Talking Point

What are the requisites for a model transport analog?

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Perhaps because we have come to accept so well the possibility of exquisite precision in biomolecular recognition in general, and by enzymes in particular, a need has arisen for re-emphasis of the limits that may apply to the possible one-to-one specificity between the mediators and substrates for the special area of membrane transport. Early studies with bacteria had brought attention to transport systems specific virtually for one particular amino acid, sugar, etc. Accordingly, early resistance was encountered to the now familiar idea of transport systems serving in parallel in mammalian cells and tissues, each system transporting a rather wide range of amino acids. The consequence of this pattern is that each system handles a substantial range, although a circumscribed one, of amino acids, and that each amino acid is moved across the plasma membrane often by two or more mediating systems. Amino acid transport systems of rather wide scope have subsequently been recognized in bacteria, and narrowly specific ones in higher animals. For the ubiquitous wide-range systems, an obvious need has arisen to discover for each transport system the substrate analog, natural or artificial, that would serve best to identify transport catalysed by that system.

Discrimination of distinct transport systems

Let me first review in an elementary way the matter of discrimination of distinct transport systems, which I have reviewed more extensively elsewhere[1,2]. The total number of such systems that may serve for amino acids in each cell type has not really been established. To simplify matters, I will limit my attention to dipolar (or 'neutral') amino acids, and to three apparently ubiquitous transport systems, A, ASC, and L. Furthermore, we will assume that each of these systems represents a homogeneous catalytic entity, i.e. that we face no complications such as L1, L2 discrimination or ASC, asc discrimination. Hence under our assumptions only these three familiar systems for catalysing the movement of dipolar amino acids across the otherwise quite effective osmotic barrier need to be considered.

The first step in assigning transport of a particular amino acid to the various transport systems is to deduct and then usually to disregard that component of transmembrane passage which fails under the conditions selected to give the usual evidence of catalysis of substrate passage. That is, its rate fails to 'saturate', but instead appears to rise linearly as the concentration of the amino acid is raised. Furthermore analogs of the test substrate are apt not to inhibit the 'non-saturable' component. For the amino acids, except under special conditions[3], most of this component cannot usually be assigned to diffusion. Whether in standard experiments it includes, as is likely, a purely diffusional component (i.e. one that is totally unmediated) has in fact not been proved (see Ref. 4).

Suppose that we test uptake of an amino acid that is a poor substrate for an available transport system. If it half-saturates that system at 30 mM, and we were to choose to test its uptake from 1 to 10 mM, the Michaelis–Menten curve would appear quite linear, and we would tend to conclude that its uptake is not saturable. For other cases where much higher levels may be tested without encountering evidence of saturation, the observed fluxes show degrees of structural selectivity among amino acids and also temperature coefficients, neither of which is consistent with movement restricted to diffusion[5]. Hence when we disregard the non-saturable component, we provisionally close our minds to unknown (although often minor) components.

If our substrate has a high affinity for System L (e.g. phenylalanine), the correction for non-saturable uptake may be negligible. Our initial assumptions should assure us that a rectangular hyperbola describing transport by System L will then be obtained on plotting flux versus phenylalanine concentration. If the substrate is a poor one for System L in the absence of Na^+ catalysis of flow (e.g. glycine), we will need to extend our study to concentrations so high that the non-saturable component requires a large correction. In general, System L favors apolar mass on the substrate molecule, whether disposed in a bulky way or not. Therefore the artificial introduction of bulk, as in the bicyclo-(2.2.1)heptane or the bicyclo(3.2.1)-octane amino acid[2], has served for the design of analogs which have widely proved to be what we boldly describe as 'model substrates' for System L, because their bulk largely blocks their entry via other transport systems. This article will examine where that description may tend to be used too optimistically.

Conventionally and under the stated assumptions, we might measure transport of a test amino acid by System A plus System ASC (both Na^+-dependent transport systems) by replacing the Na^+ in the medium with the choline cation, or by making some other iso-osmotic substitution known to produce the equivalent effect. Then we would subtract the observed flux, in the presence of choline, from the corresponding flux obtained at each substrate concentration in the presence of Na^+, to obtain the Na^+-dependent component. We have at the same time eliminated the non-saturable component, since it is not Na^+-dependent. The plot of the residual influx versus the concentration of the test amino acid may approximate a rectangular hyperbola, distorted to a greater or lesser extent by the overlap of a second rectangular hyperbola describing influx via the second of these two Na^+-dependent systems (if the two sets of kinetic parameters involved are sufficiently different to cause perceptible distortion). The reliability with which the parameters for a presumed flux by each of two or more routes can be derived from such curves may often be exaggerated, and supplementary evidence is usually desirable[1,2].

It is in the application of the procedure of the preceding paragraph to unfamiliar situation that we encounter the problems that inspired this essay. We seek to dis-

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tinguish transport by each of two or more systems by the difference in their molecular specificity. A number of dipolar amino acids are found to be recognized to similar degrees by Systems A and ASC. Alanine, serine, and 1-amino-*n*-butyric acid are examples. Methionine is, however, rather too long and glycine rather too short of side chain for System ASC, and a well-placed hydroxyl or sulfhydryl group favors transport by that system. Nevertheless differences in such features have not allowed any ordinary amino acid to serve as a model substrate fully discriminating between the A and the ASC components. True, a sufficiently large part of the Na^+-dependent uptake of labeled threonine or cysteine across the plasma membrane of several cell-types will be attributable to System ASC, and a small enough part to System A, to allow description of System ASC. Nevertheless, excesses of one of these amino acids in unlabeled form cannot be relied upon to inhibit System ASC without an appreciable parallel effect on System A transport. The smallness of their entry by System A may be due to low V_{max} values, and in any case it fails to indicate how much System A uptake by another amino acid they may inhibit.

The situation typically encountered in inhibition analysis has been simplified for our teaching purposes and represented as Fig. 1. It shows two parallel modes of vehicular access across what may be taken to represent the Detroit River. Trucks seemed an obvious choice as one of the species of vehicles because of the marked tolerance of transport by System L for molecular bulk. Whatever the real basis of molecular discrimination may be, the diagram may prove helpful to some *TIBS* readers. We will arbitrarily assume that a rate-limiting number of customs agents serve at let us say the Canadian portals, supposing we are observing the easterly flux. An observer at a distance could infer whether cars and trucks share one or both of the two routes, or whether each vehicle is, as a case of unusual simplicity, restricted to only one route. Only in the latter case should we expect no decrease in the number of cars emerging per minute as the number of trucks seeking entry into Canada increases, or vice versa. In that case we could describe each of the two modes of entry by supplying in turn each type of vehicle in excess. Such a case of perfect simplicity has, however, rarely been encountered for amino acid transport. Even given that the two routes are shared by both types of vehicle, we could describe how any type of vehicle divides its movement if we could design a vehicular analog specific to one of the routes, and another vehicular analog specific to the other route. In amino acid transport, the equivalent identifications have been attained only occasionally, after extended selections among analogs whose structures show features that favor their exclusion from one route or the other.

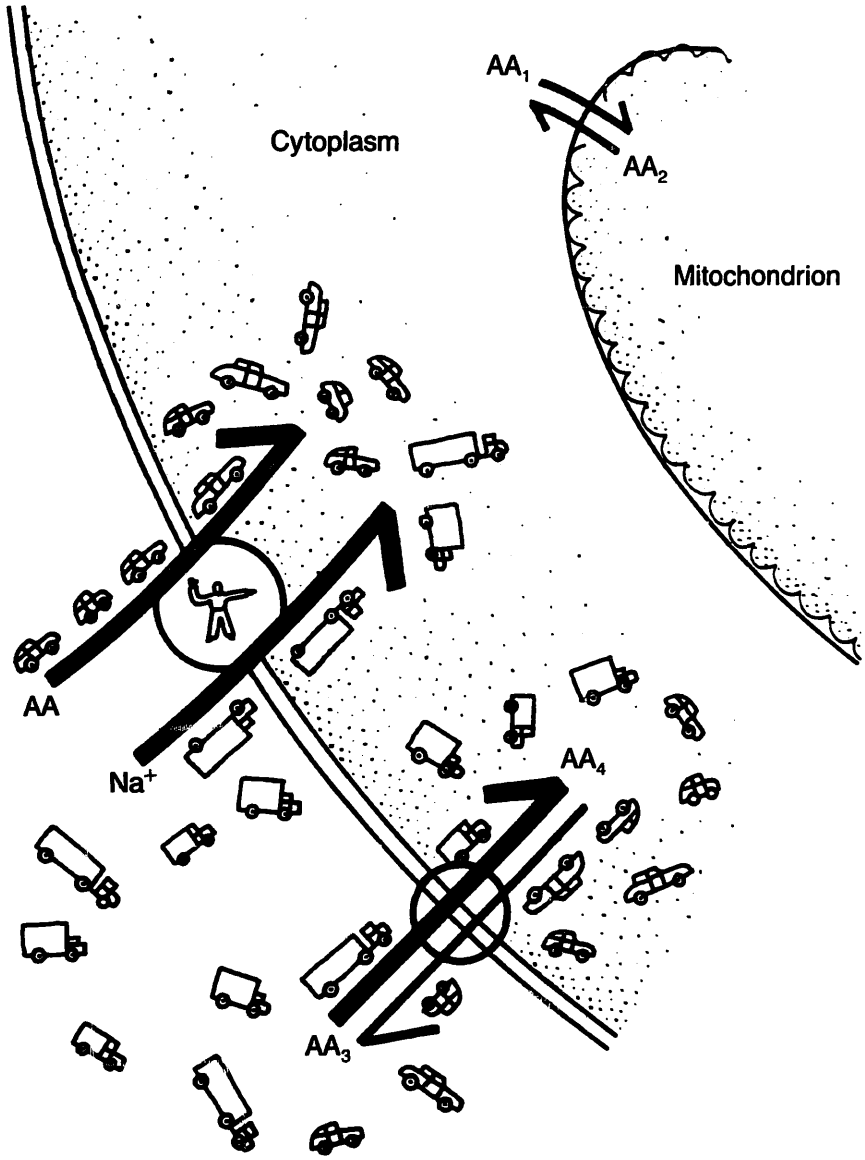

Fig. 1. Before we can assign all the interference with traffic by the trucks to one of the bridges, we must also take into account the traffic over the other bridge. 'Under the test conditions no parallel system should suffer substantial inhibition by it.' Figure drawn by Julie Wei.

What constitutes a model analog?

The current conceptual difficulty appears to lie in an undue confidence in amino acids that appear to serve adequately in inhibition analysis but that merely *prefer* one or another wide-range transport system and are actually far from being strictly limited to that system. How many casual readers suppose leucine is a model substrate for System L? The difficulty might be illustrated harshly by citing occasional published work, but more of it lies hidden in a fascinating literature doomed to extinction, namely the exchange of critiques between colleagues serving by turn as referee and as defending author. One referee felt that our observation of ~ 70% inhibition of Na^+-dependent uptake of MeAIB by leucine in excess was inconsistent with our own published work. Allowing for momentary optimism and for didactic simplification, I don't believe, however, that any of our colleagues working in this area have consistently reported evidence for a natural amino acid as a fully valid system-specific

model among these three transport systems of higher organisms. Nevertheless, that referee's criticism deserves our attention since it shows that we may have failed to be clear. Indeed the initial discrimination of Systems A and L would have been much easier had their overlap as to transport substrates not been so severe. To quote from Ref. 7 'The overlap between these two groups is so extensive that all except perhaps glycine are represented in both groups'. Even that exception proves unnecessary, as re-examination of the first *in vitro* tests of mammalian amino acid transport[8] indicate. These were tests of the inhibitory action of various amino acids on glycine uptake by the excised rat diaphragm. The inhibitory actions were limited to α-amino acids, including for the first time 2-aminoisobutyric acid, but sharply excluding two β-amino acids, an *N*-formyl amino acid, and creatine.

Furthermore, the pH sensitivity seen in 1963 for the uptake of leucine and phenylalanine (Figs 5 and 6 in Ref. 7) was intermediate between that seen for 2-aminoisobutyric acid, to represent System A, and that later seen for 2-amino*endo*bicyclo(2.2.1)heptane-2-carboxylic acid, to represent System L. A summary essay in 1966[1] was addressed to the general problem of resolving the complexity of substrate overlap for parallel transport systems. In agreement with our earlier joint findings, Oxender *et al.* more recently showed[9] that Na^+ replacement eliminated fully a third of the total initial rate of leucine uptake by BALB/3T3 cells, as would be expected from the participation of System A along with System L. Indeed, the reactivity of leucine with System A has been shown sufficient to allow this amino acid at 5 mM to exert a repressive influence on that system in cultured human fibroblasts[5]. If we could all avoid ever again referring uncritically to leucine as a 'System L substrate', or more particularly to System L as the 'leucine-preferring system', no doubt the confusion would disappear.

There are two features that an analog should meet to serve fully as a systems-discriminatory model: (1) Almost all of its mediated uptake should occur by one system; and (2) under the test conditions no parallel system should suffer substantial inhibition by it.

Resistance to metabolic modification is a further advantage but not a requisite. The first of the two conditions allows use of the analog to detect the pertinent system, but does not guarantee the often needed second feature. Even if leucine had only 5%, or only 1% of its uptake by the parallel system A (or ASC), a concentration of it might be selected (by misfortune or necessity) that inhibits say 70% of the uptake of another amino acid by the parallel route A or ASC.

A corresponding confusion may arise when an excess of threonine as an inhibitor is expected to eliminate uptake by System ASC without diminishing uptake by System A. Before undertaking such use, one needs to verify the presence of a 'window of opportunity' – a concentration of threonine high enough to eliminate almost all ASC transport, and yet not so high as to inhibit other Na^+-dependent amino acid uptake. Obviously, threonine is imperfect as a model substrate for System ASC. I suggest we should restrict our use of the term, *model substrate,* if we use it at all, to the exceptional analog meeting both our enumerated conditions, and substitute the term, *model transport analog,* for those which are no more than candidates for the full role. There are of course many conditions of the transport tests that can be modified[1,2] to reinforce limitations in the specificity of the inhibitory analogs available.

The identification of optimal discriminatory analogs is also likely to prove important to find valid tests for specific protection of a transport-mediating structure from an irreversible modification, by use of a reagent such as azidophenylalanine, for example, applied perhaps to monitor its isolation. In a recent paper[12] the authors conclude that no given system transports all five of the amino acids which are most effective in protecting CHO cells from thermal damage at 45°C (including, significantly, 2-aminoisobutyric acid). However, reference to Fig. 3 in Ref. 13 shows that these five amino acids may actually point to System A, in which case these authors prematurely dismissed their own interesting suggestion that the protective action by the amino acids was to stabilize a protein component of that transport system.

Care in understanding limitations in the evidence provided by model transport analogs will steadily become more important as efforts are made to identify the cDNA(s) and the corresponding proteins pertinent to each membrane transport system. Although my examples (understandably) come from the field of amino acid transport, corresponding cautions are likely to apply to substrate analogs proposed for other classes of transport systems, e.g. phthalate as an analog for methotrexate[14] transport, or cinnamic acid derivatives for a system exchanging anions for anions in mitochondria[15] and leucocytes[15], should the problem of parallel routes arise also for those transports. The question of selecting model substrates must also apply for other parallel catalytic processes, for example, the reactions catalysed by the several P-450s.

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