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Topical Review

Quantitative Studies of Cotransport Systems: Models and Vesicles

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Introduction

The ubiquity and importance of secondary active cotransport processes in biological systems is now well established. Over the past few years a number of review articles dealing in whole or in part with this type of transport have been published (Mitchell, 1973, 1979; Kotyk & Janacek, 1975; Crane, 1977; Hopfer, 1977, 1978; Sacktor, 1977; Schultz, 1977; Eddy, 1978; Heinz, 1978; Oxender & Fox, 1978; Poole, 1978; Rosen & Kashket, 1978; Silhavy, Ferenci & Boos, 1978; Lanyi, 1979; Silverman & Turner, 1979; Ullrich, 1979; Gunn, 1980; Kabach, 1980; Lever, 1980; Murer & Kinne, 1980; West, 1980; Aronson, 1981; Borst-Pauwels, 1981; Freel & Goldner, 1981; Geck & Heinz, 1981; Njus, Knoth & Zablakian, 1981; Kanner, 1983). In the present topical review I would like to discuss several aspects of the study of such transport mechanisms which have not been extensively treated in these earlier publications. These topics center around the determination of reliable kinetic, stoichiometric, and other quantitative data for cotransport systems and their interpretation in terms of various kinetic models. Such considerations are particularly pertinent at the present time since the results obtainable with the experimental preparations and techniques currently available have reached the degree of sophistication required to test and distinguish between many of these models. Because of the special problems involved in measuring coupled fluxes (*see* later) and the obvious advantages of being able to control both cis and trans membrane conditions, vesicle preparations are an ideal experimental system for quantitative studies of this type. For this reason, in what follows I will concentrate mainly on experimental work with vesicles, although much of the discussion applies equally well to more intact preparations.

This article is divided into three parts. In the first I discuss the current status and understanding of cotransport models. The treatment is for the most part nonmathematical and attempts to clarify some problems of interpretation that have appeared in the literature. The second section deals with the experimental aspects of obtaining reliable quantitative data for coupled transport systems. The remainder of the article discusses various approaches to the measurement of the stoichiometry and order of binding of cotransport systems. Studies of this type represent much of the current focus of attempts to understand the mechanism of these transport processes.

Kinetic Models of Cotransport Systems

The purpose of this section is to give the reader who is not directly involved in working with cotransport models a feeling for their significance and the significance of their predictions. Perhaps it is appropriate to begin by briefly discussing the apprehensions that some investigators seem to have about applying arguments based on kinetic models to their results. These apprehensions arise mainly from a concern about the model dependence of conclusions arising from kinetic analyses. Although it is, of course, desirable to be able to interpret data in a manner that is as model-independent as possible, one must bear in mind that in the last analysis all interpretations of experimental results are based on some conceptualization (model) of the process under examination and thus are subject to some degree of model dependence. The major strength of a kinetic model and its atten-

dant mathematical analysis over a purely mental conceptualization of a transport (or enzymatic) process is that in the former all assumptions are necessarily clearly stated and the resulting predictions can be rigorously and quantitatively derived.

In this regard it should be stressed that there are obviously many ways of attacking the fundamental problem of how transport proteins function. Our final understanding of these systems will certainly be based on a synthesis of information from a number of methodologies. The power of the kinetic approach for distinguishing between and characterizing various reaction mechanisms is well known from classical enzymology (Segal, 1975). Kinetic analyses have played and continue to play a major role in unraveling the mechanism of action of functional proteins, *including those for which a large amount of detailed structural and biochemical information is already available.* Kinetic techniques are only beginning to be applied to transport systems in general and to cotransport systems in particular. For this reason a broad basis of theory and expertise associated with the particular problems of treating transport as distinct from enzymatic processes is not yet fully established.

Cotransport models are necessarily somewhat complex since they must incorporate the association and dissociation of at least two ligands with the carrier on either side of the membrane as well as the translocation events of the free and loaded carrier species. Solving these models can be tedious, and the resulting flux equations are often cumbersome. However, owing to the complexity of the cotransport process, it is difficult to arrive at an understanding of the behavior of such a model without the benefit of a rigorous mathematical analysis. There are a number of examples in the literature where authors have attributed incorrect predictions to various kinetic schemes on the basis of qualitative (nonmathematical) arguments. It has also been common to attempt to deduce the predictions of transport models from the properties of similar kinetic schemes which have been applied to enzymatic systems. This procedure is also risky since the correspondence between transport mechanisms and enzymatic mechanisms is not always good (e.g., transport phenomena necessarily involve reactants in two pools whereas enzymatic phenomena involve reactants in a single pool).

The Carrier Model of Cotransport

Although a number of kinetic schemes have been suggested and analyzed for facilitated diffusion systems (Lieb & Stein, 1970, 1974; Naftalin, 1970; LeFevre, 1973; Deves & Krupka, 1978), to date, with a few notable exceptions (Borst-Pauwels, 1973; Lombardi, 1981), only models of the carrier type have been analyzed for cotransport. As will be discussed further below, the carrier model of cotransport is one of the simplest kinetic schemes which incorporates the required features of a secondary active transport system. Accordingly, the carrier model will be used here as the basis of the discussion to follow. A schematic representation of a carrier model is given in Fig. 1. Here the two cotransported species are referred to as the substrate, S, and the activator, A. The free carrier on side n of the membrane is represented by C_n, the carrier plus bound substrate by CS_n, the carrier plus bound activator by CA_n and the fully loaded carrier by CAS_n. The external and internal sides of the membrane are labeled $n=1$ and $n=2$, respectively. The rate constants for the translocation of the various free and loaded carrier species are designated k_{nm}, h_{nm}, etc. The dissociation constants, K_{A_n}, K_{S_n}, etc., characterize the binding events at the membrane faces and are equal to the ratios of the "off" and "on" rate constants for the appropriate reactions.

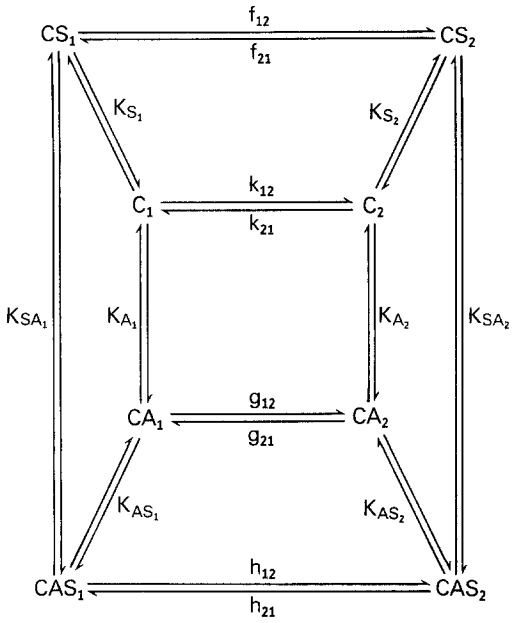

Fig. 1. The carrier model of cotransport (*see* text for details). The case of random binding of the substrate (S) and activator (A) to the carrier (C) is illustrated. (Reproduced from Turner, 1981)

In order to retain generality it has been assumed in Fig. 1 that the partially loaded forms of the carrier, CA_n and CS_n, are mobile (i.e., that f_{nm} and $g_{nm} \neq 0$). However, if the transporter is to maintain efficiency of energy coupling it is ex-

pected that either $[CA_n]$ and $[CS_n]$ are small, owing to cooperative effects of ligand binding, or that f_{nm} and g_{nm} are small relative to h_{nm}. It should also be noted that all of the parameters of the model shown in Fig. 1 are not independent of one another. These parameters are necessarily related by three thermodynamic constraints arising from the requirement that there be no net flux via the transporter when the electrochemical potential differences for both substrate and activator across the membrane are zero (Turner, 1981).

As has been frequently noted, the carrier model is not limited to the mobile carrier interpretation in which a transport protein shuttles back and forth across the membrane. The only assumptions made about the mechanism of the translocation event in Fig. 1 are that the activator and substrate binding sites are simultaneously exposed to the solution at one or the other side of the membrane and that the transition between these two configurations is characterized by the rate constants k_{nm}, g_{nm}, etc. There are many physical representations of the abstract kinetic scheme shown in Fig. 1 (*see*, e.g., Turner & Silverman, 1980).

Cotransport and Energy Transduction

A coupled transport system may be thought of as an energy transducer whose function is to convert free energy stored as an activator electrochemical gradient to free energy stored as a substrate electrochemical gradient (the reverse process is also possible since the distinction between the activator and substrate is a physiologic rather than a thermodynamic one). There has been some speculation regarding both the step in the cotransport process where this transfer of energy takes place and the mechanism by which this is achieved. However, as has been stressed by Hill (Hill, 1977; Hill & Eisenberg, 1981), free energy transduction can only be accomplished by complete biochemical cycles and cannot be associated with an individual step or event in a cycle. This point can be more readily appreciated by considering a concrete example:

Suppose that a membrane containing transporters of the type shown in Fig. 1 separates two identical solutions of A and S, i.e., $[A_1]=[A_2]$, $[S_1]=[S_2]$ and $\Delta\psi=0$ ($\Delta\psi=\psi_2-\psi_1$ is the transmembrane electrical potential difference). For simplicity, let us also assume that except for these transporters the membrane is impermeant to A and S and that the partially loaded forms of the carrier, CS_n and CA_n are immobile, i.e., $f_{nm}=g_{nm}=0$ in Fig. 1. In this system A and S can only cross the membrane in a 1:1 tightly coupled fashion. Under the above "equilibrium exchange" conditions the unidirectional fluxes of A and S from either side of the membrane to the other must be equal so that there is no net flux of either species. If $[A_1]$ is now increased the law of mass action requires that $[CAS_1]$ increases and hence that the unidirectional flux of A and S from side 1 to side 2 of the membrane (which is proportional to $h_{12}[CAS_1]$) also increases. Thus S will be transported against its concentration gradient until the concentrations of A and S in the two compartments are such that their net fluxes across the membrane are again zero (the solutions separated by the membrane are assumed to be of finite size). It can easily be shown from thermodynamic arguments that this condition is given by

$$[S_2]/[S_1]=[A_1]/[A_2]. \quad (1)$$

At this new steady-state, free energy initially present as an activator gradient has been converted by the coupled transporter into free energy stored as a substrate gradient.

In this example there is obviously no one step in the coupled transport cycle where energy is physically transferred from an activator to a substrate molecule. In fact the existence of such a step would be impossible since the energy stored in the activator gradient is not really a property of the individual activator ions at all but rather a property of the ensemble of ions as a whole. Thus free energy transduction occurs simply as a result of the stochastic cycling of the transporter, a process which can be accounted for and understood solely in terms of conventional reaction kinetics (the law of mass action) and equilibrium thermodynamics (Eq. (1)).

In order to discuss the implications of free energy transduction for cotransport models it is useful to distinguish between "catalytic" and "energetic" activation of transport processes. This distinction arises because the activator can influence substrate flux in two different ways. By virtue of its binding to the transporter the activator can induce a change in substrate binding affinity or translocation rate which leads to a modification of transport properties (usually an increase in substrate flux). This effect is referred to as catalytic activation. In order to produce catalytic activation the activator need not be transported. Energetic activation, on the other hand, refers to the actual coupling of activator and substrate fluxes via a transport protein. Thus energetic activation involves free energy transduction. These two types of activation can occur independently or simultaneously in the same model (see below).

The concept of secondary active transport incorporates energetic activation. From a thermodynamic point of view this is essential since coupling of activator and substrate fluxes is required for free energy transduction. In fact, the most common method for demonstrating the existence of a secondary active transport mechanism is to show that an activator gradient alone can drive the concentrative uptake of substrate, i.e., to demonstrate free energy transduction. An example of such an experiment is shown in Fig. 2. Here the uptake of D-glucose into renal outer cortical brush border membrane vesicles is studied in the presence and absence of an initial extravesicular to intravesicular sodium gradient. In the presence of a sodium gradient the intravesicular glucose concentration is seen to transiently rise above (overshoot) its equilibrium value then fall back toward equilibrium as the sodium gradient dissipates. In the absence of a gradient no accumulation of D-glucose above equilibrium is evident. Thus the overshoot phenomenon is not simply due to the presence of sodium but rather to the presence of a sodium gradient. (Note also that the overshoot cannot be explained by osmotic changes in vesicle size or by the effects of membrane potentials, which have been short circuited in this experiment – *see* Figure legend.) Figure 2 provides strong evidence for the existence of a secondary active transport system which couples the fluxes of sodium and D-glucose in this vesicle preparation.

It is worth emphasizing that the active accumulation of D-glucose seen in Fig. 2 can be completely accounted for on thermodynamic grounds. In their theoretical simulations of vesicle uptake experiments using carrier models, Babcock, Garvey and Berman (1979) and Weiss, McNamara and Segal (1981) account for the existence of the overshoot on the basis of kinetic arguments. More specifically they point out that in their models the (apparent) affinity of the transporter for the substrate at either side of the membrane increases with increasing activator concentration on that side. Thus under initial activator gradient conditions influx is enhanced by the high extravesicular activator concentration while efflux remains low until the intravesicular activator concentration rises. The result is a net influx of substrate into the vesicles which continues even after the substrate has reached chemical equilibrium, provided an activator gradient is still present. Although this explanation is certainly valid for the models these authors consider, it is important to realize that the effect of activator concentration on the apparent substrate affinity which "leads to" the overshoot by the above

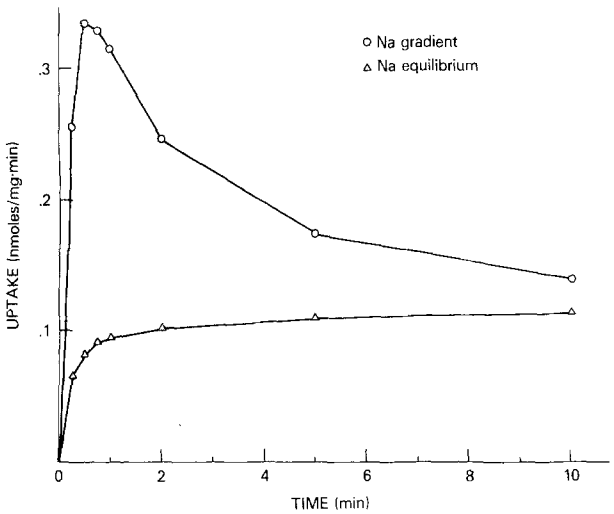

Fig. 2. Demonstration of the "overshoot"phenomenon for D-glucose in rabbit outer cortical brush border membrane vesicles. Vesicles were prepared in 10 mM Tris-HEPES (pH 7.4) buffer containing 100 mM mannitol, 12.5 μg valinomycin/mg protein and 100 mM KSCN along with 90 mM choline chloride (o) or 60 mM NaCl plus 30 mM choline chloride (Δ). In each case the composition of the incubation medium was such that the final extravesicular solution was 10 mM Tris-HEPES, 100 mM mannitol, 100 mM KSCN, 30 mM choline chloride and 60 mM NaCl

argument is itself a result of the assumption of a cotransport mechanism and its attendant thermodynamic constraints. A thermodynamically consistent model which incorporated an increase in substrate affinity with activator concentration but did not involve cotransport of activator could not predict an overshoot phenomenon since energy transduction would not be possible. Thus it is the physical coupling of fluxes which is the primary cause of the overshoot. Any thermodynamically consistent cotransport model would predict such a phenomenon provided it did not incorporate substantial leak pathways.

The fact that a transporter is capable of energy transduction imposes certain limitations on its physical properties. For example, as discussed above it must be able to actually transport the activator. However, this is not enough. A transporter with an activator binding site which is simultaneously exposed to the solutions on both sides of the membrane (e.g., located in a transmembrane channel) is capable of translocating the activator but is nevertheless incapable of energy transduction. Such a transporter is necessarily insensitive to an activator gradient because it does not distinguish between activator ions originating from different sides of the membrane. Thus it has no means of coupling substrate and activator fluxes and must function in a purely catalytic activation mode. By a similar argument the substrate binding

site cannot be simultaneously exposed at both sides of the membrane. The carrier model of cotransport is one of the simplest kinetic realizations of a secondary active transport mechanism since it incorporates both a direct coupling between substrate and activator fluxes and a means of distinguishing between the solutions at either side of the membrane.

Solutions of the Rapid Equilibrium Carrier Model of Cotransport

Most theoretical analyses of carrier models of cotransport have assumed rapid equilibrium of association and dissociation events at the membrane faces or, equivalently, that the transmembrane translocation events are rate limiting. This assumption greatly reduces the labor involved in solving the models and in most cases simplifies the form of the resulting flux equations. The general steady-state solution to the model shown in Fig. 1 under rapid equilibrium conditions is given in Turner (1981). For the purposes of testing such a model experimentally it is desirable to introduce some further simplications. These typically take the form of certain experimentally convenient substrate and activator conditions. Three of the most commonly treated cases are

(i) *zero trans* conditions, i.e., $[S_2]=[A_2]=0$;

(ii) *equilibrium exchange* conditions, i.e., $[S_1]=[S_2]$, $[A_1]=[A_2]$, $\Delta\psi=0$;

(iii) *infinite trans* conditions, i.e., $[S_2]\to\infty$, $[A_2]\to\infty$ (*see* Turner (1981) for a more precise definition).

It can be shown (Turner, 1981) that, for each of these three experimental conditions, the unidirectional substrate flux from side 1 to side 2 of the membrane predicted by the model in Fig. 1 may be written in the following Michaelis Menten form

$$J_S^{1\to 2}=V_S^{1\to 2}[S_1]/(K_S^{1\to 2}+[S_1]) \tag{2}$$

where $V_S^{1\to 2}$, the maximum velocity of transport, and $K_S^{1\to 2}$, the apparent Michaelis constant, can be written as functions of $[A_1]$ as follows:

$$V_S^{1\to 2}=(V_{S_1}^{o}K_{A_1}^{\infty}+V_{S_1}^{\infty}[A_1])/(K_{A_1}^{\infty}+[A_1]) \tag{3}$$

$$\begin{aligned}K_S^{1\to 2}&=K_{S_1}^{\infty}(K_{A_1}^{o}+[A_1])/(K_{A_1}^{\infty}+[A_1])\\&=K_{S_1}^{o}K_{A_1}^{\infty}(K_{A_1}^{o}+[A_1])/K_{A_1}^{o}(K_{A_1}^{\infty}+[A_1]).\end{aligned} \tag{4}$$

The kinetic constants in the above expressions have the following significance:

V_S^o = maximum flux of S from side 1 to side 2 of the membrane when $[A_1]=0$

$V_{S_1}^{\infty}$ = as above when $[A_1]\to\infty$.

$K_{S_1}^{o}$ = apparent Michaelis constant for the flux of S from side 1 to side 2 of the membrane when $[A_1]=0$

$K_{S_1}^{\infty}$ = as above when $[A_1]\to\infty$.

$K_{A_1}^{o}$ = apparent Michaelis constant for the flux of A from side 1 to side 2 of the membrane when $[S_1]=0$

$K_{A_1}^{\infty}$ = as above when $[S_1]\to\infty$.

The above kinetic constants can be simply expressed in terms of the rate constants and dissociation constants shown in Fig. 1 (Turner, 1981). These expressions differ for zero trans, equilibrium exchange, and infinite trans conditions. Note also that $K_S^{1\to 2}$ defined in Eqs. (2) and (4) is an *apparent* binding constant and in general is different from both K_{S_1} and K_{AS_1}.

There are several points I would like to make about the solution of the rapid equilibrium carrier model of cotransport represented by Eqs. (2)–(4). The first is that, although the dependence of flux on activator concentration is not simple, it is not so complex as to be unrealistic to test experimentally. Experimental strategies for testing this model and several related models are discussed in Turner (1981). Secondly, it is worth emphasizing the advantages of writing the flux equations in terms of constants with well-defined kinetic interpretations such as V_S^o . K_S^{∞}, etc., defined above. These constants not only provide a meaningful way to group rate and dissociation constants in the flux equations but also make immediately obvious the number of parameters of the model which can be determined from a steady-state kinetic analysis. A related approach has been used to derive rejection criteria for this model (Turner, 1982). Finally, note that the carrier model of cotransport incorporates both energetic and catalytic activation, the former by virtue of the assumption of coupled transport and the latter because the binding constants for the substrate to the carrier (K_{S_n} and K_{AS_n}) and the translocation rate constants for the carrier with bound substrate (f_{nm} and h_{nm}) may differ according to whether or not the activator is also bound.

Some simplification of Eqs. (2)–(4) can be obtained by assuming that the binding sequence of the substrate and activator is ordered rather than random as shown in Fig. 1. Four such models which differ according to the order and symmetry of the binding events at the two membrane faces are discussed at length in Turner (1981). A number of authors have made the assumptions of symmetry of translocation rate constants ($h_{12}=h_{21}$,

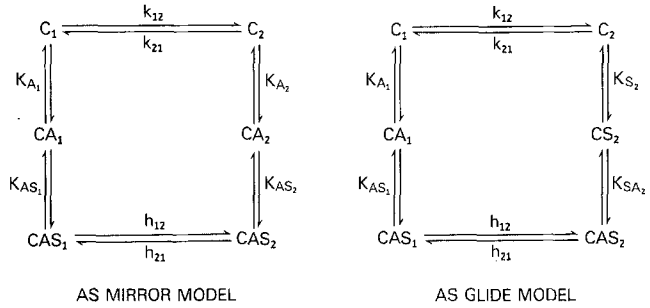

Fig. 3. Two ordered models of cotransport. The figures illustrate "mirror symmetry" where the order of binding of A and S is the same on each side of the membrane and "glide symmetry" where the order of binding of A and S is reversed on the two sides of the membrane. (Reproduced in part from Turner, 1981)

$k_{12}=k_{21}$, etc.), symmetry of binding events at the two membrane faces ($K_{A_1}=K_{A_2}$, $K_{AS_1}=K_{AS_2}$, etc.), or lack of mobility of the partially loaded forms of the carrier ($f_{nm}=g_{nm}=0$). However, these additional assumptions seldom lead to any significant simplifications of the form of Eqs. (2)–(4) (Turner, 1981).

Heinz, Geck and Wilbrandt (1972) and Geck and Heinz (1976) have considered two special cases of the model shown in Fig. 1. These are the so-called "velocity-type" and "affinity-type" models. In velocity-type models it is assumed that the activator increases the mobility of the carrier without changing its affinity for the substrate (i.e., $h_{nm}>k_{nm}$ but $K_{AS_n}=K_{S_n}$), while in affinity-type models the activator increases affinity rather than mobility (i.e., $k_{nm}=g_{nm}=f_{nm}=h_{nm}$ but $K_{AS_n}<K_{S_n}$). Unfortunately, the significance of the terms *velocity-type model* and *affinity-type model* have been misinterpreted in some cases. It is often assumed that in velocity-type models $K_S^{1\to 2}$ is independent of $[A_1]$ or, in other words, that the activator affects only the maximum velocity of transport. However, this is not the case. In fact no simplification of the form of Eqs. (2)–(4) is obtained by making the velocity-type model assumptions. Also, although $V_S^{1\to 2}$ is independent of $[A_1]$ for the affinity-type model assumptions as stated above, this is only true provided $f_{nm}=h_{nm}$. If $k_{nm}=h_{nm}\neq f_{nm}$ (e.g., if $f_{nm}\ll h_{nm}$ as is suspected by many investigators) no simplification of the form of Eqs. (2)–(4) is obtained from the affinity-type model assumptions.

Non-Rapid Equilibrium Models of Cotransport

Without the rapid equilibrium assumption discussed above, the labor involved in solving transport models and the complexity of the resulting flux equations increases substantially. Relatively little work has been published on nonrapid equilibrium (steady state) solutions of cotransport models (Stein, 1976*a*, *b*; Hill, 1977; Hopfer & Liedtke, 1981). However, this is not primarily due to mathematical difficulties. Systematic methods for solving problems of this type have been available for some time and a number of techniques requiring little mathematical sophistication have been developed by enzyme kineticists. The real problem here is what to do with the solution once one has it. The general steady-state solution of the model shown in Fig. 1 will include linear, quadratic, and mixed terms in $[S_n]$ and $[A_n]$ in both the numerator and denominator. Although some simplification may be possible by further mathematical manipulations, the difficulties involved in dealing with such a complex expression are obvious. Theoretical and experimental advances will be required to devise methods and/or reasonable approximations to reduce such flux equations to testable and interpretable forms.

Hopfer and his collaborators (Hopfer & Groseclose, 1980; Hopfer & Liedtke, 1981) have devised an experimental test which can indicate whether the rapid equilibrium assumption is invalid for a given cotransport system. This test is based on the observation that in nonrapid equilibrium kinetic schemes where an ordered binding sequence of substrate and activator is required the equilibrium exchange flux of at least one of the cotransported species is expected to exhibit a biphasic dependence on the concentration of the other. Consider, for example, the model shown on the left in Fig. 3 where A necessarily binds before S on both sides of the membrane (*AS Mirror Model*). It can be shown that the equilibrium exchange flux of S has a simple hyperbolic dependence on A, whereas the equilibrium exchange flux of A first increases with increasing S then subsequently decreases, approaching zero as $[S]\to\infty$. The explanation for this latter phenomenon is the following. The flux of A initially increases with $[S]$ because S is required for transport; however, as $[S]$ increases further the dominant form of the carrier becomes CAS_n, from which A cannot dissociate. Accordingly, at large $[S]$ the equilibrium exchange flux of A is inhibited. In the model shown on the right in Fig. 3 where the order of binding of A and S is reversed at the two membrane faces (*AS Glide Model*) the equilibrium exchange fluxes of *both A and S* are expected to show a biphasic dependence on the concentration of the other cotransported species. The introduction of random binding schemes (Fig. 1) and/or mobile partially

loaded carriers (f_{nm}, $g_{nm} \neq 0$) can modify or eliminate the biphasic behavior predicted for the models shown in Fig. 3 (Hopfer & Liedtke, 1981).

The above biphasic dependence of the equilibrium exchange flux of one cotransported species on the concentration of the other does not occur for rapid equilibrium models since here the association/disassociation events at the two membrane surfaces are assumed to be at equilibrium at all times. Thus, provided other modes of substrate inhibition can be ruled out, the observation of such a biphasic phenomenon indicates that the translocation of the fully loaded carrier is not the rate limiting step in the transport process.

Determining Quantitative Properties of Cotransport Systems Using Vesicles

A number of methods have been employed to measure uptake of solutes into vesicles. These include monitoring solute fluxes using flow dialysis (Ramos & Kaback, 1977*a*; Kinsella & Aronson, 1980), monitoring transport-related changes in membrane potential or proton concentration using various dyes (Beck & Sacktor, 1978; Lee & Forte, 1978; Rabon, Chang & Sachs, 1978; Waggoner, 1979; Reenstra, Warnock, Yee & Forte, 1981; Wright, Krasne, Kippen & Wright, 1981; Burnham, Munzesheimer, Rabon & Sachs, 1982), monitoring membrane permeabilities using light scattering (Busse & Steinmaier, 1974; Gier et al., 1978; Sachs, Jackson & Rabon, 1980) and recording transport-related changes in the composition of the extravesicular medium using ion sensitive electrodes (Murer, Hopfer & Kinne, 1976; Liedtke & Hopfer, 1977; Rabon et al., 1978). However, the most commonly applied and generally applicable method for studying coupled transport systems is the "rapid filtration" technique. For this reason I will mainly concentrate on data obtained using this method in what follows.

The basic procedure for the rapid filtration technique is the following. Vesicles are combined with an incubation medium containing radioactively labeled ligands and other constituents as required. After an appropriate time a stop solution is added and the vesicles are collected on a filter which is subsequently washed and counted for radioactivity. Efflux studies can be carried out in the same way using vesicles preloaded with labeled substrate.

The Stop Solution

The quantitative interpretation of data obtained using the rapid filtration technique depends critically on the ability of the stop solution to prevent any further uptake or efflux of labeled substrate from the vesicles. Figure 4 illustrates some of the problems which can arise from a poorly chosen stop solution. In this experiment renal outer medullary brush border membrane vesicles were preequilibrated with a solution containing 10 mM NaCl and 0.1 mM labeled D- and L-glucose. These are typical intravesicular sodium and glucose concentrations which arise during the study of the sodium-coupled D-glucose transport system found in this membrane. Aliquots of these vesicles were diluted 10-fold into various cold stop solutions then filtered immediately or after 10 or 20 sec delays. The stop solutions tested were chosen specifically to demonstrate certain problems; however, similar stop solutions have been used in studies of various sodium-coupled transport systems reported in the literature.

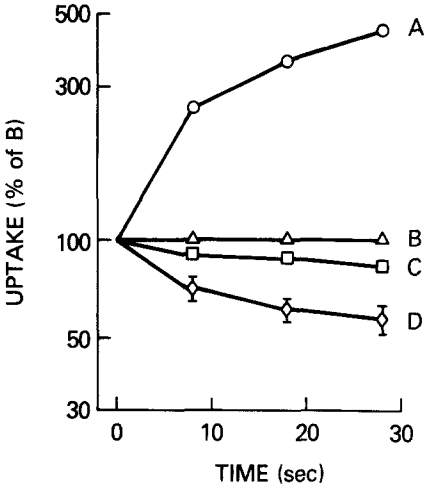

Fig. 4. Tests of the effectiveness of various stop solutions for renal brush border membrane D-glucose transport. Rabbit renal outer medullary brush border membrane vesicles were prepared in 10 mM Tris-HEPES (pH 7.4) plus 100 mM mannitol, 10 mM NaCl, and 0.1 mM ^{14}C-D- and ^{3}H-L-glucose. At time zero 100 μl of this vesicle suspension were added to 900 μl of cold stop solution then filtered immediately or after 10 or 20 sec delays. The filters (Millipore HAWP) were subsequently washed with a further 4.5 ml of stop solution. (Samples that are filtered immediately are in contact with the stop solution for approximately 8 sec) The stop solutions were 10 mM Tris-HEPES plus 300 mM NaCl (*A*), 300 mM NaCl and 1 mM phlorizin (*B*), 300 mM NaCl and 1 mM D-glucose (*C*), or 600 mM mannitol (*D*). The measured uptake has been normalized to the average D-glucose uptake found for stop solution *B*

Solution B has the characteristics of an effective stop solution. In this case there is no significant loss of intravesicular D-glucose when the time between dilution and filtration of the vesicles is prolonged. Also, the measured uptake of D-glucose is equal to that of L-glucose (not shown). These results indicate that there is no significant change

in intravesicular D-glucose content during the stopping and washing procedure.

Stop solution B contains a relatively large concentration of phlorizin (1 mM), a specific inhibitor of sodium-coupled D-glucose transport, in addition to 300 mM NaCl added to enhance phlorizin binding to the carrier (Turner & Silverman, 1981). If phlorizin is omitted from the stop solution (curve *A* in Fig. 4) D-glucose is actually transported into the vesicles from the extravesicular medium after dilution. This is due to the large extravesicular to intravesicular sodium gradient and occurs in spite of the fact that the stop solution is at 4 °C. If one attempts to solve this problem either by removing sodium (curve *D*) or by adding unlabeled D-glucose (curve *C*) to the stop solution, intravesicular label is now lost during the stopping and washing procedure. In the former case this is presumably due to the intravesicular to extravesicular sodium and glucose gradients and in the latter case to accelerated exchange diffusion (Kotyk & Janacek, 1975).

It is clear from Fig. 4 that a poor stop solution introduces a potentially serious uncontrolled variable into an uptake experiment. With a stop solution that does not prevent transport, the magnitude of the deviation of the measured uptake from the true uptake value will depend on the composition of the stop solution, on the intravesicular substrate and activator concentrations at the time of the measurement, on the composition of the incubation medium, and also on the degree of dilution produced by the stop solution.

Zero Trans Experiments

Zero trans conditions ($[S_2]=[A_2]=0$ or in some cases simply $[S_2]=0$) are the most popular and conceptually simplest experimental arrangement for quantitative studies of cotransport systems. Owing to the existence of activator and substrate gradients, substrate fluxes are typically large and hence easily determined. However, particular care must be taken in order to obtain reliable zero trans flux measurements for quantitative studies. This is because the magnitude and/or rate of dissipation of the various gradients present under these conditions may vary with experimental circumstances (Mitchell, 1968; Hopfer, 1978, 1981; Turner & Moran, 1982*a*). Consider, for example, measurements of the sodium dependence of zero trans fluxes of substrates cotransported rheogenically with sodium in renal brush border membrane vesicles. Since there are a number of pathways for sodium in this membrane (including the transporter of interest) each with its own K_m and V_{max}, the rate at which the extravesicular to intravesicular sodium gradient is dissipated will depend on the sodium concentration. One of these pathways is a Na^+/H^+ antiporter, hence the pH gradient across the membrane and its rate of dissipation may also be a function of sodium concentration. Likewise, the magnitude and rate of dissipation of transmembrane electrical potential will depend on the flux of sodium via its various rheogenic pathways (including the transporter of interest). Finally, the behavior of all these gradients will be a function of vesicle size and the concentration of these various transporters in the vesicular membrane. Thus in vesicle populations which are nonhomogeneous with respect to size and/or membrane composition different vesicles may behave quite differently. Since substrate flux via a rheogenic sodium-coupled transporter could be a function of all these experimental parameters, appropriate care must be taken in zero trans experiments to eliminate any such artifacts. Initial rates must be measured, solutions must be adequately buffered, and membrane potentials must be controlled.

By measuring initial rates, problems associated with dissipation of transmembrane chemical gradients can be avoided. What is required here is that there exists an early time period during which these gradients have not changed sufficiently from their initial values to significantly affect substrate uptake. A plot of uptake *vs.* time will be linear during this time period and the slope of this line will give the initial uptake rate. Except in a few special cases, other methods of estimating zero trans flux parameters (e.g., integrated rate equations) are impractical for cotransport systems owing to lack of knowledge of the functional form of the flux equations as well as to complex volume changes usually associated with zero trans conditions over longer time periods.

Membrane potentials are typically controlled using a valinomycin/K^+ voltage clamp. The principle of this procedure can be understood from the following analysis. Consider a vesicle (or cell) in the presence of various ion gradients. Assuming that the Goldman-Hodgkin-Katz equations (Kotyk & Janacek, 1975) are valid for this system the transmembrane potential $\Delta\psi$, is given by

$$\Delta\psi=(RT/F)\ln\frac{P_K[K_1]+\Sigma P_a[a_2]+\Sigma P_c[c_1]}{P_K[K_2]+\Sigma P_a[a_1]+\Sigma P_c[c_2]}.$$

Here P_K, P_a and P_c are the conductive permeabilities for potassium and the various anions (a) and

cations (c) present. Inspection of this equation reveals that if the terms $P_K[K_1]$ and $P_K[K_2]$ can be made sufficiently large they will dominate the numerator and denominator of the argument of the natural logarithm and $\Delta\psi$ will be simply given by the potassium diffusion potential, i.e.,

$$\Delta\psi = (RT/F)\ln([K_1]/[K_2]).$$

$\Delta\psi$ is typically clamped at the potassium diffusion potential by increasing the potassium permeability of the membrane by adding the ionophore valinomycin. The effectiveness of this procedure may be enhanced by ensuring that the other ionic species present have low permeabilities when it is desirable to clamp $\Delta\psi$ at some finite value or by including a permeant anion with K if it is desired to clamp $\Delta\psi$ at zero (Turner & Moran, 1982*a*).

Equilibrium Exchange Experiments

Equilibrium exchange experiments are technically more difficult to do and to some degree conceptually more difficult to appreciate than zero trans experiments. Equilibrium exchange experiments are typically carried out with substrate and activator at chemical and electrical equilibrium across the vesicle membrane. The unidirectional flux of labeled substrate may be measured from the extravesicular to intravesicular space or vice versa. Since there are no gradients present in this type of experiment the problems discussed above for measuring zero trans fluxes are not relevant. However, fluxes are frequently smaller under equilibrium exchange conditions than under zero trans conditions owing to the absence of driving forces. Thus the direct measurement of initial rates of tracer uptake are more difficult. This problem is less serious in homogeneous vesicle populations since here the form of the flux equation is known and the following integrated rate approach can be used.

The equilibrium exchange uptake of labeled tracer into a homogeneous vesicle population is given by the equation

$$u(t) = u(\infty)[1 - \exp(-\sigma J_S t/V[S])] \qquad (5)$$

where $u(t)$ is the uptake of tracer at time t, $u(\infty)$ is the uptake of tracer at equilibrium ($t \to \infty$), σ is the surface area of a vesicle, J_S is the flux of S per unit surface area at concentration $[S]$, and V is the volume of a vesicle. The derivation of this equation requires no assumptions regarding the form of J_S. Rewriting Eq. (5) as

$$1 - u(t)/u(\infty) = \exp(-\sigma J_S t/V[S])$$

one can see directly that the rate constant for tracer uptake (i.e., the slope of a $\ln[1-u(t)/u(\infty)]$ *vs.* t plot) is directly proportional to $J_S/[S]$ and hence contains the equilibrium exchange flux information. Equivalently, the half time[1] for tracer equilibrium at substrate concentration $[S]$, τ_S, may be taken as a measure of the equilibrium exchange flux since it is proportional to $[S]/J_S$.

This latter result also holds for vesicles that are identical in all respects except size (Hopfer, 1981). In this case Eq. (5) becomes

$$1 - u(t)/u(\infty) = [1/u(\infty)]\Sigma u_i(\infty)\exp(-\sigma_i J_S t/V_i[S])$$

where the sum is over all vesicles. Setting $t = \tau_S$ and rewriting the above equation we have

$$\Sigma u_i(\infty)\exp(-\sigma_i J_S \tau_S/V_i[S]) = u(\infty)/2.$$

The right-hand side of this equation is obviously independent of $[S]$. Thus the left-hand side must also be independent of $[S]$. This can only be true if τ_S is proportional to $[S]/J_S$ as found above for the case of homogeneous vesicles[1]. Note, however, that this relation between τ_S and J_S cannot be derived if J_S is not identical for all vesicles. Thus if the vesicles are heterogeneous with respect to substrate transport properties, i.e., if J_S, the substrate flux per unit membrane area, is a function of i, the above argument breaks down. In this case initial rates must be measured directly.

Hopfer (1981) has suggested an empirical test for determining if one is working with a vesicle preparation that is heterogeneous with respect to size but homogeneous with respect to membrane type. This is based on the observation that if τ_S is proportional to $[S]/J_S$, as expected for such a preparation, a plot of $\ln[1-u(t)/u(\infty)]$ *vs.* t/τ_S should be independent of $[S]$. Hence such plots obtained for different values of $[S]$ should be superimposable.

Infinite Trans and Other Experimental Conditions

Most kinetic studies of cotransport fluxes have been carried out under zero trans or equilibrium exchange conditions. Infinite trans and other less common experimental arrangements provide further opportunities to test various kinetic models and to characterize the transport process (*see* be-

[1] The half time is taken as a matter of convenience. In fact, the time for any fixed fractional uptake must be proportional to $[S]/J_S$.

low). Since these experiments typically involve substrate and/or activator gradients and net flux measurements, similar precautions to those described for zero trans experiments must be used to obtain reliable flux data.

Some Current Experimental Applications

In this Section various methods for determining the stoichiometry and order of binding of cotransport systems are discussed. Studies of this type provide valuable information concerning the mechanism of the coupled transport process and are the focus of much of the current research in this area. Such quantitative studies are good examples of measurements requiring the procedures and precautions described above.

Stoichiometric Studies of Cotransport Systems

The activator/substrate stoichiometry, n, of a cotransport system is of particular interest since it figures heavily into the understanding of both the mechanism of the cotransport event and the concentrating capacity of the transporter. Equilibrium thermodynamics predicts the following relationship between a given activator electrochemical gradient and the substrate gradient which can be produced by a cotransport system.

$$\ln([S_1]/[S_2]) \leqq n(\ln([A_2]/[A_1]) + F\Delta\psi/RT). \quad (6)$$

The inequality arises from the possible existence of two types of dissipative processes – internal and external leaks. External leaks are unrelated to the transporter of interest and represent other pathways in the membrane through which the substrate gradient may be dissipated (including simple diffusion). Internal leaks arise when flux via the carrier itself is not tightly coupled, i.e., when f_{nm}, $g_{nm} \neq 0$ (*cf.* Fig. 1). In the absence of internal and external leaks the inequality in Eq. (6) becomes an equality. Three independent methods have been employed to measure the activator/substrate stoichiometry; we refer to these as the activation method, the direct method, and the static head method (Turner & Moran, 1982*b*, *c*). Each of these is discussed individually below.

The Activation Method

The activation method of determining the activator/substrate stoichiometry consists of measuring substrate flux as a function of activator concentration (the complimentary experiment of measuring activator flux as a function of substrate concentration is less common but equally valid). Zero trans or equilibrium exchange experimental conditions are typically employed. It can be shown that a hyperbolic relationship between activator-dependent substrate flux and activator concentration is expected for rapid equilibrium carrier-type models with a 1:1 coupling ratio (Turner, 1981). In fact, it is likely that this result holds for a broad class of models so that a hyperbolic dependence of flux on activator concentration is a good indication of a 1:1 stoichiometry. The experiment shown in Fig. 5 demonstrates that the outer cortical brush border membrane D-glucose transporter exhibits this type of behavior.

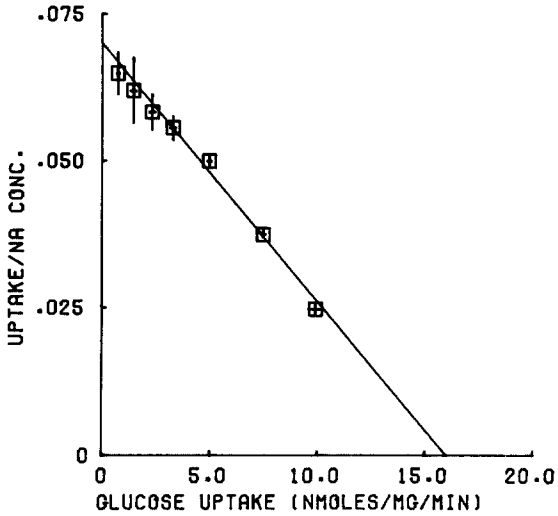

Fig. 5. The results of an activation method experiment for the renal outer cortical brush border membrane D-glucose transporter. The initial rate of sodium-dependent D-glucose uptake was measured as a function of sodium concentration over the range 0–400 mM with choline replacing sodium isosmotically. Effects of membrane potentials were controlled by including 100 mM KSCN in all solutions and adding valinomycin at a concentration of 12.5 μg/mg membrane protein. The linearity of this plot indicates that glucose flux is a hyperbolic function of sodium concentration as expected for a 1:1 coupled system. (Reproduced from Turner & Moran, 1982*b*)

A sigmoidal dependence of substrate flux on activator concentration as shown in Fig. 6*a* for the outer medullary brush border membrane D-glucose transporter is indicative of the involvement of multiple activator ions per substrate translocation event (i.e., $n>1$). In order to determine the activator/substrate stoichiometry we must fit the data shown in Fig. 6*a* to the appropriate flux equation; i.e., we must know how substrate flux is expected to depend on activator concentration when $n>1$. The derivation of this equation requires information about the details of the transport mechanism which are not yet available. It may be shown, how-

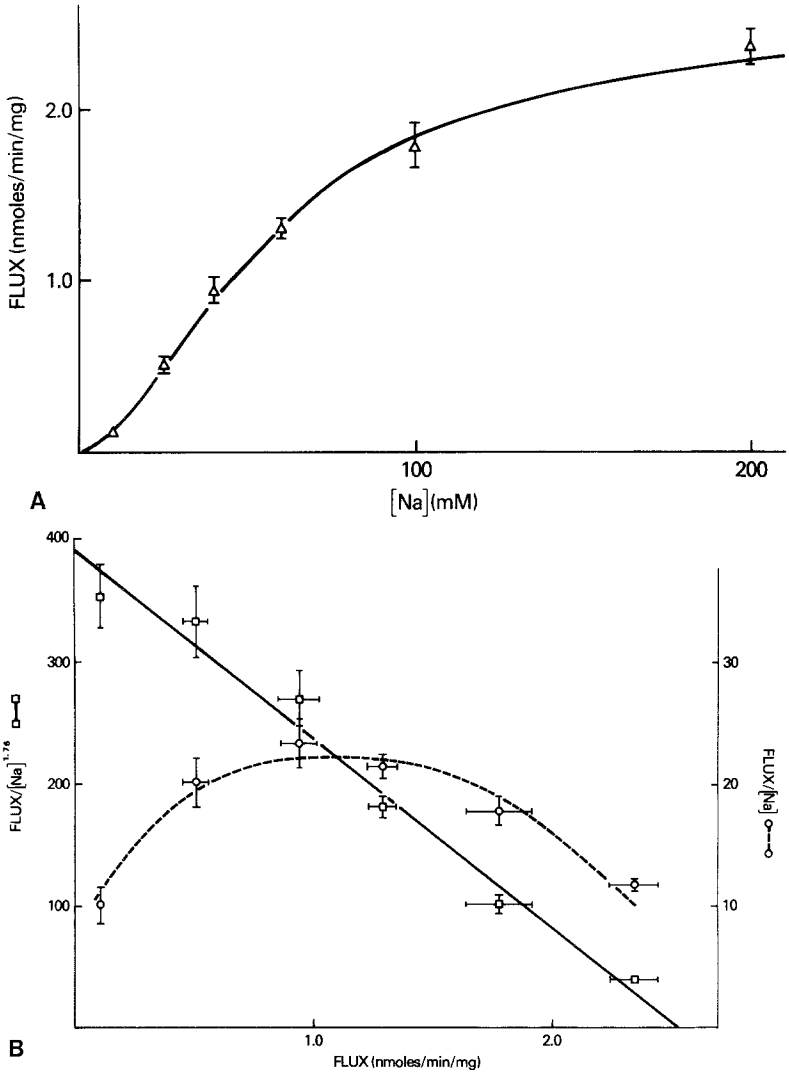

Fig. 6. The results of an activation method experiment for the renal outer medullary brush border membrane D-glucose transporter. Initial D-glucose uptake was measured as a function of sodium concentration over the range 0–200 mM with choline replacing sodium isosmotically. Effects of membrane potentials were controlled by including 100 mM KSCN in all solutions and adding valinomycin at a concentration of 12.5 μg/mg membrane protein. (*a*): A plot of flux *vs.* sodium concentration. (*b*): Plots of flux/[Na] *vs.* flux (○ and dashed line) and flux/$[Na]^{1.76}$ *vs.* flux (□ and solid line). The latter plot was found by fitting the data to Eq. (7) by the method of least squares. The linearity of this plot is indicative of the involvement of approximately 1.8 sodium ions per glucose transport event (*see* text). (Reproduced from Turner & Moran, 1982*c*)

ever, that one might expect the following Hill-type equation to hold as a first approximation (*cf.* Segal (1975) for the analogous case in enzyme kinetics)

$$\text{flux} = V_{\max}[A]^n/(K^n_{0.5}+[A]^n). \quad (7)$$

This equation assumes the existence of n essential cooperative activator binding sites per substrate site (Segal, 1975).

Data may be fit to the Hill equation by the method of least squares or by eye (by plotting flux/$[A]^n$ *vs.* flux for various values of n). A least squares fit to the data shown in Fig. 6*a* yields an n value of approximately 1.8 (Fig. 6*b*). Owing to present uncertainties regarding the functional form of the flux equation, the value of n obtained from the Hill analysis must be regarded as approximate. Since different kinetic models may predict different dependences of flux on $[A]$, particularly for $n>1$, the activation method could be used to distinguish between these models.

It should also be noted that the activation method does not distinguish between catalytic and energetic activation since there is no requirement that the activator is cotransported with the substrate. Thus the activation method allows one to determine the total number of activator ions (energetic plus catalytic) involved in the transport event. Methods for determining the number of activator ions actually cotransported are given below.

The Direct Method

The direct method for measuring the activator/substrate stoichiometry relies on the actual determination and comparison of the simultaneously measured activator-dependent substrate flux and substrate-dependent activator flux. This method has the advantage of demonstrating the direct (en-

ergetic) coupling of substrate and activator fluxes; it suffers, however, from the practical limitation that the substrate-dependent activator flux may be small relative to the background activator flux. Interpretation of this type of experiment can be complicated by more complex behavior of the transporter. For example, in a nontightly coupled system (internal leaks) it is possible for the carrier-activator complex (CA_n) to recycle repeatedly across the membrane so that one activator ion acts to catalyze the transport of several substrate molecules. External leaks do not affect the validity of this stoichiometric determination. A direct stoichiometry experiment for the outer cortical brush border membrane D-glucose transporter illustrating a 1:1 coupling ratio is shown in Fig. 7.

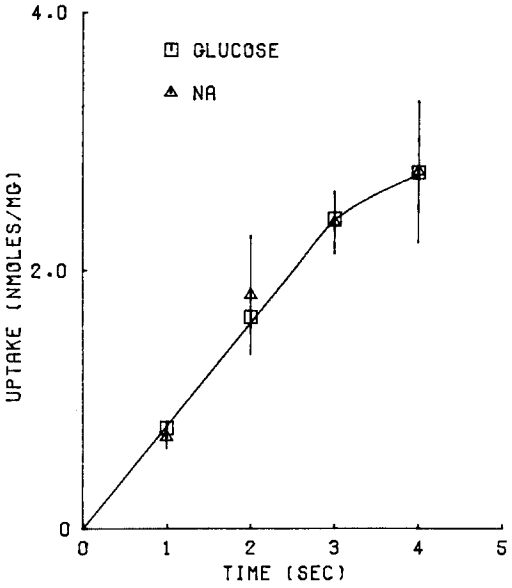

Fig. 7. Comparison of the sodium-dependent component of D-glucose flux and the glucose-dependent component of sodium flux in outer cortical brush border membrane vesicles. The 1:1 relationship between these fluxes is indicative of a 1:1 sodium/glucose coupling ratio. (Reproduced from Turner & Moran, 1982*b*)

The Static Head Method

The static head method of determining the activator/substrate stoichiometry was introduced as a means of circumventing some of the practical limitations of the direct method (Turner & Moran, 1982b, *c*). The static head method is based on the following argument. Consider a tightly coupled transport system where n activator ions are transported per substrate molecule. The thermodynamic condition that there is no net flux of either substance via the transporter, or equivalently that the thermodynamic driving forces for activator and substrate fluxes are balanced, is given by

$$\ln([S_1]/[S_2]) = n[\ln([A_2]/[A_1])]. \quad (8)$$

Here the effects of membrane potentials (*cf.* Eq. (6)) have been omitted since it is assumed that $\Delta\psi$ can be set to zero by an appropriate voltage clamping procedure. Equation (8) is a thermodynamic relation which involves only cotransported substrate and activator. The static head method of determining stoichiometric ratios essentially consists of determining substrate and activator gradients for which Eq. (8) holds (static head conditions); n can then be calculated directly.

An example of a static head experiment for the outer cortical brush border membrane D-glucose transporter is shown in Fig. 8. In this experiment vesicles were preequilibrated with given concentrations of sodium and labeled glucose then diluted 1:6 into appropriate glucose-free media, thus establishing an intravesicular-to-extravesicular glucose gradient of 6:1. The glucose retained in these vesicles was measured as a function of time and as a function of extravesicular sodium concentration. A control run was also carried out in the absence of sodium to measure efflux via unrelated sodium-independent pathways (external leaks). The static head condition is characterized by that external sodium concentration that causes the test points to superimpose on the control. The results shown in the figure illustrate again that the stoichiometry of the renal outer cortical brush border membrane D-glucose transporter is 1:1.

Like the direct method of measuring stoichiometry, the interpretation of results obtained from the static head method is complicated by the existence of internal leaks, which allow some uncoupling of driving forces and thus change the form of Eq. (8). External leaks do not affect the validity of the method since these are compensated for by including the control run (Fig. 7).

A technique similar in principle to the static head method has been employed to measure the activator/substrate stoichiometry in bacterial and other systems capable of maintaining an activator gradient over time (Ramos & Kaback, 1977*b*; Lanyi, 1978; Johnson, Carty & Scarpa, 1981; Knoth, Zallakian & Njus, 1981; van der Broek, Christianse & van Steveninck, 1982; Pastuszko, Wilson & Ericinska, 1982). In these experiments steady-state accumulation ratios of substrate in response to known activator gradients are measured and n is calculated as above from Eq. (8). This procedure is impractical for systems that cannot maintain an activator gradient since in this case

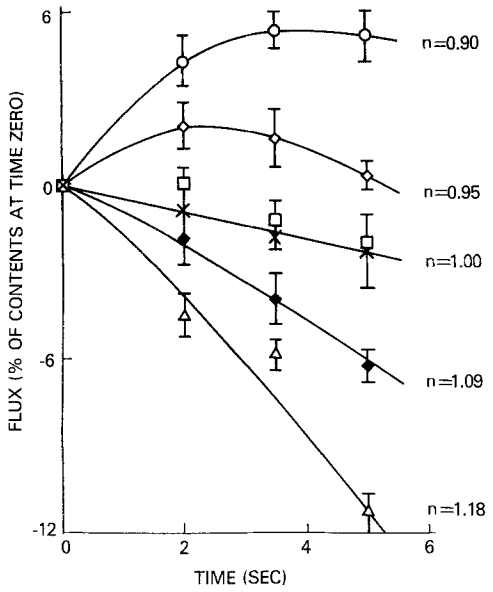

Fig. 8. The results of a static head experiment for the outer cortical brush border membrane D-glucose transporter. Vesicles were loaded with 20 mM NaCl, 180 mM choline chloride, and 0.5 mM labeled glucose then diluted 1:6 into isosmotic media containing various concentrations of sodium. The values of n indicated on the figure are the sodium/glucose stoichiometries, which would be predicted were that run to result in static head conditions. A control run (×) was carried out in the absence of sodium to measure efflux via unrelated sodium-independent pathways. The static head condition is characterized by that external sodium concentration which causes the test points to lie on the control. Membrane potentials were clamped at zero by including 100 mM KSCN in all solutions and adding the K^+ ionophore valinomycin at a concentration of 12.5 μg/mg membrane protein. (Redrawn from Turner and Moran, 1982*b*)

such a sustained steady state never occurs. Also this method assumes that the observed substrate gradient is in thermodynamic equilibrium with the activator gradient. This assumption has recently been questioned (Booth, Mitchell & Hamilton, 1979) since it neglects the existence of external leaks. If these leaks are significant the observed accumulation ratio of substrate will represent a kinetic steady state between the cotransporter and the leak rather than a thermodynamic equilibrium between activator and substrate gradients. In this case the calculated value of n will be a lower limit on the true activator/substrate stoichiometry. As stated above, the existence of external leaks is taken into account in the static head method.

Order of Binding of Substrate and Activator

Knowing the order of binding of the cotransported species is of fundamental importance to the determination of transport mechanism. There are several approaches to this problem. The most quantitative and most difficult is by detailed analysis of kinetic properties. Various ordered and random binding schemes may be distinguished by their predictions of the dependence of $K_S^{1\to 2}$ and $V_S^{1\to 2}$ on activator concentration (Turner, 1981). For example, with the rapid equilibrium assumption and appropriate experimental conditions (*see* above) the random model shown in Fig. 1 predicts that $V_S^{1\to 2}$ has the dependence on $[A_1]$ given in Eq. (3), whereas the *AS* Mirror Model of Fig. 3 predicts that $V_S^{1\to 2}$ is independent of $[A_1]$. In many cases comparison of results obtained under a variety of experimental conditions (zero trans, equilibrium exchange, infinite trans) can also be useful (Turner, 1981). Although this approach is the most definitive, it is also potentially somewhat model dependent. Several simpler and less model-dependent approaches are given below.

As already discussed above in a different context, Hopfer and his collaborators have shown that various ordered models can be distinguished on the basis of the dependence of the equilibrium exchange flux of one transported species on the concentration of the other (Hopfer & Groseclose, 1980; Hopfer & Liedtke, 1981). Thus, for example, the *AS* Mirror Model in Fig. 3 predicts a hyperbolic dependence of the equilibrium exchange flux of S on $[A]$ and a biphasic dependence of the equilibrium exchange flux of A on $[S]$, while the *AS* Glide Model predicts that the equilibrium exchange flux of both A and S will be biphasic functions of $[S]$ and $[A]$, respectively (*see* above). An important feature of these ordered models is that when such biphasic behavior is observed the associated equilibrium exchange flux must necessarily approach zero as the concentration of the cotransported species becomes large. A biphasic approach to a finite (non-zero) equilibrium exchange flux is indicative of a random binding scheme. A similar approach to that of Hopfer and collaborators which does not require the use of equilibrium exchange conditions has been suggested by Stein and collaborators (Stein, 1967*a*, *b*; Stein & Honig, 1977).

A somewhat more straightforward but less definitive approach, which has received some experimental application (Kanner & Bendahan, 1982), is the following. It is generally thought or suspected that the slowest step in the transport cycle is the translocation of the unloaded carrier, C_n, across the membrane. Thus, for example, in the *AS* Mirror Model of Fig. 3 one would expect that $J_S^{1\to 2}$ might be stimulated by the presence of trans substrate, i.e., that $J_S^{1\to 2}([S_2]\neq 0) > J_S^{1\to 2}([S_2]=0)$, since this would allow the fully loaded carrier sim-

ply to exchange (labeled) substrate from side 1 for (unlabeled) substrate from side 2 and recycle as CAS_n rather than dissociating both A and S and recycling a C_n. Accordingly, the observation of trans stimulation of $J_S^{1\to 2}$ by S_2 when $[A_2]=0$ argues against any model where S cannot dissociate from CAS_2 before A (continuous recycling of the transporter as CAS_n while exchanging labeled substrate from side 1 for unlabeled substrate from side 2 is not possible in such a model when $[A_2]=0$). Experiments of this type are most valuable when combined with other methods for determining the order of binding of ligands.

Turner and Silverman (1980, 1981) have shown that the binding properties of nontransported competitive inhibitors of cotransport systems may also be used to determine the order of binding of substrate (inhibitor) and activator.

Concluding Remarks

The large number of cotransport systems now available for experimental study offer a tremendous opportunity for testing various kinetic models. Although a number of significant theoretical papers have been published and at least some of these have not received the attention they merit, I feel that considerably more effort should be devoted to theoretical studies and attempts to put these results in a form in which they can be widely understood and used by experimenters. Among the areas which require attention are:

(i) a detailed analysis of nonrapid equilibrium models to determine further tests for the breakdown of the rapid equilibrium assumption, the significance of this breakdown for the interpretation of experimental data, and possible ranges of validity of the rapid equilibrium assumption in otherwise nonrapid equilibrium systems (recall that the rapid equilibrium assumption leads to considerable simplification of the derivation and final form of the flux equations).

(ii) a more general analysis of transport phenomena in order to allow one to determine whether a certain prediction is a characteristic of a given class of models or set of assumptions regarding transport mechanism. For example, Turner (1982) gives six rejection criteria for the carrier model of cotransport; it would be useful to know whether any of these rejection criteria are unique to carrier models.

(iii) detailed analytical (as opposed to numerical) analyses of transport models in order to provide a better understanding of their properties and predictions.

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References

Aronson, P.S. 1981. *Am. J. Physiol.* **240**:*F*1–*F*11
Babcock, A.K., Garvey, T.Q., Berman, M. 1979. *J. Membrane Biol.* **49**:157–169
Beck, J.C., Sacktor, B. 1978. *J. Biol. Chem.* **253**:7158–7162
Booth, I.R., Mitchell, W.J., Hamilton, W.A. 1979. *Biochem. J.* **182**:687–696
Borst-Pauwels, G.W.F.H. 1973. *J. Theor. Biol.* **40**:19–31
Borst-Pauwels, G.W.F.H. 1981. *Biochim. Biophys. Acta* **650**:88–127
Broek, P.J.A. van den, Christianse, K., Steveninck, J. van 1982. *Biochim. Biophys. Acta* **692**:231–237
Burnham, C., Munzesheimer, C., Rabon, E., Sachs, G. 1982. *Biochim. Biophys. Acta* **685**:260–272
Busse, D., Steinmaier, G. 1974. *Biochim. Biophys. Acta* **345**:359–372
Crane, R.K. 1977. *Rev. Physiol. Biochem. Pharmacol.* **78**:99–159
Deves, R., Krupka, R.M. 1978. *Biochim. Biophys. Acta* **513**:156–172
Eddy, A.A. 1978. *In:* Current Topics in Membrane Transport. F. Bronner and A. Kleinzeller, editors. Vol. 10, pp 279–360. Academic Press, London
Freel, R.W., Goldner, A.M. 1981. *Am. J. Physiol.* **241**:*G*451–*G*460
Geck, P., Heinz, E. 1976. *Biochim. Biophys. Acta* **443**:49–53
Geck, P., Heinz, E. 1981. *In:* Membrane Transport. S.L. Bonting and J.J.H.H.M. de Pont, editors. pp. 285–310. Elsevier/North Holland Biomedical, New York
Gier, J. de, Blok, M.C., Dijck, P.W.M. van, Mombers, C., Verkley, A.J., Neut-Kok, E.C.M. van der, Deenen, L.L.M. van. 1978. *Ann. N.Y. Acad. Sci.* **308**:85–100
Gunn, R.B. 1980. *Annu. Rev. Physiol.* **42**:249–259
Heinz, E. 1978. Mechanics and Energetics of Biological Transport. Springer-Verlag, Berlin
Heinz, E., Geck, P., Wilbrant, W. 1972. *Biochim. Biophys. Acta* **255**:442–461
Hill, T.L. 1977. Free Energy Transduction in Biology. Academic Press, New York
Hill, T.L., Eisenberg, E. 1981. *Q. Rev. Biophys.* **14**:463–511
Hopfer, U. 1977. *Am. J. Physiol.* **233**:*E*445–*E*449
Hopfer, U. 1978. *Am. J. Physiol.* **234**:*F*89–*F*96
Hopfer, U. 1981. *Fed. Proc.* **40**:2480–2485
Hopfer, U., Groseclose, R. 1980. *J. Biol. Chem.* **255**:4453–4462
Hopfer, U., Liedtke, C.M. 1981. *Membrane Biochem.* **4**:11–29
Johnson, R.G., Carty, S.E., Scarpa, A. 1981. *J. Biol. Chem.* **256**:5773–5780
Kaback, H.R. 1980. *Ann. N.Y. Acad. Sci.* **339**:53–60
Kanner, B.I. 1983. *Biochim. Biophys. Acta (in press)*
Kanner, B.I., Bendahan, A. 1982. *Biochemistry* **21**:6327–6330
Kinsella, J.L., Aronson, P.S. 1980. *Am. J. Physiol.* **238**:*F*461–*F*469
Knoth, J., Zallakian, M., Njus, D. 1981. *Biochemistry* **20**:6625–6629
Kotyk, A., Janacek, K. 1975. Cell Membrane Transport: Principles and Techniques. (2nd ed.). Plenum Press, New York
Lanyi, J.K. 1978. *Biochemistry* **17**:3011–3018
Lanyi, J.K. 1979. *Biochim. Biophys. Acta* **559**:377–397
Lee, H.C., Forte, J.G. 1978. *Biochim. Biophys. Acta* **508**:339–356
LeFevre, P.G. 1973. *J. Membrane Biol.* **11**:1–19
Lever, J.E. 1980. *CRC Crit. Rev. Biochem.* **7**:187–246

Lieb, W.R., Stein, W.D. 1970. *Biophys. J.* **10**:585–609
Lieb, W.R., Stein, W.D. 1974. *Biochim. Biophys. Acta* **373**:165–177
Liedtke, C.M., Hopfer, U. 1977. *Biochem. Biophys. Res. Commun.* **76**:579–585
Lombardi, F.J. 1981. *Biochim. Biophys. Acta* **649**:661–679
Mitchell, P. 1968. Chemiosmotic Coupling and Energy Transduction. Glynn Research Ltd. Bodmin, England
Mitchell, P. 1973. *In:* Membrane Structure and Mechanism of Biologial Energy Transduction. J. Avery, editor. pp. 265–293. Plenum Press, New York
Mitchell, P. 1979. *Eur. J. Biochem.* **95**:1–20
Murer, H., Hopfer, U., Kinne, R. 1976. *Biochem. J.* **154**:597–604
Murer, H., Kinne, R. 1980. *J. Membrane Biol.* **55**:81–95
Naftalin, R.J. 1970. *Biochim. Biophys. Acta* **211**:65–78
Njus, D., Knoth, J., Zallakian, M. 1981. *Curr. Top. Bioenerg.* **11**:107–147
Oxender, D., Fox, C.F. 1978. Molecular Aspects of Membrane Transport. Alan R. Liss, New York
Pastuszko, A., Wilson, D.F., Erecinska, M. 1982. *J. Biol. Chem.* **257**:7514–7519
Poole, R.J. 1978. *Annu. Rev. Physiol.* **29**:437–460
Rabon, E., Chang, H., Sachs, G. 1978. *Biochemistry* **17**:3345–3353
Ramos, S., Kaback, H.R. 1977*a*. *Biochemistry* **16**:848–854
Ramos, S., Kaback, H.R. 1977*b*. *Biochemistry* **16**:4271–4275
Reenstra, W.W., Warnock, D.G., Yee, V.J., Forte, J. 1981. *J. Biol. Chem.* **256**:11663–11666
Rosen, B.P., Kashket, E.R. 1978. *In:* Bacterial Transport. B.P. Rosen, editor. pp. 559–620. Marcel Dekker, Basel
Sachs, G., Jackson, R.J., Rabon, E.C. 1980. *Am. J. Physiol.* **238**:*G*151–*G*164
Sacktor, B. 1977. *Curr. Top. Bioenerg.* **6**:39–81
Schultz, S.G. 1977. *Am. J. Physiol.* **233**:*E*249–*E*254
Segal, I.H. 1975. Enzyme Kinetics. John Wiley and Sons, New York
Silhavy, T.J., Ferenci, T., Boos, W. 1978. *In:* Bacterial Transport. B.P. Rosen, editor. pp. 127–169. Marcel Dekker, Basel
Silverman, M., Turner, R.J. 1979. *In:* Biomembranes, L.A. Manson, editor. Vol. 10, pp. 1–76, Plenum Press. New York
Stein, W.D. 1976*a*. *J. Theor. Biol.* **62**:467–478
Stein, W.D. 1976*b*. *In:* Intestinal Permeation. M. Kramer and F. Lauterbach, editors. pp. 262–274. Excerpta Medica, Amsterdam
Stein, W.D., Honig, B. 1977. *Mol. Cell. Biochem.* **15**:27–44
Turner, R.J. 1981. *Biochim. Biophys. Acta* **649**:269–280
Turner, R.J. 1982. *Biochim. Biophys. Acta* **689**:444–450
Turner, R.J., Moran, A. 1982*a*. *Am. J. Physiol.* **242**:*F*406–*F*414
Turner, R.J., Moran, A. 1982*b*. *J. Membrane Biol.* **67**:73–80
Turner, R.J., Moran, A. 1982*c*. *J. Membrane Biol.* **70**:37–45
Turner, R.J., Silverman, M. 1980. *Biochim. Biophys. Acta* **596**:272–291
Turner, R.J., Silverman, M. 1981. *J. Membrane Biol.* **58**:43–55
Ullrich, K.J. 1979. *Annu. Rev. Physiol.* **41**:181–195
Waggoner, A.S. 1979. *Annu. Rev. Biophys. Bioeng.* **8**:47–68
Weiss, S.D., McNamara, P.D., Segal, S. 1981. *J. Theor. Biol.* **93**:597–608
West, I.C. 1980. *Biochem. Biophys. Acta* **604**:91–126
Wright, S.H., Krasne, S., Kippen, I., Wright, E.M. 1981. *Biochem. Biophys. Acta* **640**:767–778

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