM solution of the amino acid in a Ringer solution containing 32 mM- Na^+ and 8 mM- K^+ . The presence of ouabain (0.3 mM) and valinomycin (10 $\mu\text{g}/\text{ml}$) prevented the sodium pump from expelling Na^+ . In (a), $\square$ represents the cellular content of Na^+ , assayed as $^{22}\text{Na}^+$, at various times after the addition of 40 mM-isoleucine to the cell suspension. The tumour cells had previously been equilibrated with $^{22}\text{Na}^+$. $\circ$ Represents the time course of the uptake of [^{14}C]isoleucine in the presence of 32 mM- Na^+ . $\triangle$ Represents the cumulative basal flow of isoleucine computed from a knowledge of the relevant first-order rate constant, k , and the observed uptake of isoleucine ($\circ$) (see the Materials and methods and Results sections). (b) Combines the results of the assays depicted in (a) with a further similar set. Eqn. (4) used for that purpose relates the total Na^+ absorbed in the interval from time zero to time t (Na^t) to the total amount of isoleucine absorbed through the symport ($I^t - I'$) in the same interval, t . The slope of the line is a measure of symport stoichiometry, n (eqn. 7).

a 10 mM solution; (2) membrane depolarization occurred, exhibiting a K_m value of 26 mM with respect to [isoleucine]; and (3) a relatively fast uptake of isoleucine occurred in the absence of Na^+ , a process which appeared to explain why only a small amino acid gradient developed. We have now studied the flow of Na^+ entering the tumour cells in the presence of isoleucine

and 32 mM- Na^+ (Fig. 2). Fig. 2(a) illustrates the time course of the gross uptake of both Na^+ ($\square$) and isoleucine ($\circ$). The increase in Na^+ uptake was about 30–40% of the uptake in the absence of the amino acid (results not shown). In order to apply eqn. (4), an estimate of S' as a function of time is required. Hacking & Eddy (1981) assayed the first-order rate constant for isoleucine uptake in the absence of Na^+ . Because the conditions used were somewhat different from those to which Fig. 2(a) refers, we repeated the assays in the presence of 40 mM-isoleucine, 0.3 mM-ouabain and valinomycin (10 $\mu\text{g}/\text{ml}$), Na^+ being replaced by K^+ to give 40 mM- K^+ altogether. Two series of assays corresponded to a mean value for k of 0.0187 min^{-1} . The corresponding predicted basal flow of isoleucine (I') in the presence of Na^+ is illustrated in Fig. 2(a) ($\triangle$). The observations were next combined by means of eqn. (4) to give the result illustrated in Fig. 2(b). Linear regression analysis of the 18 pairs of observations showed that the overall Na^+ uptake (Na^t) at time t and the estimated isoleucine uptake through the symport ($I^t - I'$) were related by the equation:

$$\frac{\text{Na}^t}{t} = (1.136 \pm 0.120) \left(\frac{I^t - I'}{t} \right) + 1.341 \pm 0.696 \quad (7)$$

with a coefficient of linear correlation of 0.926.

The observations are thus consistent with the view that the flow of isoleucine through the symport is coupled to the passage of about 1 equiv. of Na^+ . Although methionine and glycine behave similarly (Eddy, 1985), the tumour cells form relatively large gradients of methionine or glycine in comparison with those formed with isoleucine. This is because the basal flow of amino acid, which probably occurs through a separate carrier system, is faster in the latter instance (Hacking & Eddy, 1981).

DISCUSSION

Although the models represented by eqn. (2) and eqn. (4) satisfactorily describe the net uptake of co-substrate in the two systems examined, we wish to stress the implications of assuming in either case that parameter B is constant. The use of the regression analysis to distinguish between the two components of the co-substrate flow requires that the rate of flow through the symport (i) changes relative to the basal flow (ii). Relevant observations therefore extend beyond the period in which studies of initial rates are conducted, a constant rate of uptake being represented by a constant value of, for instance, S^t/t . The very fact that the relative velocity of i and ii changes means, however, that their interactions change. An increase in the magnitude of i will tend to lower ii by (1) depolarizing the cell membrane, (2) accelerating the intracellular accumulation of co-substrate (Philo & Eddy, 1978) and (3), as pointed out by

an anonymous referee in relation to an earlier version of this paper, by depleting cellular $[\text{K}^+]$. The first-named factor affects ii immediately, whereas the other actions are cumulative. Similarly a decrease in i would be associated with an increase in ii . It is thus highly relevant that the assays of co-substrate uptake into the mouse ascites-tumour cells were conducted in the presence of valinomycin. This compound would tend to clamp the membrane potential, thereby lessening the interaction between i and ii . Indeed, the experimental observations are consistent with the idea that the basal flow of Na^+ into the mouse ascites-tumour cells, in the presence of isoleucine, was driven by a fairly constant electrochemical gradient of Na^+ and that depletion of cellular K^+ was not a significant factor. The mechanism of outflow of protons from the bacteria is uncertain, but in that case, too, the presence of KSCN (West & Mitchell, 1973) would tend to clamp the membrane voltage. The extent to which proton outflow affects values of n deduced from initial-rate studies deserves further study (cf. Page, 1987).

The above considerations suggest that, in systems such as those of yeast, where voltage clamping is not feasible, regression analysis based on eqn. (2) can lead to apparent values of n (n') that are smaller than the true value as a result of the negative correlation between i and ii . Ideally one would wish to find experimental conditions where ii was zero. The factor $(n - n')$ would then tend to zero. We examine this proposal experimentally in the following paper (Eddy & Hopkins, 1988).

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