Experimental analysis of ion/solute cotransport by substrate binding and facilitated diffusion

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An ion/solute cotransporter can be studied in the absence of a transmembrane gradient of the electrochemical potential of the ion. Inspection of the appropriate equations discloses that basic parameters of the cotransport cycle can be obtained by measuring cosubstrate binding and the initial-velocity kinetics of four modes of facilitated diffusion as a function of the concentration of the cotransported ion. The following information can be derived: estimates of the affinities of both cosubstrates, the number of binary intermediates participating in cotransport (equivalent to determining the order of cosubstrate binding and release), and the rate constants for the reorientation of the binding sites during cotransport. In general, both maximal velocities and half-saturation constants for the facilitated diffusion of one cosubstrate depend upon the concentration of the other. In some cases, the maximal velocities of influx and efflux do not increase monotonically with the concentration of the ion but pass through a maximum and decrease. If direct binding studies are not possible, affinities of the cosubstrates can be estimated from data for equilibrium exchange or countertransport. Also, an approximate description of the time course of the transient accumulation (overshoot) during countertransport is derived. Under certain circumstances, the height of the overshoot is proportional to the concentration of the cotransported ion. Thus, countertransport can be employed as a simple test to establish if a solute is cotransported with a particular ion. This treatment allows many effects noted in galactoside countertransport in *Escherichia coli* to be explained.

Introduction

The membrane of a cell or organelle represents a barrier to the passive diffusion of most solutes. The presence of certain proteins in the membrane selectively permits the passage of molecules between the compartments formed by the membrane. The facilitated (i.e. carrier-mediated or -catalyzed) diffusion of a solute across the membrane is typified by the glucose or hexose transporter of erythrocytes [1] or by the glycerol facilitator of *Escherichia coli* [2]. Facilitated diffusion leads only to the equilibration of the solute between the two compartments.

The transport of some solutes is frequently coupled to the transport of a second solute, e.g. lactose/H^+ cotransport in *E. coli* (Fig. 1, for reviews see Refs. 3–7). The transmembrane gradient of the electrochemical potential of this second solute (usually an ion, e.g. H^+ or Na^+) is used to drive the uptake of the first solute above the level of mere equilibration [8–10].

The kinetic mechanism of active transport by an ion/solute cotransporter is described by (1) the intrinsic affinities of the transport protein for the cosubstrates (the dissociation constants K_H and K_G in Fig. 1), (2) the rate constants for the reorientations of the binding sites (k_o and k_c), (3)
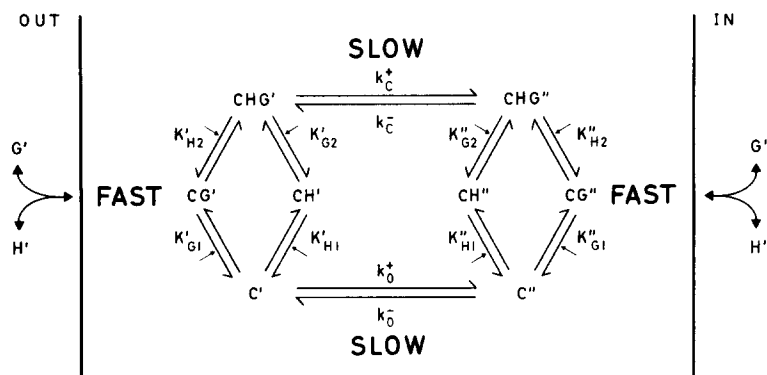
OUT

IN

SLOW

Fig. 1. Transport cycle for a carrier (C) catalyzing H^+-galactoside (G) cotransport.

the number of intermediate complexes (CH and CG) participating in the catalytic cycle, and (4) the points in the cycle where the imposition of the driving electrochemical gradient elicits changes. The problem with analyzing active transport alone to obtain all this information is that the kinetics can be quite complex. While there are already exact descriptions of such kinetics [11,12], the complexity of the effects of the concentrations of ion and solute and of the gradient of the electrochemical potential on the experimental parameters hinders an intuitive interpretation of experimental results. Also, many theoretical descriptions of cotransport assume a particular order of cosubstrate binding or do not explicitly discuss the dependence of the experimentally determined binding and kinetic parameters on the concentration of the cotransport ion.

One strategy to study the transduction of free energy by a cotransporter is to define the basic parameters of cotransport (points 1 to 3 above) in the absence of a driving gradient of the ion by measuring binding and facilitated diffusion and then to observe where changes in the cycle occur during active transport. The purpose of this analysis is to examine the equations for substrate binding and four modes of facilitated diffusion derived for a cotransport system and to determine some general rules useful in a non-mathematical evaluation of experiments. These theoretical results are employed in the analysis of H^+/galactoside cotransport in *E. coli* [4].

The model

The cotransport cycle consists of steps for the binding and release of cosubstrates (identified by the various intrinsic dissociation constants for the ion H^+ and a neutral solute G in Fig. 1) and for the reorientation of unloaded and fully loaded carriers (with rate constants k_o^+, k_o^- and k_c^+, k_c^-, respectively) between two compartments ('out' designated by $'$ and 'in' by $''$). In this treatment, the binary complexes are not permitted to reorient. The indispensible species are the reorienting forms of the carrier C$'$, CHG$'$, C$''$ and CHG$''$. The conversion of C to CHG may involve one or two binary complexes (CH or CG) as intermediate(s).

In this analysis of binding and transport the following assumptions are made: the binding and release of cosubstrates are rapid and carrier reorientations are slow, there is no transmembrane gradient of the electrical potential ($\Delta\psi = 0$) and solute fluxes are measured in the absence of a concentration gradient of the symported ion. Equations are derived for random binding of cosubstrates to the carrier, the most general case, where all four binary complexes are present. Mechanisms with partially or fully ordered binding are derived from the general expression by a simple procedure.

Irrespective of which precise kinetic scheme is discussed, thermodynamic considerations (the principle of detailed balance) require that when no source of energy is coupled to symport the following relationship between the parameters of the model obtains at equilibrium ([H$'$] = [H$''$] and [G$'$]

$= [G''])$

$$\frac{K''_{G1}}{K'_{G1}} \cdot \frac{K''_{H2}}{K'_{H2}} \cdot \frac{k_c^+}{k_c^-} \cdot \frac{k_o^-}{k_o^+} = 1 \qquad (1)$$

This restriction arises from the requirement that the net fluxes of the cosubstrates be zero at equilibrium.

Cotransport: The general rate equation

Cosubstrates bind in random order

The general rate equation for cotransport can be derived from the following considerations (cf. Refs. 11–13), conservation of mass:

$$C_T = [C'] + [CH'] + [CG'] + [CHG'] + [C''] + [CH'']$$

$$+ [CG''] + [CHG''] \qquad (2)$$

steady-state assumption:

$$k_o^+ \cdot [C'] + k_c^+ \cdot [CHG'] = k_o^- \cdot [C''] + k_c^- \cdot [CHG''] \qquad (3)$$

and the appropriate rate law for net flux (v_N)

$$v_N = k_c^+ \cdot [CHG'] - k_c^- \cdot [CHG''] \qquad (4a)$$

or unidirectional flux $(v$, here from left to right in Fig. 1)

$$v = k_c^+ \cdot [CHG'] \qquad (4b)$$

Using the shorthand notation $\alpha' = [G']/K'_{G1,2}$, $\beta' = [H']/K'_{H1,2}$, $\alpha'' = [G'']/K''_{G1,2}$, and $\beta'' = [H'']/K''_{H1,2}$ (where the appropriate subscripts (e.g. H1 or H2) are selected as required for each case), the equation for the net flux of solute or ion with completely random cosubstrate binding is

$$v_N = [k_c^+ \alpha'\beta'(k_o^- + k_c^- \alpha''\beta'') - k_c^- \alpha''\beta''(k_o^+ + k_c^+ \alpha'\beta')]$$

$$\times [(k_o^- + k_c^- \alpha''\beta'')(1 + \alpha' + \beta' + \alpha'\beta')$$

$$+ (k_o^+ + k_c^+ \alpha'\beta')(1 + \alpha'' + \beta'' + \alpha''\beta'')]^{-1} \qquad (5)$$

For random binding of cosubstrates, the individual dissociation constants (see Fig. 1) are related, e.g.

$$K'_{G1} \cdot K'_{H2} = K'_{G2} \cdot K'_{H1} \qquad (6)$$

and

$$K''_{G1} \cdot K''_{H2} = K''_{G2} \cdot K''_{H1} \qquad (7)$$

The corresponding unidirectional fluxes contain only the first or second term in the numerator of Eqn. 5.

Partially or fully ordered substrate binding

Not all of the four binary complexes are required during cotransport. Binding and transport measurements can determine which of these intermediates are present. The binary complexes CG', CH', CG'', and CH'' appear as α', β', α'' and β'', respectively, in the elements $(1 + \alpha + \beta + \alpha\beta)$ in the denominator of Eqn. 5. The product $\alpha\beta$ and 1 represent the essential species CHG and C, respectively. If, for instance, the binding of cosubstrates at the outer face of the membrane is ordered so that the galactoside binds first and the ion second, no binary CH' complex appears. Consequently, Eqn. 5 is altered by omitting β' (not as $\alpha'\beta'$, however, which represents CHG') in the denominator, which is equivalent to [CH'] = 0. In general, rate equations for particular experiments are obtained by defining G', H', G'', H'', the nature of the flux measured (net or unidirectional) and the binary carrier complexes present. Eqn. 5 is then rearranged to the familiar form

$$V = V_{max} \frac{S}{S + K_T} \qquad (8)$$

where V_{max} is the maximal velocity, S is the concentration of the substrate (H', G', H'', or G'') which is varied, and K_T the half-saturation constant for this substrate. Dividing V_{max} by the total carrier concentration C_T (cf. Eqn. 2) yields the catalytic constant or turnover number k_{cat}, representing the maximal velocity as the number of carrier cycles per time unit (e.g. s^{-1}); for convenience, K_T is referred to as the transport constant. To avoid re-deriving a new rate equation for every different mechanism (1 to 9 in Table I), the relevant equation for the most general case (random binding) appears in the text. The terms corresponding to the binary complexes are indicated by the subscripts CH and CG. The appropriate terms can, therefore, be readily eliminated for the mechanisms 2 to 9 in Table I.

Substrate binding

In the case of a cotransport system, the description of the binding of one of the cosubstrates to the carrier is complicated by the presence of the other cosubstrate and by the existence of two compartments (the spaces designed by ' (out) and " (in)). Solute is normally added to the outer compartment ('), cotransport occurs, and at equilibrium $[G'] = [G'']$ and $[H'] = [H'']$. Solute bound to the carrier is present in four cotransport intermediates: CG', CHG', CG'' and CHG''. The distribution of the carrier among the various species will depend upon the value of rate and dissociation constants.

The observed, overall or macroscopic dissociation constant for the solute is given by

$$K_{G,obs} = \frac{(G)\cdot(\text{all carrier forms without solute})}{(\text{all carrier forms with solute})} \quad (9)$$

which for the mechanism with completely random substrate binding (Fig. 1) becomes

$$K_{G,obs} = \frac{[G]\cdot([C']+[CH']+[C'']+[CH''])}{([CG']+[CHG']+[CG'']+[CHG''])} \quad (10)$$

This expression can be reduced in its most general form to

$$K_{G,obs} = \frac{\left(1+\dfrac{[H^+]}{K'_{H1}}\right)+\dfrac{k_o^+}{k_o^-}\left(1+\dfrac{[H^+]}{K''_{H1}}\right)}{\left(1+\dfrac{[H^+]}{K'_{H2}}\right)\dfrac{1}{K'_{G1}}+\dfrac{k_o^+}{k_o^-}\left(1+\dfrac{[H^+]}{K''_{H2}}\right)\dfrac{1}{K''_{G1}}} \quad (11)$$

The observed dissociation constant for one cosubstrate is determined by several factors: $K_{G,obs}$ depends on the affinity of the carrier for solute on both sides of the membrane; K'_{G1} and K''_{G1} need not be equal. Second, the concentration of the symported ion as well as its affinities for the carrier on both sides of the membrane determine $K_{G,obs}$. Next, the binding of ion and solute may not be independent. For example, the solute may bind to the carrier-ion binary complex with higher affinity than to the unloaded carrier so that $K'_{G1} > K'_{G2}$. In this case, the affinity of the ion for the unloaded carrier and carrier-solute binary complex differ by the same factor ($K'_{H1} > K'_{H2}$, cf. Eqn. 6). This behavior is frequently designated positive (or

negative) cooperativity. Lastly, the order of binding of the cosubstrates influences $K_{G,obs}$. The coupled binding equilibria in Fig. 1 can be displaced by the mass action of one cosubstrate when binding and release of cosubstrates are ordered.

Observed dissociation constants depend on the order of substrate binding: the diagnostic value of binding studies

The dependence of the observed dissociation constant for one cosubstrate upon the concentration of the other can be used to determine the order of substrate binding. Possible effects arising from unequal affinities for substrates on both sides of the membrane or cooperative binding of cosubstrates are not considered here (see Discussion). Simplifying Eqn. 11 by assuming $k_o^+ = k_o^-$ and $K'_{G1} = K'_{G2} = K''_{G1} = K''_{G2} = K_G$, yields

$$K_{G,obs} = K_G \frac{(1+\beta'_{CH})+(1+\beta''_{CH})}{(a'_{CG}+\beta')+(a''_{CG}+\beta'')} \quad (12)$$

where the subscripts CH and CG indicate terms arising from the presence of the four binary complexes (Fig. 1); a'_{CG} and $a''_{CG} = 1$ when CG is present in the respective compartments, a'_{CG} and $a''_{CG} = 0$ when CG is absent in the respective compartments (see Table I).

The general way in which the observed dissociation constant varies with the concentration of the symported ion for each of the nine possible modes of binding and release of the two cosubstrates can be obtained by inspection of this simplification. There are four experimentally distinguishable classes (Table I).

Type A. $K_{G,obs}$ does not vary with the concentration of the symported ion (Mechanism 1; random, independent binding of cosubstrates.

Type B. $K_{G,obs}$ decreases slightly with increasing concentration of the ion (Mechanisms 2–5, 8, 9).

Type C. $K_{G,obs}$ decreases from a finite value to zero (infinite affinity) with increasing ion concentration (Mechanism 6). This behavior is due to the mass action of the ion binding to the carrier second on both sides of the membrane.

Type D. $K_{G,obs}$ decreases from an infinite value (no affinity) to a finite value with increasing ion

concentration (Mechanism 7). This behavior is due to the fact that solute only binds to the carrier-ion complexes.

Importantly, all binding sites are accessible to solute for every mechanism. Except for Mechanism 7 (no CG', CG'') where the presence of the symported ion is absolutely necessary for binding, the total number of binding sites can be measured even in the absence of the symported ion. The carriers can always reorient via the steps k_o^+ and k_o^- (Fig. 1) from one side of the membrane to the other. The analysis in the following section permits $K_{G,obs}$ and half-saturation constants for facilitated diffusion to be compared.

Facilitated diffusion

Experimental conditions

A symport system can translocate cosubstrates in the absence of an energy source. In contrast to active transport, the conditions [H'] = [H''] and [G'] = [G''] are fulfilled at equilibrium. Four modes of facilitated diffusion can be measured. During influx or efflux, substrate is initially present only on one side of the membrane (i.e. [G''] or [G'] = 0). Net flux of solute occurs. During equilibrium exchange, substrate is present at identical concentrations on both sides of the membrane (i.e. [G'] = [G'']); however, only substrate on one side of the membrane is labelled. The unidirectional flux during exchange is observed by following the movement of the label. The net fluxes of solute and ion are zero at all times. During countertransport, as treated here, the inner compartment contains saturating levels ($> K_G''$) of unlabelled substrate. The vesicles or cells are diluted into a solution containing another or the same substrate which is labelled.

Influx and efflux: rate equations and dependence on symported ion

The basic kinetic parameters for the influx of galactoside are obtained by rearranging Eqn. 5 in the form of Eqn. 8 under the condition $\alpha'' = 0$ (no solute in compartment ''). This yields the turnover number for influx

$$k_{cat} = \frac{k_c^+ k_o^- \beta'}{(a'_{CG} + \beta')k_o^- + (1 + \beta''_{CH})(k_c^+ \beta')} \quad (13)$$

and a normalized transport constant ($\tilde{K}_T' = K_T'/K_G'$)

$$\tilde{K}_T' = \frac{k_o^- (1 + \beta'_{CH}) + k_o^+ (1 + \beta''_{CH})}{k_o^- (a'_{CG} + \beta') + k_c^+ \beta'(1 + \beta''_{CH})} \quad (14)$$

The partially random or strictly ordered models are described by equations generated by removing the terms in Eqns. 13 and 14 corresponding to the binary complexes (CH', CG', CH'', or CG'') which are absent in each case.

The maximal velocity is a function of the concentration of the symported ion ($\beta = [H]/K_H$). This is not unexpected, because H^+ is the cosubstrate. When $\beta = 0$, k_{cat} should also be 0, as seen by inspection of Eqn. 13. The dependence of k_{cat} on the ion concentration is influenced by the presence of CG' and CH''. Just as H'(β') is the substrate of the symport reaction, H''(β'') may be considered the product. While in the case of a Na^+/solute cotransporter, the concentration of Na^+ in compartment '' may be, in principle, lowered as much as needed so that $\beta'' \ll 1$; for a H^+/solute symporter, this may not be feasible. Therefore, a H^+/solute symporter is liable to exhibit product inhibition. When β' and β'' become large, k_{cat} becomes small because only the denominator of Eqn. 13 contains the product $\beta' \cdot \beta''$.

From Eqn. 14 the nonidentity of K_T' and $K_{G,obs}$, in general, is obvious. Thus if $K_{G,obs}$ is known (see above), the deviation of K_T from this value (or $\tilde{K}_T$ from 1) can be interpreted in terms of Eqn. 14. The value of K_T for influx is determined by the translocation rate constants k_o^-, k_o^+, and k_c^+ as well as by H' and H'' and by the presence of CH', CH'' and CG'. The expressions for k_{cat} and K_T can be derived for the nine possible variants of the general model (Fig. 1), and the dependence of these kinetic parameters on the concentration of the symported ion can be determined by inspection of the equations. The following general rules can be derived and are summarized in Table I.

The dependence of k_{cat} on the concentration of the symported ion β, allows the models to be divided into four classes.

Type 1. The value of k_{cat} increases from a value of zero at low β (H^+ or Na^+) to a maximum at $\beta = 1$, (pH or pNa = pK) and decreases to zero with increasing β (Mechanisms 1, 2, 5 and 8).

TABLE I

DEPENDENCE OF BINDING AND TRANSPORT PARAMETERS ON THE CONCENTRATION OF THE COTRANSPORTED ION

For definition of Types see Text. The symbols in parenthesis indicate whether the indicated parameter increases ($+$), decreases ($-$), goes through a maximum (m), or is invariant (0) with increasing concentration of the cotransported ion. The effects of non-independent binding of the ion and solute (positive or negative cooperativity) are not considered here.

Mechanism	Binding $K_{G,obs}$	Influx		Efflux		Equilibrium exchange		Countertransport	
		k_{cat}	K_T	k_{cat}	K_T	k_{cat}	K_T	k_{cat}	K_T
1. Random	A(0)	1(m)	A($-$)	1(m)	A($-$)	1($+$)	A(0)	1($+$)	A($-$)
2. No CH'	B($-$)	1(m)	A($-$)	3($+$)	D($-$)	1($+$)	A(0)	1($+$)	B($-$)
3. No CG'	B($-$)	2($-$)	B($-$)	1(m)	A($-$)	1($+$)	A(0)	1($+$)	A($-$)
4. No CH''	B($-$)	3($+$)	D($-$)	1(m)	A($-$)	1($+$)	A(0)	1($+$)	A($-$)
5. No CG''	B($-$)	1(m)	A($-$)	2($-$)	B($-$)	1($+$)	A(0)	1($+$)	C($+$,0$-$)
6. No CH', CH''	C($-$)	3($+$)	A($-$)	3($+$)	A($-$)	1($+$)	B($-$)	1($+$)	B($-$)
7. No CG', CG''	D($-$)	2($-$)	B($-$)	2($-$)	B($-$)	2(0)	C($-$)	2(0)	A($-$)
8. No CH', CG''	B($-$)	1(m)	A($-$)	4(0)	C($-$)	1($+$)	A(0)	1($+$)	B($-$)
9. No CG', CH''	B($-$)	4(0)	C($-$)	1(m)	A($-$)	1($+$)	A(0)	1($+$)	A($-$)

Type 2. The value of k_{cat} decreases from a maximal value at low β to zero with increasing β (Mechanisms 3 and 7).

Type 3. The value of k_{cat} increases with increasing β (Mechanisms 4 and 6).

Type 4. The value of k_{cat} is independent of β (Mechanism 9).

Type 1 behavior is due to the inhibiting effect of the symported ion at high ion concentrations. In each of these mechanisms the binary CH'' complex is present. Type 2 behavior in the range of small β is ascribable to the ordered addition of substrate (H$^+$ or Na$^+$ first, solute second). At infinite solute concentration where k_{cat} is defined, the carrier will appear to have infinite affinity for the symported ion. At higher concentrations of the symported ion, the formation of CH'' inhibits influx. Both Mechanisms 3 and 7 exhibit a pronounced dependence of K_T on β (see below). Type 3 behavior is due to the absence of CH'', the source of product (H'') inhibition in influx. Type 4 behavior is due to a combination of the mass action seen in Mechanism 3 and 7 (Type 2) and the absence of product inhibition (i.e. CH'') seen in Mechanisms 4 and 6 (Type 3).

For all mechanisms, K_T decreases with increasing β, and the magnitude of the decrease depends on the relative values of the rate constants. Based upon the variation of K_T between low and high

concentrations of the symported ion, the following classification is possible.

Type A. K_T decreases from a finite value to zero (infinity affinity) with increasing β (Mechanisms 1, 2, 5, 6 and 8).

Type B. K_T decreases from infinity (no affinity) to zero (infinite affinity) with increasing β (Mechanisms 3 and 7).

Type C. K_T decreases from infinity (no affinity) to a finite value with increasing β (Mechanism 9).

Type D. K_T decreases from one finite value to another with increasing β (Mechanism 4). Depending on the relative values of k_o^- and k_c^+, Type D may resemble Type A.

The equation for efflux is derived from Eqn. 5 by setting $\alpha' = 0$ and rearranging in the form of Eqn. 8 with $S = [G'']$. The expression for k_{cat} and K_T are the same as those for influx after interchanging both ' and '' and $^+$ and $^-$. The expected dependence of k_{cat} and K_T for influx and for efflux upon the concentration of the symported ion can be tabulated for all mechanisms according to the types previously defined. The classification of each mechanism is unique (Table I). However, to interpret data for the influx and efflux of solute unambiguously, the dissociation constant for the ion must be known. The following analysis demonstrates that measurements of the kinetics of

solute or ion exchange can provide estimates of this dissociation constant.

Equilibrium exchange

When the symport of ion and solute is not coupled to an energy source, at equilibrium the concentrations of solute on both sides of the membrane are equal as are also the concentrations of the symported ion. No net movement of solute or ion occurs; two fluxes of equal magnitude and opposite direction between the two compartments exist. If the solute (or ion) in one of these compartments is labelled, the unidirectional flux from one compartment to the other under conditions of equilibrium can be measured. The expression for the turnover number and transport constant of equilibrium exchange are derived from Eqn. 5. Because a unidirectional flux is measured, either the left-hand or the right-hand term in the numerator is used. If the labelled galactoside is in the external (') compartment, rearrangement of Eqn. 5 in the form of Eqn. 8 with $S = [G']$, yields

$$k_{cat} = \frac{k_c^+ \beta'}{(a'_{CG} + \beta') + (a''_{CG} + \beta'')\frac{k_o^+}{k_o^-} \cdot \frac{K'_G}{K''_G}} \qquad (15)$$

and

$$\tilde{K}'_T = \frac{(1 + \beta'_{CH}) + (1 + \beta''_{CH})k_o^+/k_o^-}{(a'_{CG} + \beta') + (a''_{CG} + \beta'')\frac{k_o^+}{k_o^-} \cdot \frac{K'_G}{K''_G}} \qquad (16)$$

where the subscripts indicate the terms representing the binary complexes CG', CG", CH' and CH". The values of k_{cat} and K_T can be calculated for each of the possible nine variants of the basic model.

The variations of k_{cat} and K_T for equilibrium exchange with the concentration of the symported ion is less dependent on the precise mechanism than in the cases of influx and efflux (Table I). For the variation of k_{cat} with the ion concentration, only one major and one minor class are discerned for equilibrium exchange. Type 1. The value of k_{cat} increases with increasing concentration of the symported ion. Type 2. The value of k_{cat} is independent of the concentration of the symported ion (Mechanism 7).

Likewise, there is not much diagnostic value in the dependence of K_T on β with respect to the exact mechanism of symport. While there is a tendency for K_T to decrease with increasing β, the modest changes might be difficult to detect experimentally. The classes which emerge are: Type A. K_T is not or only slightly dependent on β (Mechanisms 1–5, 8, 9). Type B. K_T decreases to 0 (infinite affinity) at high β (Mechanism 6). Type C. K_T decreases from infinity (no affinity) at low β to a finite value at high β (Mechanism 7).

The relative insensitivity of the kinetic parameters to the mechanism in several cases can, in fact, be exploited. For symport systems with Type 1 behavior for k_{cat} and Type A for K_T, measurement of equilibrium exchange can provide two valuable parameters: From the variation of k_{cat} the pK (i.e. K_H) of the symported ion can be obtained, and K_T will have the value of $K_{G,obs}$. If the unidirectional flux of the symported ion (e.g. H$^+$) can be measured, an independent confirmation of these values may be obtained. Here, the dependence of k_{cat} on the concentration of the other cosubstrate will yield an estimate of K_G, and K_T will be $K_{H,obs}$. When the symported ion is H$^+$, problems arise due to the ambiguity in the interpretation of changes in carrier function at low or high pH values. The following generalizations apply: If the k_{cat} of equilibrium exchange does not vary appreciably in the physiological pH region, then the pK of the symported H$^+$ is greater than the highest pH tested. Alternatively, Mechanism 7 may be operative. However, this should be obvious from other measurements. If the k_{cat} of equilibrium exchange increases with decreasing pH, then the pK of the symported H$^+$ lies within or below the pH range tested.

Countertransport

Countertransport or counterflow is included in this treatment of facilitated diffusion because data for countertransport complement those from influx and equilibrium exchange, and especially because the initial velocity of countertransport can be more readily or more accurately measured than the other modes of facilitated diffusion due to the initial accumulation of substrate. Countertransport may be performed in many variations, one of which is the following: Vesicles or cells are loaded

with a substrate, generally at saturating levels $[(G'') > K_G'']$, and then diluted into a solution of the same or a second, labelled substrate. The initial conditions are saturating substrate internally and variable levels of labelled substrate externally. The labelled substrate is transported into the cell or vesicles but cannot exit as long as the unlabelled substrate is saturating. Thus, the labelled substrate is transiently accumulated (overshoot). As the concentration of the internal unlabelled substrate sinks, the inhibition of the exit of the labelled substrate is relaxed. At longer times equilibrium is established: the concentrations of labelled and unlabelled substrates on both sides of the membrane become equal.

The analysis of the time-course of the transient accumulation during countertransport is difficult. The overshoot may have some diagnostic value, as at this maximum $d[G'']/dt = 0$ for the labelled substrate [14,15]. Rosenberg and Wilbrandt, studying hexose transport in erythrocytes, demonstrated and theoretically described countertransport [16]. The treatment offered here is initially an analysis of the initial rates of countertransport catalyzed by a solute: ion carrier, under the conditions that the concentration of the symported ion is equal on both sides of the membrane and that the imposition of $\Delta\psi$ across the membrane due to the flux of the symported ion is prohibited.

To analyze the most general type of countertransport with different external, labelled substrate (identified by the subscript 1 and $\alpha' = [G_1']/K_1$) and internal, unlabelled substrate (identified by the subscript 2 and $\gamma'' = [G_2'']/K_2$), the basic Eqns. 2 to 7 must be expanded to include additional carrier complexes and translocation steps. Thus, the equation for conservation of mass becomes (cf. Eqn. 2).

$$[C_T] = [C'] + [CH'] + [CG_1'] + [CG_2'] + [CHG_1']$$

$$+ [CHG_2'] + [C''] + [CH''] + [CG_1''] + [CG_2'']$$

$$+ [CHG_1''] + [CHG_2''] \tag{17}$$

and that for the steady-state assumption (cf. Eqn. 3) is

$$k_o^+ \cdot [C'] + k_1^+ \cdot [CHG_1'] + k_2^+ \cdot [CHG_2']$$

$$= k_o^- \cdot [C''] + k_1^- \cdot [CHG_1''] + k_2^- \cdot [CHG_2''] \tag{18}$$

where k_1 and k_2 are the appropriate values of k_c for each type of ternary complex. The expression for the net influx of G_1 (Eqn. 4a) under initial-velocity conditions is

$$\frac{v_N}{C_T} = [k_1^+ \alpha'\beta'(k_o^- + k_1^- \alpha''\beta'' + k_2^- \gamma''\beta'')$$

$$- k_1^- \alpha''\beta''(k_o^+ + k_1^+ \alpha'\beta' + k_2^+ \gamma'\beta')]$$

$$\times [(1 + \alpha' + \beta' + \gamma' + \alpha'\beta' + \gamma'\beta')$$

$$\times (k_o^- + k_1^- \alpha''\beta'' + k_2^- \gamma''\beta'')$$

$$+ (1 + \alpha'' + \beta'' + \gamma'' + \alpha''\beta'' + \gamma''\beta'')$$

$$\times (k_o^+ + k_1^+ \alpha'\beta' + k_2^+ \gamma'\beta')]^{-1} \tag{19}$$

Usually countertransport is measured under the initial conditions of negligible unlabelled substrate externally ($\gamma' \approx 0$; e.g. cells are loaded with unlabelled substrate at $10 \times K_G$ and diluted 100-fold so that $\gamma' = 0.1$) and no labelled substrate internally ($\alpha'' = 0$). Eqn. 19 simplifies to

$$\frac{v}{C_T} = [k_1^+ \alpha'\beta'(k_o^- + k_2^- \gamma''\beta'')]$$

$$\times [(k_o^- + k_2^- \gamma''\beta'')$$

$$\times (1 + \alpha_{CG}' + \beta_{CH}' + \alpha'\beta') + (k_o^+ + k_1^+ \alpha'\beta')$$

$$\times (1 + \beta_{CH}'' + \gamma_{CG}'' + \gamma''\beta'')]^{-1} \tag{20}$$

Rearrangement in the form of Eqn. 8 with $S = [G_1']$ yields

$$k_{cat} = [k_1^+ \beta'(k_o^- + k_2^- \gamma''\beta'')][(k_o^- + k_2^- \gamma''\beta'')$$

$$\times (a_{CG}' + \beta') + k_1^+ \beta'(1 + \gamma_{CG}'' + \beta_{CH}'' + \gamma''\beta'')]^{-1} \tag{21}$$

and

$$\bar{K}_T' = [(1 + \beta_{CH}')(k_o^- + k_2^- \gamma''\beta'')$$

$$+ k_o^+ (1 + \gamma_{CG}'' + \beta_{CH}'' + \gamma''\beta'')]$$

$$\times [(a_{CG}' + \beta')(k_o^- + k_2^- \gamma''\beta'')$$

$$+ k_1^+ \beta'(1 + \gamma_{CG}'' + \beta_{CH}'' + \gamma''\beta'')]^{-1} \tag{22}$$

To emphasize the fact that the internal un-

labelled galactoside (G_2) may not be identical to the labelled external galactoside, the symbol $\gamma'' = [G_2'']/K_G''$ is used, and the translocation rate constants for the substrates are designated by the subscripts 1 (for the external, labelled substrate) and 2 (for the internal, unlabelled substrate) instead of c. As the translocation rates of the ternary complexes (k_1^+, k_1^-, k_2^+ or k_2^-; cf. k_c^+ and k_c^- in Fig. 1) may have characteristic values for each galactoside, k_{cat} may be different for different substrates. The terms arising from the presence of the binary CG', CH', CG'', CH'' complexes are designated by the appropriate subscripts. The exact expressions for k_{cat} and K_T can be derived for the nine possible mechanisms (Table I). The dependence of these parameters upon the concentration of the symported ion ($\beta = [H]/K_H$) contains information about the order of substrate binding and the affinity of the carrier for the symported ion.

The variation of k_{cat} reveals two classes. Type 1: The value of k_{cat} increases with increasing concentration of the ion. Type 2: The value of k_{cat} is independent of the concentration of the ion. These are the same classes observed for equilibrium exchange (Table I). Therefore, with exception of Mechanism 7, the variation k_{cat} for countertransport with β is thus a method of determining the pK of the symported ion. This method for estimating the pK of the symported ion is often experimentally more feasible than the use of equilibrium exchange, because the overshoot in countertransport facilitates the measurement of true initial velocities. For H^+/solute symport, an appropriate generalization would be that the pH independence of k_{cat} for countertransport is an indication that the pK of the symported H^+ is greater than the highest pH tested.

The dependence of the K_T for countertransport on the concentration of the symported ion evinces three classes: Type A. K_T decreases from an infinite value (low affinity) to a finite value with increasing ion concentration (Mechanisms 1, 3, 4, 7 and 9). Type B. K_T decreases from an infinite value to zero (infinite affinity) (Mechanisms 2, 6, 8). Type C. K_T varies between two finite values. K_T can increase, decrease, or pass through a maximum depending on the magnitude of k_2^-, k_o^+, and k_1^+.

Semiquantitative description of the time course of countertransport

The net velocity v of the labelled substrate (here G_1) at any given instant is given by Eqn. 19. At the peak of the overshoot, v is zero. Rearranging the numerator of Eqn. 19 yields

$$\frac{[G_1'']}{[G_1']} = \frac{K_G''}{K_G'} \cdot \frac{k_1^+ k_o^- \beta'}{k_1^- k_o^+ \beta''} \cdot \frac{(k_o^- + k_2^- \gamma'' \beta'')k_o^+}{(k_o^+ + k_2^+ \gamma' \beta')k_o^-} \tag{23}$$

Recognizing that for both solutes,

$$\frac{[G_1'']}{[G_1']} = \frac{[(H^+)']}{[(H^+)'']} \cdot \frac{K_G'' K_H''}{K_G' K_H'} \cdot \frac{k_c^+ k_o^-}{k_c^- k_o^+}$$

$$= \exp(-\Delta\tilde{\mu}_{H^+}/RT) = A \tag{24}$$

at equilibrium, and designating the accumulation ratio predicted by the chemiosmotic theory as A, in Eqn. 23 may be rewritten as

$$\frac{[G_1'']}{[G_1']} = A\frac{(k_o^- + k_2^- \gamma'' \beta'')k_o^+}{(k_o^+ + k_2^+ \gamma' \beta')k_o^-} \tag{25}$$

In general, the accumulation ratio of the labelled substrate is a kinetically determined quantity.

If the concentrations of ion and solute are saturating on both sides of the membrane so that the carrier reorients exclusively as ternary complexes ($k_2^- \beta'' \gamma'' > k_o^-$ and $k_2^+ \beta' \gamma' > k_o^+$), then after applying Eqn. 24 for the unlabelled substrate, the accumulation ratio of the labelled substrate corresponds exactly to that of the unlabelled substrate

$$\frac{[G_1'']}{[G_1']} = A \cdot A^{-1} \cdot \frac{[G_2'']}{[G_2']} = \frac{[G_2'']}{[G_2']} \tag{26}$$

This requires that both the symported ion and the unlabelled cosubstrate be saturating on both sides of the membrane. At the peak of the overshoot, the concentration ratio of the unlabelled substrate $[G_2'']/[G_2']$ will probably be smaller than the gradient originally imposed due to the efflux of this substrate.

Frequently, countertransport is performed with the external concentration of the unlabelled substrate at subsaturating levels ($[G_2'] < K_G'$). In this case, the accumulation ratio of the labelled sub-

strate at the peak of the overshoot is

$$\frac{[G_1'']}{[G_1']} = A \frac{(k_o^- + k_2^- \gamma''\beta'')}{k_o^-} \tag{27}$$

The peak accumulation increases with increasing H″ and G_2'' (generally smaller than G_2'' at $t = 0$) and depends also on the value of $\Delta\bar{\mu}_{H^+}$ and of k_2^- and k_o^-. Therefore, the variation of the overshoot peak with the concentration of an ion is a simple test for ion/solute symport.

If the symported ion is saturating, the following limiting values can be derived for the fully random mechanism

$$k_{cat} = \frac{k_1^+ \cdot k_2^-}{k_1^+ + k_2^-} \tag{28}$$

$$\tilde{K}_T' = \frac{k_2^-}{k_1^+ + k_2^-} \tag{29}$$

and

$$\frac{k_{cat}}{\tilde{K}_T'} = k_1^+ \tag{30}$$

If the external concentration of labelled substrate is subsaturating, the initial velocity is (cf. Eqn. 8 for $S < K_T$)

$$v = [C_T]\frac{k_{cat}}{\tilde{K}_T'} \cdot \alpha' = [C_T]k_1^+ \frac{[G_1']}{K_1'} \tag{31}$$

Lastly, the decrease in the internal concentration of labelled substrate after the overshoot can be treated in a simple approximation. Integration of an efflux equation in the form of Eqn. 8 with respect to time discloses that efflux is, in general, a biphasic process: At saturating internal concentration efflux is linear (zero order) with time; as the concentration sinks below the effective K_T for efflux, partially determined by the level of internal unlabelled substrate, the loss of internal labelled substrate is exponential with time (first order). The half-time for efflux in this latter region is

$$t_{1/2} = \frac{\ln 2}{V_{max}''/\tilde{K}_T''} \tag{32}$$

For the case that the external concentrations of labelled and unlabelled substrates are subsaturat-

ing and that the symported ion is saturating, the expression

$$\frac{V_{max}''}{\tilde{K}_T''} = \frac{[C_T]k_1^-}{1 + 2\gamma''} \tag{33}$$

is a good approximation. Therefore, the efflux of the labelled substrate, after the peak of the overshoot is attained, is characterized a half-time roughly given by

$$t_{1/2}^{-1} \approx \frac{[C_T]k_1^-}{1 + 2\gamma''} \tag{34}$$

An approximate description of the time-course of countertransport can be assembled on the basis of the above considerations (Fig. 2).

This analysis clarifies some heretofore unexplained observations about countertransport by the lactose/H$^+$ carrier of *E. coli* [15,17–19]. Initial rates at fixed substrate concentrations or maximal velocities of countertransport in cells, vesicles, or reconstituted proteoliposomes are nearly independent of pH in the physiological region. If the external concentration of labelled substrate is subsaturating, the peak of the overshoot decreases with increasing pH. If the external concentration is near saturation, the peak of the overshoot hardly varies with pH. In recent examinations of the effect of $\Delta\bar{\mu}_{H^+}$ on galactoside binding and transport, the binding sites for the symported H$^+$ were demonstrated to be saturated at physiological pH [4,13]. Under these conditions the velocity of countertransport is not expected to increase with increasing concentration of H$^+$ (Table I). At subsaturating external concentrations of the labelled substrate, Eqn. 27 applies, and the height of the overshoot increases with the concentration of H$^+$. At saturating concentrations, Eqn. 26 applies, and the height of the overshoot does not vary with the concentration of the symported ion. Finally, the time course of countertransport (Fig. 2) was observed to flatten as the level of carrier in the membrane decreased [14]. The approximation described in Fig. 2 predicts that the slopes of ascending and descending regions of the overshoot are proportional to the carrier level [C$_T$]. As [C$_T$] decreases, the entire curve becomes flatter. The overshoot appears later, because the solute flux across the membrane is slower. As the overshoot is
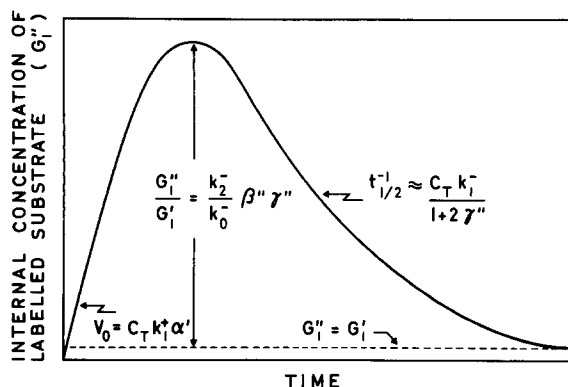
Fig. 2. Semiquantitative description of the time-course of countertransport catalyzed by an ion/solute carrier. The values for the initial rate (v_0), the overshoot of the labelled substrate (G_1''/G_1'), and the approximate half-time $(t_{1/2})$ for the return to equilibrium are expressed in terms of the basic parameters of the model. The initial conditions are: Low external concentrations of the labelled $(\alpha' < 1)$ and unlabelled $(\gamma' < 1)$ solutes and high internal concentrations of the ion and labelled solute $(k_2^- \gamma'' \beta'' > k_o^-)$. Note that the steepness of the two sides of the curve increases with increasing carrier level (C_T) and rate of the reorientation of the ternary complex containing the labelled solute $(k_1^+$ and $k_1^-)$ and that the overshoot increases with increasing concentration of the ion.

shifted to later times, the internal concentration of unlabelled substrate will be lower; and the height of the overshoot decreases (Eqn. 25). Lastly, mutants (designated Y^{UN} for energy-uncoupled phenotype) defective in the active transport of galactosides have been examined to determine if the movement of galactoside were uncoupled from that of H^+ (cf. Ref. 19). Kinetic studies including countertransport demonstrated that changes in the rates of carrier reorientation and not a decrease in coupling are responsible for the phenotype [20]. For instance, the observation that the overshoot during countertransport in the mutant exhibits the same pH dependence as in the wild type [19] demonstrates that symport still occurs in the mutant (cf. Eqn. 27). The ability of this simple treatment of countertransport to explain these diverse observations bespeaks its basic correctness.

Discussion

Systems catalyzing secondary active transport may be studied at the less complicated levels of binding and facilitated diffusion. Once a kinetic model for the transport process is selected, the effects of imposing driving gradients $(\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+})$ can be more readily interpreted as changes in an already well-defined model. In this analysis, experimental parameters for substrate binding and facilitated diffusion have been defined in terms of the basic model for cotransport.

The observed dissociation constant for one cosubstrate is a function of the concentration of the other cosubstrate and of the order of substrate binding and differs from most transport constants (K_T). Thus, binding studies complement transport studies and may be of diagnostic value in selecting particular models.

In general, the values of k_{cat} and K_T for facilitated diffusion of one cosubstrate (e.g. galactoside) depend upon the concentration of the other cosubstrate and the order of substrate binding. Determination of the pH (pNa) dependence of k_{cat} and K_T for influx and efflux allows an unambiguous assignment of the kinetic mechanism, if the pK of the symported ion is known. In many cases, high concentrations of the symported ion evoke a form of product inhibition, so that the value of k_{cat} for influx or efflux may exhibit a maximum near $[H^+] = K_H$ for a proton symporter (Table I).

Measurement of equilibrium exchange leads to an estimate of K_G from the value of K_T for the solute. This value is identical to that from binding measurements. The pK of the symported ion can be obtained from the variation of k_{cat} with the ion concentration (Table I). The advantage of using equilibrium exchange as opposed to influx or efflux to estimate K_H and K_G is that the interpretation of the kinetic data is nearly independent of the kinetic mechanism.

Countertransport is a form of facilitated diffusion in the exchange mode. The dependence of k_{cat} for solute on the concentration of the symported ion is similar to that for equilibrium exchange, and an estimate of the pK of the symported ion can be obtained. The value of K_T for countertransport evinces a more pronounced dependence upon the concentration of the symported ion than the K_T for equilibrium exchange, and hence this variation may be of additional diagnostic value.

The dependence of $K_{G,obs}$ and the various K_T

on the concentration of the cotransported ion in Table I is due to mass action. Positive or negative cooperativity in the binding of the cosubstrates is not considered here for simplicity. This fact does not detract from the usefulness of this analysis. Strong negative cooperativity is immediately obvious from the tendency of $K_{G,obs}$ or K_T to increase instead of decrease with the concentration of the cosubstrate. Weak negative or positive cooperativity would not reverse the general tendencies derived from the simpler analysis.

The results obtained here can be used to interpret binding and kinetic data obtained for the lactose/H^+ carrier of *E. coli* in both a qualitative and quantitative manner [4].

Acknowledgement

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