Biochem. J. (1989) 260, 885–891 (Printed in Great Britain)

Testing transport models and transport data by means of kinetic rejection criteria

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In the case of a transport system obeying Michaelis–Menten kinetics, completely general relationships are shown to exist between the final ratio of internal and external substrate concentrations, α, and the V/K_m ratios found in zero-trans-entry, zero-trans-exit and equilibrium-exchange experiments (where V is a maximum substrate flux and K_m a substrate half-saturation constant). The proof depends on a new method of derivation proceeding from the form of the experimental data rather than, as has been the practice in kinetic analysis, from a hypothetical reaction scheme. These general relationships, which will be true of all mechanisms giving rise to a particular type of behaviour (here Michaelis–Menten kinetics), provide a test for internal consistency in a set of experimental data. Other relationships, which are specific, can be derived from individual reaction schemes, with the use of traditional procedures in kinetic analysis. The specific relationships include constants for infinite trans entry and exit in addition to constants involved in the general relationships. In conjunction, the general and specific relationships provide a stringent test of mechanism. A set of results that fails to satisfy the general relationships must be rejected; here systematic error or unexpected changes in the transport system in different experiments may have distorted the calculated constants, or the system may not actually obey Michaelis–Menten kinetics. Results in accord with the general relationships, on the other hand, can be applied in specific tests of mechanism. The usefulness of the theorem is illustrated in the cases of the glucose-transport and choline-transport systems of erythrocytes. Experimental results taken from several studies in the literature, which were in accord with hyperbolic substrate kinetics, had previously been shown to disagree with relationships derived for the carrier model, and the model was rejected. The new analysis shows that the data violated the general relationships and therefore cannot decide the issue. More recent results on the glucose-transport system satisfy the general relations and agree with the carrier model.

INTRODUCTION

The mechanism of even the simplest membrane transport systems is still uncertain, despite a multitude of experimental studies that have been reported in the literature. One established fact is that integral membrane proteins, in some cases of defined amino acid sequence, are involved. The proteins are thought to create channels through the membrane and to contain one or more substrate-binding sites, which confer the high specificity often seen. As permanently open channels would not explain the kinetics of transport, it is necessary to postulate that the channels open and close intermittently, presumably as a result of conformational changes in the membrane-spanning protein. The substrate-binding sites could be arranged in various ways. One possibility is a row of substrate-binding regions stretching from one side of the membrane to the other, with sites exposed simultaneously on both surfaces. Another possibility is a single substrate-binding site that, as a result of conformational changes in the protein, is alternately exposed on opposite sides; this is the ordinary carrier model. All that we can deduce with some certainty is that transport proteins contain channels of limited length and substrate-binding sites; how many sites, how they are interconnected and how the sites and channels function remain to be decided. These questions are being addressed through studies of the structure of the proteins and of the kinetics of transport.

Aside from its applications in characterizing substrates and inhibitors, the method of kinetics can be very useful in determining the mechanism of a reaction. Kinetic studies can establish the characteristic properties of a reaction, and can show that these properties are in accord with a hypothetical mechanism. In addition, they can unequivocally refute a proposed mechanism, for all mechanisms predicting kinetic behaviour at variance with that observed must be abandoned. This last-mentioned approach to transport studies was pioneered by Lieb and co-workers (Lieb & Stein, 1971, 1972, 1974*a*,*b*; Hankin *et al.*, 1972). Building on earlier work on the kinetics of carrier systems (Patlak, 1957; Britton, 1964; Regen & Morgan, 1964; Miller, 1971; Geck, 1971; Hoare, 1972), these authors pointed out that for a particular transport mechanism definite relationships should hold among the experimentally measured constants, independent of the rates of individual reactions in in the transport process. Such relations were proposed as specific rejection criteria for the simple pore and carrier models.

The analysis was applied to four facilitated transport systems in human erythrocytes, namely those for leucine, uridine, glucose and choline (Lieb, 1982). The criteria for the ordinary carrier model included the requirement that the V/K_m ratios for entry, exit and equilibrium exchange should be identical (V being a maximum flux and K_m a substrate half-saturation constant). The results on leucine and uridine agreed with the prediction. Those on glucose and choline did not, and the carrier model was rejected outright.

An unexamined assumption in the analysis was that some more complex model would be consistent with the differing V/K_m ratios reported in the glucose-carrier and choline-carrier systems. If in fact V/K_m ratios for a reversible reaction, such as a transport system, are subject to completely general constraints, independent of the mechanism, then discordant results would have to be incorrect. As I show below, definite constraints on the relative values of some of the experimental constants emerge from the pattern inherent in the experimental observations. This pattern reflects a simple mathematical relationship between dependent and independent variables, referred to as Michaelis–Menten kinetics. Not all the constants are constrained in this way, of course, and those that are not may be used to characterize a particular mechanism. As will become apparent, extending the theorem in this way makes the test introduced by Lieb and co-workers more, not less, useful.

The customary method of kinetic analysis starts out with a definite reaction scheme. On the basis of this scheme rate equations are derived, as well as relationships among the parameters of the equations. Haldane relationships for both enzyme catalysis (Haldane, 1930) and transport (Segel, 1975; Heinz, 1978), as well as the rejection criteria for the simple pore and carrier models, were derived in this way. This method cannot be used now, because the results would apply specifically to some particular reaction scheme.

To discover general relationships, a departure from the usual methods of kinetic analysis is required. The procedure must in a sense reverse the traditional one. Instead of beginning with a hypothetical reaction scheme, I begin with the observed pattern of experimental behaviour. From it I deduce certain necessary relationships between the fluxes measured in different types of experiment, and thence relationships among transport parameters. The introduction of this approach to kinetic analysis, which differs fundamentally from that followed in the past, makes it possible to establish relationships among the experimental parameters that are true for every possible mechanism giving rise to simple saturation kinetics. Hence data at variance with the general relationships cannot be invoked either to prove or to disprove a particular mechanism. A new kind of test emerges, in which the data are assessed for internal consistency before being applied to specific transport models.

THEORY

General relationships

In the analysis I assume, as is commonly reported, that the transport system exhibits simple saturation kinetics, the flux of substrate across the membrane, v, approaching an upper limit as the substrate concentration, [S], rises. A test for such behaviour is linearity in a double-reciprocal plot, i.e. a graph of $1/v$ against $1/[S]$ takes the form of a straight line with a positive intercept and a positive slope. The equation defining the straight line is:

$$1/v = a + b/[S] \quad (1)$$

where a is the intercept and b the slope. The equation can be re-arranged into the familiar Michaelis–Menten form:

$$v = V/(1 + K_m/[S]) \quad (2)$$

where $V = 1/a$ and $K_m = b/a$. As in enzyme kinetics, V is a maximum velocity, the limiting flux at high substrate concentrations, and K_m is the substrate concentration at which the flux is half this maximum. The slope of the double-reciprocal plot is equal to K_m/V, a constant that plays a central role in the analysis.

Transport rates may be measured under various conditions. For example, the entry of labelled substrate, over a range of concentrations, may be monitored into cells that contain either no substrate at all, or unlabelled substrate at a concentration equal to that outside, or substrate, labelled or unlabelled, at a saturating concentration. Exit may be studied in similar ways. In each type of experiment initial rates can be measured and, given simple saturation kinetics, a maximum rate constant and a substrate half-saturation constant can be calculated. Further, in the final steady state or equilibrium, there will be a definite experimentally measurable ratio, α, of internal and external substrate concentrations under the conditions of the experiment:

$$([S_i]/[S_o])_{final} = \alpha \quad (3)$$

At high substrate concentrations the measured value of α could be incorrect, either because of leakage across the membrane by simple diffusion, or because of cellular regulatory mechanisms limiting the internal concentration. For these reasons α is best measured at a low substrate concentration. It may be noted that, if α varied in the concentration range where substrate constants are determined, double-reciprocal plots would be non-linear.

The various transport experiments are described in Table 1. Those of immediate interest, because they are found to involve general relationships, are zero-trans entry, zero-trans exit and equilibrium exchange. 'Zero trans' refers to the flux of labelled substrate when no substrate is present in the opposite compartment. 'Equilibrium exchange' is taken to include exchange in the final steady state in active-transport systems. The exchange experiment may be envisaged as follows. The substrate, initially present in the suspending medium, is allowed to attain its final concentration inside; the external pool is then labelled with a minute quantity of radioactive substrate, and the initial rate of appearance of the label inside the cell is measured.

Assuming that the rates at various substrate concentrations are accurately described by eqn. (1), flux equations may be conveniently written in terms of constants analogous to those used in enzyme kinetics, following a convention suggested previously (Devés & Krupka, 1979):

(i) Zero trans efflux:

$$\bar{v}_{si} = \frac{\bar{V}_{si}}{1 + \bar{K}_{si}/[S_i]} \quad (4)$$

(ii) Zero trans influx:

$$\bar{v}_{so} = \frac{\bar{V}_{so}}{1 + \bar{K}_{so}/[S_o]} \quad (5)$$

(iii) Equilibrium exchange, unidirectional efflux of radioactive substrate:

$$\bar{\bar{v}}_{si} = \frac{\bar{\bar{V}}_{si}}{1 + \bar{\bar{K}}_{si}/[S_i]} \quad (6)$$

Table 1. Distribution of substrates in various transport experiments

In equilibrium-exchange and infinite-trans experiments the unidirectional flux of labelled substrate is measured, independently of the flux in the opposite direction of unlabelled substrate from the trans compartment. In infinite-cis experiments the net flux is measured, i.e. the difference between opposite unidirectional fluxes. 'Equilibrium exchange' includes exchange in the final steady state in active transport. S_i^* and S_i indicate labelled and unlabelled substrate, inside; S_o^* and S_o indicate labelled and unlabelled substrate, outside. Separate symbols are used for inward and outward unidirectional exchange fluxes, $\bar{\bar{V}}_{so}$ and $\bar{\bar{V}}_{si}$, though $\bar{\bar{V}}_{so}$ is necessarily equal to $\bar{\bar{V}}_{si}$, both under equilibrium conditions and in the final steady state in active transport.

Experiment	Labelled/unlabelled substrates		Constants determined
	Intracellular	Extracellular	
Zero-trans entry	$[S_i] = 0$	S_o^*	$\bar{K}_{so}, \bar{V}_{so}$
Zero-trans exit	S_i^*	$[S_o] = 0$	$\bar{K}_{si}, \bar{V}_{si}$
Equilibrium exchange, unidirectional entry	$[S_i] = \alpha[S_o]$	S_o^*	$\bar{\bar{K}}_{so}, \bar{\bar{V}}_{so}$
Equilibrium exchange, unidirectional exit	S_i^*	$[S_o] = [S_i]/\alpha$	$\bar{\bar{K}}_{si}, \bar{\bar{V}}_{si}$
Infinite-trans entry	$[S_i] \to \infty$	S_o^*	$\tilde{K}^s_{so}, \bar{\bar{V}}_{so}$
Infinite-trans exit	S_i^*	$[S_o] \to \infty$	$\tilde{K}^s_{si}, \bar{\bar{V}}_{si}$
Infinite-cis net exit	$[S_i] \to \infty$	S_o	$\tilde{K}^s_{so}, \bar{V}_{si}$
Infinite-cis net entry	S_i	$[S_o] \to \infty$	$\tilde{K}^s_{si}, \bar{V}_{so}$

(iv) Equilibrium exchange, unidirectional influx of radioactive substrate:

$$\bar{\bar{v}}_{so} = \frac{\bar{\bar{V}}_{so}}{1 + \bar{\bar{K}}_{so}/[S_o]} \tag{7}$$

V, v and K, each with an attached subscript and overbar form to denote a particular experimental arrangement, represent a maximum flux, the measured flux and a substrate half-saturation constant respectively. The measured flux, v, is the initial rate in the steady state. The overbar '$^-$' denotes a zero-trans experiment and the double overbar '$^=$' exchange at equilibrium or in the final steady state. The subscripted terms S_o and S_i designate the labelled substrate and its location, outside and inside the cell respectively, in the experiment. Thus $\bar{v}_{si}$ is the measured exit rate, $\bar{V}_{si}$ the maximum exit rate and $\bar{K}_{si}$ the substrate half-saturation constant inside, all in zero-trans-exit experiments. $[S_i]$ and $[S_o]$ are the internal and external substrate concentrations respectively. The term 'unidirectional' refers to the flow of labelled substrate under conditions where unlabelled substrate may be moving in the opposite direction.

At substrate concentrations approaching zero the rate equations reduce to an expression that is first-order in substrate, and in which a single pseudo-first-order rate constant governs the rate. Thus, setting $[S_i] \to 0$ and $[S_o] \to 0$, eqns. (4)–(7) become:

$$\bar{v}_{si} = (\bar{V}_{si}/\bar{K}_{si})[S_i] \tag{8}$$

$$\bar{v}_{so} = (\bar{V}_{so}/\bar{K}_{so})[S_o] \tag{9}$$

$$\bar{\bar{v}}_{si} = (\bar{\bar{V}}_{si}/\bar{\bar{K}}_{si})[S_i] \tag{10}$$

$$\bar{\bar{v}}_{so} = (\bar{\bar{V}}_{so}/\bar{\bar{K}}_{so})[S_o] \tag{11}$$

The pseudo-first order rate constant is seen to be a V/K_m ratio.

Relationship between equilibrium exchange constants and α. In the final steady-state where $[S_i] = \alpha[S_o]$, the unidirectional rates of exit and entry, $\bar{\bar{v}}_{si}$ and $\bar{\bar{v}}_{so}$, are equal, by definition. In the limit, as $[S_i] \to 0$ and $[S_o] \to 0$, the rates are given by eqns. (10) and (11), and therefore:

$$(\bar{\bar{V}}_{si}/[\bar{\bar{K}}_{si}])[S_i] = (\bar{\bar{V}}_{so}/\bar{\bar{K}}_{so})[S_o] \tag{12}$$

Substitution of $[S_i] = \alpha[S_o]$ (eqn. 3) into eqn. (12) yields:

$$\alpha = \frac{\bar{\bar{V}}_{so}/\bar{\bar{K}}_{so}}{\bar{\bar{V}}_{si}/\bar{\bar{K}}_{si}} \tag{13}$$

The derivation of eqn. (13) is seen to be purely algebraic, and involves no assumptions about mechanism.

The maximum rates of unidirectional exit and entry are actually identical ($\bar{\bar{V}}_{si} = \bar{\bar{V}}_{so}$), as shown by the following reasoning. At equilibrium or in the final steady state, the flux of labelled substrate, initially confined to one compartment, is equal to the flux in the opposite direction of unlabelled substrate from the opposite compartment. As these opposing rates are equal at all substrate concentrations, the extrapolated maximum rates must also be equal (i.e. the $1/v$ intercepts in double-reciprocal plots). The asymmetry in active transport is therefore expressed, in the final steady state, as a difference in inner and outer affinities: substitution of $\bar{\bar{V}}_{si} = \bar{\bar{V}}_{so}$ into eqn. (13) yields the relationship $\alpha = \bar{\bar{K}}_{si}/\bar{\bar{K}}_{so}$.

Relationship between zero trans constants and α. The relationship between the experimental constants for entry and exit follows from the Law of Mass Action and a first-order dependence of rates on substrate concentration. The experimental proof of the first-order dependence is linearity in a double-reciprocal plot. Linear behaviour is governed by eqn. (1), and at $[S] \to 0$ eqn. (1) reduces to $v = [S]/b$. The first-order rate constant, $1/b$, is the reciprocal of the slope, which in terms of Michaelis–Menten parameters (eqn. 2) is V/K_m.

Given that the rates are first-order in substrate concentration, the transport reaction is formally equivalent

to a chemical reaction in which one molecule, S_o, reacts reversibly to form another, S_i:

$$S_o \underset{k_r}{\overset{k_f}{\rightleftharpoons}} S_i \qquad (14)$$

From the Law of Mass Action, the equilibrium constant for the reaction is equal to the ratio of k_f and k_r, the first-order rate constants in the forward and reverse directions:

$$K_{eq.} = ([S_i]/[S_o])_{equilibrium} = k_f/k_r \qquad (15)$$

[In a simple reaction such as that defined by eqn. (14), the equilibrium constant equals the ratio of forward and reverse rate constants measured in the steady state. A simple reaction is one in which the kinetic order of the mechanism and of the overall reaction are the same; it may proceed through a single pathway or several pathways of the same kinetic order (Krupka *et al.*, 1966). In a complex reaction, the equilibrium constant can differ from the ratio of forward and reverse rate constants; parallel pathways of different kinetic order may be involved or separate sub-reactions in series, where the kinetic order of the rate-controlling sub-reaction differs from that of the overall reaction (see Manes *et al.*, 1950; Adamson, 1973). In the transport reaction, the observed first-order dependence on substrate concentration in both the forward and reverse directions (eqn. 14) agrees with the kinetic order of the overall reaction, where a substrate molecule is transferred from one side of the membrane to the other. The reaction is therefore simple, as defined above, and the Law of Mass Action applies.]

The constants k_f and k_r are equal to the pseudo-first-order rate constants for zero-trans entry (eqn. 9) and zero-trans exit (eqn. 8), i.e. $k_f = \bar{V}_{so}/\bar{K}_{so}$ and $k_r = \bar{V}_{si}/\bar{K}_{si}$, and the equilibrium constant for the reaction, $K_{eq.}$, is equal to α (eqn. 3). It follows, by substitution into eqn. (15), that:

$$\alpha = \frac{\bar{V}_{so}/\bar{K}_{so}}{\bar{V}_{si}/\bar{K}_{si}} \qquad (16)$$

In deriving eqn. (16), no assumption has been made about the nature of the catalyst converting S_o into S_i, or indeed about whether there is a catalyst. That is, no assumption is made about the transport mechanism, other than that the rate becomes first-order in substrate as its concentration approaches zero, which is implicit in Michaelis–Menten behaviour. The V/K_m ratios are determined, of course, not from a single rate at a low substrate concentration, but by analysis of double-reciprocal plots or some equivalent treatment of the data.

Eqn. (16) should be distinguished from a Haldane relationship, even though the equations may be identical. A Haldane relationship is found by substitution of the experimental parameters, $\bar{V}_{so}$, $\bar{K}_{so}$ etc., by equivalent expressions made up of individual rate constants in the reaction scheme treated. By contrast, eqn. (16) is found from the observed hyperbolic dependence of transport rates on substrate concentration. The validity of the Haldane relationship derives from the proposed reaction scheme, the validity of eqn. (16) from the experimental relationship between rates and concentration.

Relationship between equilibrium-exchange and zero-trans constants. Here, the required relationship follows from the first-order dependence on substrate concentration and from the approach of the system to an 'ideal' state unaffected by substrate, under the condition that $[S] \rightarrow 0$. The argument is as follows.

The limiting rate of equilibrium exchange can be expected to differ from the zero-trans rate, owing to the presence of unlabelled substrate in the trans (i.e. opposite) compartment. In many systems the effect of the trans substrate is to increase the unidirectional flux of labelled substrate, making the maximum rate of equilibrium exchange faster than the maximum zero-trans rate. In other systems exchange might be the slower process. The trans substrate could work in various ways, altering either the affinity of the labelled substrate, or its rate of translocation within the membrane, or both, depending on the details of the transport mechanism.

Under the conditions that concern us, however, where the substrate concentration approaches zero, neither the transport mechanism nor the effect of the trans substrate on unidirectional flux needs to be specified. Unlabelled substrate in the trans compartment can only affect the transport of labelled substrate by interacting in some way with the membrane system, and the smaller the proportion of the system interacting with molecules of the trans substrate the smaller the effect. This proportion can be made completely negligible by allowing the substrate concentration to become disappearingly low.

Consider the case of entry, i.e. zero-trans entry compared with unidirectional entry in equilibrium exchange, with S_o labelled and S_i unlabelled. In the first experiment, zero-trans entry, the internal substrate concentration is zero, and in the limit, as the external substrate concentration approaches zero, the system approaches a state free of any influence of the substrate. Here, each molecule of labelled substrate in the external solution encounters the transport system wholly in the free, equilibrium, state. In the second experiment, equilibrium exchange, the internal substrate concentration is proportional to the external substrate concentration ($[S_i] = \alpha[S_o]$), but in the limit, as both the internal and external substrate concentrations approach zero, the system likewise approaches a state unperturbed by the substrate. Again, therefore, a labelled substrate molecule in the external solution encounters the system entirely in the free, equilibrium, state. Therefore in the limit, as $[S_o] \rightarrow 0$, the labelled substrate will interact with identical systems under equilibrium-exchange and zero-trans conditions, i.e. the affinity of the substrate and its rate of translocation within the membrane, and therefore the rate of transport from one compartment to the other, will be the same. Thus $\bar{\bar{v}}_{so} \rightarrow \bar{v}_{so}$ as $[S_o] \rightarrow 0$ (the concentration of labelled substrate being set at the same value in the two experiments). From eqns. (9) and (11), therefore:

$$\bar{\bar{V}}_{so}/\bar{\bar{K}}_{so} = \bar{V}_{so}/\bar{K}_{so} \qquad (17)$$

Similar reasoning for exit (zero-trans efflux compared with equilibrium-exchange undirectional efflux) shows that $\bar{\bar{v}}_{si} \rightarrow \bar{v}_{si}$ as $[S_i] \rightarrow 0$. From eqns. (8) and (10), therefore:

$$\bar{\bar{V}}_{si}/\bar{\bar{K}}_{si} = \bar{V}_{si}/\bar{K}_{si} \qquad (18)$$

From eqns. (17) and (18), together with eqn. (13), the following relationship, which includes all the ratios, is found:

$$\frac{\bar{\bar{V}}_{so}}{\bar{\bar{K}}_{so}} = \frac{\bar{V}_{so}}{\bar{K}_{so}} = \frac{\alpha\bar{\bar{V}}_{si}}{\bar{\bar{K}}_{si}} = \frac{\alpha\bar{V}_{si}}{\bar{K}_{si}} \qquad (19)$$

The validity of eqn. (19) is confirmed by eqn. (16), the relationship between the constants for zero-trans entry and zero-trans exit, which is one of the equalities in eqn. (19), and which was demonstrated through an independent argument.

As the V/K_m ratios are the reciprocals of the slopes of double-reciprocal plots (eqn. 1), the slopes of the plots for zero-trans influx and equilibrium-exchange influx should be the same, though the intercepts, the reciprocals of the maximum flux, can differ. Similarly, the slopes, but not necessarily the intercepts, should be the same in zero-trans efflux and equilibrium-exchange efflux. However, the slopes for the exit and entry experiments should differ by the factor α.

Deviation from the general relationships

As no assumptions have been made about the transport mechanism, the equations will be valid under all circumstances. They remain valid, for example, if the system is made asymmetric, either by a competitive inhibitor present on only one side of the membrane, which causes the maximum rates of entry and exit to be unequal (Krupka & Devés, 1979), or by a pre-existing gradient of a second substrate, which by countertransport produces a concentration gradient in the substrate being studied. Any departure from the general relationships therefore signals some kind of error. Imprecise measurement is not likely to be responsible, since it can be detected by statistical analysis, but systematic error, which statistics may fail to expose, could be. Systematic error could arise in various ways. The kinetics may not be truly hyperbolic, with a curvature in extended double-reciprocal plots undetected over the limited concentration range studied. Sampling may be too slow to give true initial rates. If pretreatment of the cells differs in various experiments, the transport systems compared in exit, entry and exchange may not be identical. The internal substrate may be compartmentalized, with the result that its concentration at the cell membrane differs from the calculated value. It is also possible for α to be mistaken, as noted above. Conversely, where the general relationships hold, the results should be free of such error. Here it is probable that the kinetics are truly hyperbolic, that the systems studied in exit, entry and exchange are identical, that there is no compartmentalization of the internal substrate, that the experimental methods give true initial rates and that α has been properly measured.

Specific relationships

The general relationships were found under conditions where the substrate concentration in the trans compartment was either zero or approaching zero, and where, therefore, the trans substrate could only interact with an insignificant proportion of the carrier. Specific relationships applicable to a particular transport model can be expected at high concentrations of the trans substrate, for the ease of formation of a complex on the opposite side of the membrane, and the effect of the trans complex on the measured unidirectional flux, depend on the character of the transport system. As an example, substrate sites are simultaneously exposed on both sides of the membrane in some transport models, whereas a single site is alternately exposed on opposite sides in the conventional carrier model; obviously, access to the carrier of substrate molecules on opposite sides of the membrane differs in these cases. As another example, substrate molecules being translocated in opposite directions within the membrane would necessarily interfere with one another in a simple pore but not in a carrier model; hence the effect of the trans substrate on unidirectional flux depends on the mechanism.

Specific relationships, then, involve constants determined with a saturating concentration of substrate in the opposite compartment: infinite trans and infinite cis constants (Table 1). That the V/K_m ratios for infinite-trans and infinite-cis experiments do not necessarily equal the other V/K_m ratios is proven by analysis of the carrier model (Lieb & Stein, 1974*b*; Devés & Krupka, 1979).

Involvement of the general relationships in the rejection criteria

In his careful analysis of experimental data on the glucose-transport system of erythrocytes, Lieb (1982) employed the following rejection criteria for the carrier model, derived for the case where $\alpha = 1$:

$$\frac{\bar{K}_{si}V_s}{\bar{V}_{si}} = \frac{\bar{K}_{so}V_s}{\bar{V}_{so}} = \frac{\bar{\bar{K}}_s V_s}{\bar{\bar{V}}_s} = \frac{\tilde{K}^s_{so}\bar{V}_{si}}{\bar{\bar{V}}_s} = \frac{\tilde{K}^s_{si}\bar{V}_{so}}{\bar{\bar{V}}_s} \qquad (20)$$

where $\bar{\bar{V}}_s = \bar{\bar{V}}_{si} = \bar{\bar{V}}_{so}$, $\bar{\bar{K}}_s = \bar{\bar{K}}_{si} = \bar{\bar{K}}_{so}$ and $V_s^{-1} = \bar{V}_{si}^{-1} + \bar{V}_{so}^{-1} - \bar{\bar{V}}_s^{-1}$. $\tilde{K}^s_{so}$ is the half-saturation constant for external substrate in either infinite-trans-entry or infinite-cis-net-exit experiments, and $\tilde{K}^s_{si}$ is the corresponding constant for internal substrate found in either infinite-trans exit or infinite-cis net entry (see Table 1). The first three terms in eqn. (20), which define the relationships between equilibrium-exchange and zero-trans-entry and -exit constants, are equivalent to eqn. (19), given that $\alpha = 1$, $\bar{\bar{K}}_s = \bar{\bar{K}}_{si} = \alpha\bar{K}_{so}$ and $\bar{\bar{V}}_s = \bar{\bar{V}}_{si} = \bar{\bar{V}}_{so}$.

All the test relationships in eqn. (20) are seen to involve constants from zero-trans-entry and zero-trans-exit experiments. The zero-trans constants bear a fundamental relationship to one another given in eqn. (16), this equation being a corollary of Michaelis–Menten kinetics that follows from the Law of Mass Action, independent of the transport mechanism. Disagreement with eqn. (16) presents a fatal objection to the data, and leaves no independent criteria with which to test a model.

Applying the rejection criteria

Formerly, when the experimental results deviated from the rejection criteria in eqn. (20), there was no way of deciding whether to reject the model or the data. Once the requirement for self-consistency in the measured transport constants has been recognized, the difficulty can be overcome. The test then proceeds in three stages. A set of data is examined, first, for comformance with hyperbolic kinetics, and then for internal consistency, i.e. for agreement with the general relationships. Data that fail in either respect are disqualified for purposes of testing the mechanism. Data that are internally consistent and therefore likely to be free of systematic error are compared with the specific relationships. Disagreement at this stage could confidently be taken to rule out the model being tested.

The rejection criteria listed by Lieb and co-workers apply to the case where α is equal to unity (eqn. 3). Even in simple facilitated-transport systems this condition may not be met. Consider what happens in the presence of a concentration gradient of a second

substrate: by countertransport the first substrate is driven against its own gradient, and in the final steady state its concentrations across the membrane are unequal, i.e. $([S_i]/[S_o])_{final} = \alpha \neq 1$. An example is the choline-transport system of erthrocytes (see below). The rejection criteria for the carrier model previously applied to this system, which assume $\alpha = 1$, were actually inappropriate. Relationships for the carrier model that do include α have since been worked out (Devés & Krupka, 1979).

TESTING TRANSPORT SYSTEMS

The advantage in distinguishing the general from the specific relationships is brought out by a re-examination of the data on the erythrocyte transport systems for leucine, uridine, glucose and choline. In Lieb's (1982) earlier analysis, in which all of the relationships were on an equal footing, the systems for leucine and uridine, but not those for glucose or choline, were judged to be consistent with the carrier model. All four systems showed trans acceleration ($\bar{V}_s > \bar{V}_{si}$), which unequivocally ruled out the simple pore model.

Leucine-transport and uridine-transport systems

In these systems, where α had been shown to be unity, the V/K_m ratios for zero trans entry, zero trans exit and equilibrium exchange were equal, as required by the general relationships. In the leucine-transport system, neither infinite-trans nor infinite-cis constants, which are involved in the specific relationships, were available. Hence this test cannot be construed as supporting any particular model. In the uridine-transport system, infinite-cis constants had been measured, and their demonstrated agreement with the theory does lend support to the carrier model.

Glucose-transport system

In this system α is equal to unity (Laris, 1958; Miller, 1964), and simple hyperbolic substrate saturation curves are observed in all experiments: zero trans, equilibrium exchange, infinite trans and infinite cis (Sen & Widdas, 1962; Harris, 1964; Miller, 1968, 1971; Karlish *et al.*, 1972; Eilam & Stein, 1972; Lacko *et al.*, 1972; Hankin *et al.*, 1972; Edwards, 1974; Eilam, 1975; Weiser *et al.*, 1983; Brahm, 1983; Lowe & Walmsley, 1986). The single report of deviation from hyperbolic kinetics, in equilibrium exchange (Holman *et al.*, 1981), could not be confirmed in another laboratory (Weiser *et al.*, 1983).

The data analysed by Lieb (1982), which were taken from several studies in the literature, failed to satisfy the criteria for the carrier model. Most important, we now see, was the wide disparity in V/K_m ratios for zero-trans entry and zero-trans exit, as well as equilibrium exchange. These ratios should be equal if $\alpha = 1$, according to the general relationships (eqns. 16 and 19). The disparity was far greater than the reported uncertainty in the values of the constants: the V/K_m ratios for zero-trans exit and entry differed by 4-fold, and the ratio for equilibrium exchange, which was intermediate, differed from either by 2-fold. The results cannot be explained by a value of α different than unity, either. First, the ratios for unidirectional entry and exit in equilibrium exchange should differ by the factor α, but were reported not to differ. Secondly, the ratios for entry (zero trans and equilibrium exchange) should be equal, whatever the value of α, and so should the ratios for exit (eqn. 19), but this was not observed either.

Since the experimental results failed to conform with the general relationships, and since the constants involved in the general relationships enter into all the rejection criteria, it was inevitable that the disagreement between theory and experiment should have been so definite. We can only conclude that some of the results used in this particular analysis were faulty. The experiments can neither confirm nor refute the carrier model.

Not surprisingly, other studies have given constants in accord with the general relationships. The transport of galactose by the glucose-transport system was found by Miller (1965, 1971) to obey the general relationship $\bar{V}_{si}/\bar{K}_{si} = \bar{V}_{si}/\bar{K}_{si}$, and moreover it was possible to predict the time course of counterflow, a process that depends on the specific relationships for the carrier model, involving infinite-trans constants [the attempt to account for the counterflow of glucose had been unsuccessful (Miller, 1968)]. Using techniques in which initial rates could be measured in a fraction of a second, Brahm (1983) demonstrated Michaelis–Menten kinetics with glucose, and found that $\bar{V}_s/\bar{K}_{si} \simeq \bar{V}_{si}/\bar{K}_{si}$ at 38 °C, 25 °C and 10 °C, and Lowe & Walmsley (1986) found that $\bar{V}_{si}/\bar{K}_{si} \simeq \bar{V}_{so}/\bar{K}_{so} \simeq \bar{V}_{so}/\bar{K}_{so}$ at 0 °C and 20 °C.

Lowe & Walmsley (1986) showed that the values obtained for the transport constants depend on the experimental method. An analysis of the whole time course of exit, involving integrated rate equations, gave different constants from those obtained by using the simpler and more reliable analysis based on initial rates. On the other hand, initial rates of zero-trans entry are hard to measure because net uptake is rapidly diminished by back-flux, as Miller (1975) showed. In the test reported by Lieb (1982), the available zero-trans-exit constants were derived from time-course experiments and integrated rate equations (Karlish *et al.*, 1972), and the zero-trans-entry experiments (Lacko *et al.*, 1972) involved periods of time (up to 10 s) that are probably too long to give true initial rates (Miller, 1975). Both sets of constants would therefore be suspect, apart from disagreement with the general relationships.

Recently, Wheeler & Whelan (1988) remeasured the infinite-cis-entry constant, $\tilde{K}^s_{si}$, taking precautions to avoid systematic error. The value was consistent with the carrier model, unlike previous reports. By assembling other constants reported in the literature, it was shown that no important differences remain between the model and the observed kinetics of glucose transport.

Choline-transport system

In this system α is not equal to unity: the presence in erythrocytes of a gradient of another substrate of the system, K^+ ion, induces a net uptake of choline by countertransport (Martin, 1972). The K^+ concentration gradient is stable, not only because of the presence of a $Na^+ + K^+$-dependent ATPase in the membrane, but because the internal K^+ concentration (95 mM) is more than 1000 times the concentration of choline (50 μM); hence the amount of K^+ countertransported against incoming choline is a minute fraction of the total. Under the conditions of the experiments, α was approx. 2, but the measured ratios of V/K_m for zero-trans entry and exit differed by nearly 5-fold, instead of 2-fold, the predicted factor (eqn. 16). The discrepancy cannot be used to rule out the carrier model, because it is the general relation-

ship, involving zero-trans constants, that is violated. [The experimental constants actually used in the analysis were the half-saturation constants for zero-trans entry and exit, together with the flux ratios for entry and exit $(\tilde{v}^s/\bar{v})_{so\to 0}$ and $(\tilde{v}^s/\bar{v})_{si\to 0}$; the ratio of the latter constants is equal to the ratio of the maximum rates of zero-trans entry and exit, $\bar{V}_{so}/\bar{V}_{si}$, according to the carrier model (Devés & Krupka, 1979).] Again, at least some of the experimental measurements must be queried.

CONCLUDING REMARKS

It is widely assumed that virtually any set of data on a reaction system could be explained by a kinetic model of sufficient complexity. This is certainly not true of a reversible reaction in which there is some definite pattern in the values of dependent and independent variables, as in the case of Michaelis–Menten kinetics. Here, strict limits may be imposed on the relative values of the system constants measured in certain experiments. A failure to conform to these constraints would indicate a lack of self-consistency in the data. Such data would necessarily disagree with the predictions worked out for a particular model, but the data and not the model would have to be rejected.

The result is useful in several ways. It spares us the effort of trying to explain data that involve fundamental inconsistencies. It forces us to re-examine such data, considering both the experimental methods and the experimental conditions. Where the measured constants are consistent with one another, it validates assumptions made in the experiments, for example the assumption that true initial rates have been determined, that the system obeys hyperbolic kinetics, that intracellular substrate is not compartmentalized or metabolized, and so on. Finally, it removes an element of uncertainty, due to possible systematic error, in interpreting kinetic tests of transport mechanisms.

I thank Dr. R. Devés, Dr. S. D. Dunn, Dr. D. G. Leaist, Dr. D. M. Miller, and Dr. N. O. Petersen for helpful discussions and comments on the manuscript.

REFERENCES

Adamson, A. W. (1973) A Textbook of Physical Chemistry, pp. 714–717, Academic Press, New York
Brahm, J. (1983) J. Physiol. (London) **339**, 339–354
Britton, H. G. (1964) J. Physiol. (London) **170**, 1–20
Devés, R. & Krupka, R. M. (1979) Biochim. Biophys. Acta **556**, 533–547
Edwards, P. A. W. (1974) Biochim. Biophys. Acta **345**, 373–386
Eilam, Y. (1975) Biochim. Biophys. Acta **401**, 364–369
Eilam, Y. & Stein, W. D. (1972) Biochim. Biophys. Acta **266**, 161–173
Geck, P. (1971) Biochim. Biophys. Acta **241**, 462–472
Haldane, J. B. S. (1930) Enzymes, pp. 80–83, Longmans, Green and Co., London
Hankin, B. L., Lieb, W. R. & Stein, W. D. (1972) Biochim. Biophys. Acta **288**, 114–126
Harris, E. J. (1964) J. Physiol. (London) **173**, 344–353
Heinz, E. (1978) Mechanics and Energetics of Biological Transport, pp. 98–101, Springer-Verlag, Berlin, Heidelberg and New York
Hoare, D. G. (1972) J. Physiol. (London) **221**, 311–329
Holman, G. D., Busza, A. L., Pierce, E. J. & Rees, W. D. (1981) Biochim. Biophys. Acta **649**, 503–514
Karlish, S. J. D., Lieb, W. R., Ram, D. & Stein, W. D. (1972) Biochim. Biophys. Acta **255**, 126–132
Krupka, R. M. & Devés, R. (1979) Biochim. Biophys. Acta **550**, 77–91
Krupka, R. M., Kaplan, H. & Laidler, K. J. (1966) Trans. Faraday Soc. **62**, 2754–2759
Lacko, L., Wittke, B. & Kromphardt, H. (1972) Eur. J. Biochem. **25**, 447–454
Laris, P. C. (1958) J. Cell. Comp. Physiol. **51**, 273–307
Lieb, W. R. (1982) in Red Cell Membranes: A Methodological Approach (Ellory, J. C. & Young, J. D., eds.), pp. 135–164, Academic Press, London and New York
Lieb, W. R. & Stein, W. D. (1971) J. Theor. Biol. **30**, 219–222
Lieb, W. R. & Stein, W. D. (1972) Biochim. Biophys. Acta **265**, 187–207
Lieb, W. R. & Stein, W. D. (1974*a*) Biochim. Biophys. Acta **373**, 165–177
Lieb, W. R. & Stein, W. D. (1974*b*) Biochim. Biophys. Acta **373**, 178–196
Lowe, A. G. & Walmsley, A. R. (1986) Biochim. Biophys. Acta **857**, 146–154
Manes, M., Hofer, L. J. E. & Weller, S. (1950) J. Chem. Phys. **18**, 1355–1361
Martin, K. (1972) J. Physiol. (London) **224**, 207–230
Miller, D. M. (1964) J. Physiol. (London) **170**, 219–225
Miller, D. M. (1965) Biophys. J. **5**, 417–423
Miller, D. M. (1968) Biophys. J. **8**, 1339–1352
Miller, D. M. (1971) Biophys. J. **11**, 915–923
Miller, D. M. (1975) J. Biol. Chem. **250**, 3637–3638
Patlak, C. S. (1957) Bull. Math. Biophys. **19**, 209–235
Regen, D. M. & Morgan, H. E. (1964) Biochim. Biophys. Acta **79**, 151–166
Segel, I. H. (1975) Enzyme Kinetics, pp. 534–543, John Wiley and Sons, New York
Sen, A. K. & Widdas, W. F. (1962) J. Physiol. (London) **160**, 391–403
Weiser, M. B., Razin, M. & Stein, W. D. (1983) Biochim. Biophys. Acta **727**, 378–388
Wheeler, T. J. & Whelan, J. D. (1988) Biochemistry **27**, 1441–1450

Received 16 May 1988/5 January 1989; accepted 20 January 1989