

MODIFIED TRANSPORT SUBSTRATES AS PROBES FOR INTRAMEMBRANE GRADIENTS *

Halvor N. Christensen, Carlos de Cespedes,
Mary E. Handlogten, and Gunnar Ronquist

*Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan 48104*

Introductory Definition

Probes for measuring energetic gradients across biological membranes have generally been selected for their chemical inertness with respect to the membrane. For example, the distribution of the chloride ion or of the dibenzylidimethylammonium ion across the red blood cell membrane may serve to report the transmembrane potential gradient if their movements respond to that force and to no other; 5,5-dimethyl-2,4-oxazolidinedione (DMO) will report the cytoplasmic pH only if its movement responds only to the pH gradient and to no other gradient. The same requirements apply to ion-ionophore combinations that are used for similar purposes. If any of these test substances were to encounter specific uphill transport in traversing the membrane, an invalid measurement of the desired gradient would result.

Probes of this type, of course, provide only a statistical measurement of the energetic gradient, measured all the way across the membrane and averaged for its total area. For the sake of clarity, we deliberately sidestep the possibility that we could obtain information on local gradients by avoiding any energy-transferring reactivity between the probe and components of the membrane.

We will develop here the possibility of using quite another type of probe, one whose crossing of the membrane occurs only through the chemical binding and release events implicit in active transport.^{1, 2} Such probes should be hydrophilic solutes, often zwitterions, sometimes with a net charge, so that their passage by diffusion through the lipid membrane substance is negligible in relation to their passage by specific transport. Their molecular structure must combine two sets of features: (1) the structural features necessary to keep transport restricted to the given system, which may or may not supply a vectorial force in one or the other direction; and (2) a structural feature that causes a response to a membrane gradient. Control substrates that lack this feature will then provide the reference standard, by comparison with which the response is recognized.

Such test substances we will call gradient-sensing substrates for transport. Application of a membrane probe that combines these features would invite only confusion if the membrane also permitted their passive permeation. But when migration is limited to the pathway described by the process of active transport, then we may achieve probes that sense gradients specific to the interval between successive topographic points on that pathway. Rather than obtaining another

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statistical measurement of the macroscopic gradient all the way across the full area of the membrane, we may thus measure for comparison gradients that are restricted to a selected although unmapped part of the thickness and area of the membrane. The results I shall report have provisionally convinced us that local, microscopic gradients, much larger than the statistically measured ones ordinarily reported, are present within the membrane, and probably play a large role in the three-way energy transduction between the three phenomena of electron transport, material transport, and the formation and breakdown of ATP.

To What Transport Systems should Such Substrates be Applied?

If we were willing to adopt two easy, historic propositions on the energization of amino acid transport, namely (1) that energization of the Na^+ -dependent systems occurs obligatorily through down-gradient movements of the alkali-metal ions, and (2) that the Na^+ -independent systems received energy only from down-gradient movement of other amino acids, then we would perhaps have little to look for in studying the gradient-sensing substrates that are transported by these systems. Both of these propositions we have now, we believe, proved incorrect. The first proposition is one form of the alkali-metal gradient hypothesis developed in the fifties;³⁻⁷ the other has been not more than a suspicion on our part.⁸

Experiments by Wheeler and Christensen⁹ and by Thomas and Christensen¹⁰ indicated that for one of the Na^+ -dependent transport systems (ASC) the coupling stoichiometry between the amino acid and Na^+ varies over a wide range, depending on the structure of the amino acids involved. This system appears, however, to be so largely locked into exchange that it may achieve rather little net transport. Another Na^+ -dependent system, which we will call *A*, unequivocally produces strong gradients. Furthermore, study of the flux linkages for this system leave little doubt that energy is transferred between the two flows of the alkali-metal ions and the amino acid, although this energy is not enough to account for the gradients generated for the latter.¹¹

A troublesome inconsistency in the results obtained in various laboratories, however, even with the same test cells, has led us to reexamine the role of the flow of Na^+ in amino acid uptake by this system. The key question, we felt, was whether energy *must* flow through the alkali-metal ion gradient, that is, whether in FIGURE 1 we can place the amino acid transport in the position marked II, with alkali-metal transport at the position marked I, so that the two are in series and number I is essential to the energization of number II, or whether instead we should place uphill amino acid transport at III, so that the two gradients are parallel and interconvertible. The interconversion may occur either directly (dashed lines) or through a common intermediate energy storage. Although energy can in this case flow from one gradient to the other, uphill amino acid transport should then also be possible without down-gradient movement of Na^+ , presumably through direct coupling to the obligatory energized state (whatever it may be).

*Demonstration that Energization by Na^+ Flow is not Obligatory;
Effect of Altering the Amino Acid Pool*

The Ehrlich cell contains a characteristic pattern of endogenous amino acids, which manifestly influence the contribution of the several systems to uptake.

Furthermore, we have already seen that this pattern of endogenous amino acids can influence the stoichiometry of Na^+ flow to amino acid flow in another transport system that was studied in red blood cells.^{9, 10} Accordingly, we have long sought to simplify the internal amino acid composition, without in the meantime damaging the cells.

We obtained a highly informative simplification by displacing major portions of the endogenous amino acids by four successive 5-min incubations in a large volume of medium that contained a single substrate of System A, usually α -

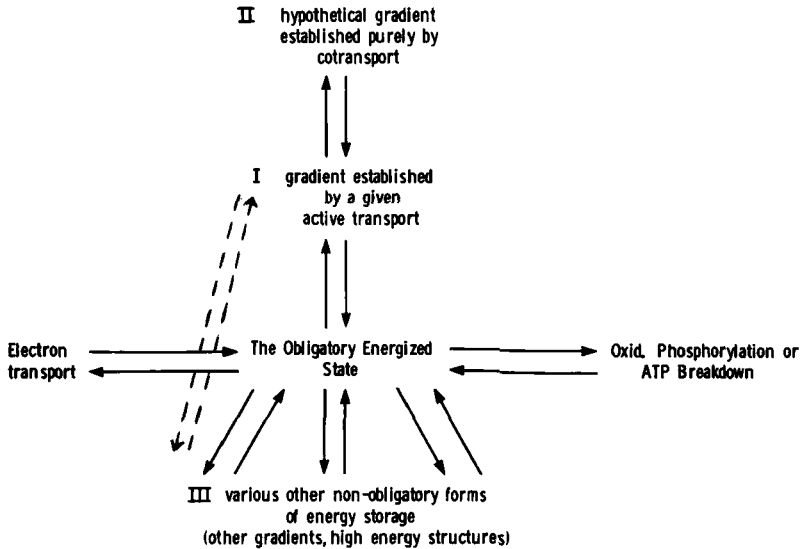


FIGURE 1. Hypothetical relations among energy gradients and flows in biological membranes. According to this diagram, an obligatory energized state, generated either by electron transport or by ATP cleavage, is necessary not only for the transduction of energy between these two but also for the generation of gradients by the membranes. These gradients can then represent additional forms of energy storage besides the essential energized state, and are to various degrees interconvertible. For example, the gradient represented by I may produce the gradient represented by III, either via the central energized state or by another route of cotransport (---). The text will consider another possibility: in certain cases gradients may be interconvertible, but one of them may have no other mode of generation. That is, the two may lie in series as shown here at upper center, rather than in parallel.

aminoisobutyric acid (AIB). Each incubation took place in a Krebs-Ringer bicarbonate (KRB) buffer, and the Na^+ concentration was reduced to the range of 6–30 mN by choline replacement. For the first two of these successive incubations, no amino acid was added; for the last two, AIB was added to the medium at 10 mM. Each 5-min incubation was terminated by a 2.5-min centrifugation, the packed cells then being resuspended in the next portion of the medium. The Ca^{2+} level was maintained at 0.25 mM throughout these experiments, and 0.5 mM dithiothreitol was included in the medium. In other respects the collection and handling of the Ehrlich cells followed our earlier descrip-

tions.¹² For the actual measurement of initial uptake rates, in all cases we used a KRB buffer with $^{22}\text{Na}^+$ at 120 mN, but with K^+ omitted to minimize Na^+ extrusion by the $\text{Na}^+\text{-K}^+$ pump. The uptake interval of 1 min was terminated as usual by dilution with ice-cold choline-containing KRB, which was followed by prompt, brief centrifugation to reisolate the cells.

As TABLE 1 illustrates, this special treatment lowered the endogenous pool of amino acids in the Ehrlich cell to less than one-third, and that of the neutral amino acids to about one-fifth, of their aggregate initial levels. The extracted amino acids were replaced almost quantitatively by AIB, which now represented 70–75% of the pool.

When the washed cells had not been treated in this way, the apparent flux stoichiometry for AIB uptake during 1 min,

$$\frac{\Delta V_{\text{Na}^+} \text{ entry due to presence of amino acid}}{\Delta V_{\text{aa}} \text{ entry due to presence of Na}^+}$$

showed a value of about 2.0 (FIGURE 2a). That is, about two ^{22}Na ions are taken up for each ^{14}C -AIB molecule that enters the cell. For sarcosine this value was about 1.35. These ratios remained approximately constant within concentration ranges of 0.3–6 mM amino acid (FIGURE 3).

When cells modified as just described ("AIB cells") were used, however, very little $^{22}\text{Na}^+$ accompanied the further uptake of AIB, which was now pre-

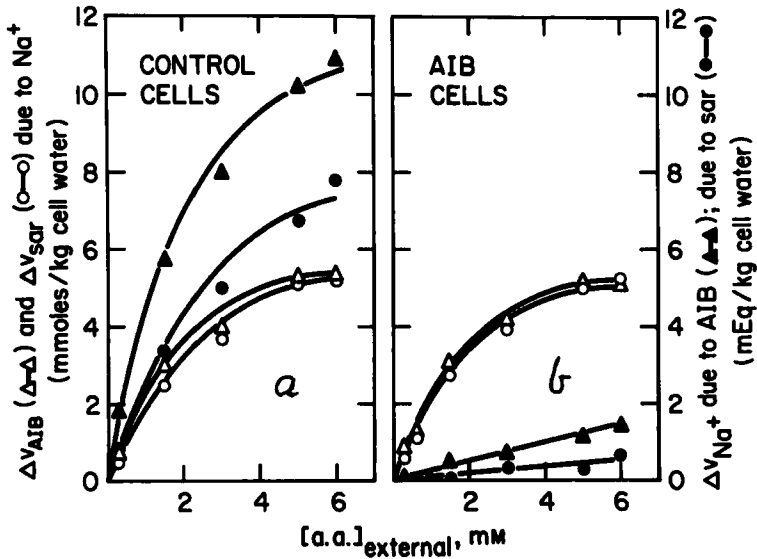


FIGURE 2. Major dissociation of the flow of Na^+ from the uphill flow of AIB or sarcosine, produced by modifying the pool of intracellular amino acids. (a) The washed Ehrlich cell takes up about 2 equivalents of Na^+ (upper curve) for 1 mole AIB, about 1.4 equivalents of Na^+ for 1 mole sarcosine. (b) When modified to contain about 75% AIB and greatly decreased levels of ordinary amino acids (TABLE 1), the cell still takes up AIB or sarcosine in a normal, Na^+ -dependent manner, but with little or no accompanying uptake of Na^+ .

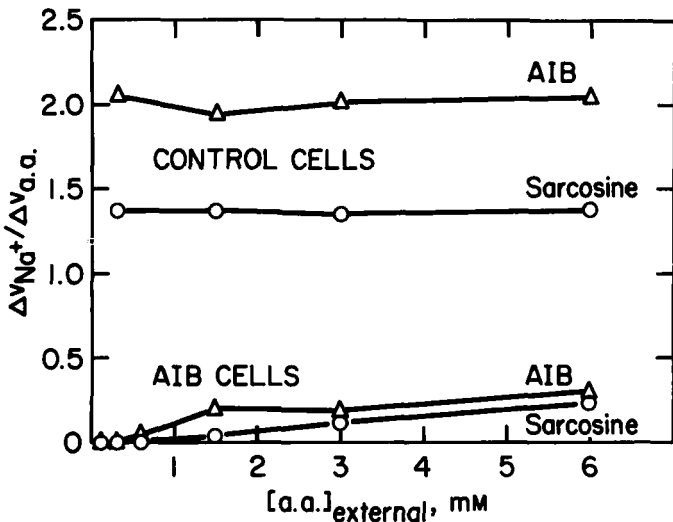


FIGURE 3. Effect of concentration of the external amino acid on the flux stoichiometry ratio (acceleration of Na^+ entry due to presence of the amino acid/acceleration of amino acid entry due to the presence of Na^+) in unmodified and AIB-enriched cells. The data used were the same as in FIGURE 2.

sented in labeled form, although the rate of amino acid uptake was essentially unaltered (FIGURE 2b, upper curves). The flux stoichiometry ratio is accordingly very low, in the range 0–0.3 (FIGURE 3, lower curves). Incidentally, note the tendency of the ratio to rise somewhat with the external substrate level. It is conceivable that the largest effect of the internal AIB is obtained when its concentration is highest relative to the external level of AIB or sarcosine used. Pre-treatment of the Ehrlich cell with sarcosine or *N*-methyl-AIB, instead of AIB, had similar tendencies largely to eliminate the uptake of Na^+ ordinarily associated with the uptake of System *A* substrates. The effect can also be seen when either of these amino acids is used instead as the test substrate with which to observe the flux stoichiometry of the modified cells.

Significantly, the uptake of AIB remained as Na^+ -dependent as ever in the modified cells. That is, the rate in the absence of Na^+ was very low and could be ascribed to difficult-to-saturate or unmediated routes rather than to System *A*. In the presence of Na^+ , uptake continued to levels as high as 95 mM, with gradients as large as 60 mM.

When instead we completed the sequence of four incubations without adding any amino acid to the media, the endogenous amino acids were decreased in the aggregate by less than one-half. On subsequent tests with labeled AIB or sarcosine, such cells showed normal, unaltered ratios of flux stoichiometry. When only 0.1 mM AIB was added (that is, only 1% of the usual amount), displacement of endogenous amino acids was almost as extensive as with 10 mM AIB, even though AIB accumulation was small (TABLE 1), but the subsequent stoichiometry of the uptake of ^{14}C -AIB and $^{22}\text{Na}^+$ remained normal (FIGURE 4). Accordingly, the dissociation here of amino acid uptake from Na^+ influx we at-

TABLE I
AMINO ACID CONTENTS IN THE EHRlich CELL AFTER VARIOUS TREATMENTS *

Treatment	Control Cells	AIB Cells	Low-AIB Cells	L-Norleucine Cells	L-Norleucine Restored Cells
mmole/kg cell water					
Aspartic + glutamic acid	8.06	3.84	3.38	6.57	3.10
Serine + threonine	3.34	0.76	1.06	1.46	1.23
Proline	2.82	0.44	0.55	0.59	0.44
Glycine	6.81	2.20	2.15	2.90	3.56
Alanine	6.14	0.39	0.44	0.49	0.44
Valine	0.93	0.14	0.28	0.35	0.36
Methionine, isoleucine, leucine, tyrosine, phenylalanine	1.73	0.47	1.06	0.93	0.97
AIB	0	21.8	0.67	0	2.27
L-Norleucine	0	0	0	14.1	12.2
Σ (amino acids)	29.8	29.3	9.59	27.4	24.6

* The analyses were made by column chromatography with an automatic amino acid analyzer. See the text for details of treatment of the cells.

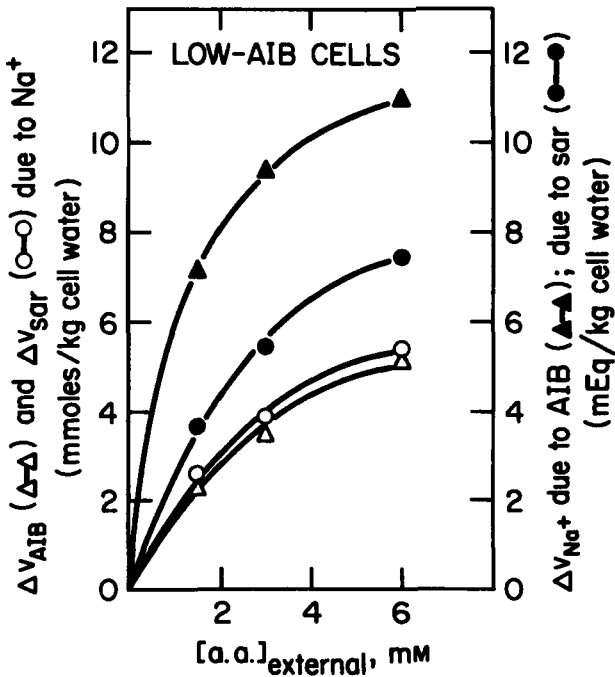


FIGURE 4. Uptake in Ehrlich cells whose endogenous amino acids have been depleted by incubation at low AIB levels; they show no significant changes in the flux stoichiometry ratio. Compare this with FIGURE 2a. The composition of the cellular amino acid pool is shown in TABLE 1.

tribute to the high intracellular AIB level combined with the lowered level of endogenous amino acids, not to the latter factor alone.

When we used instead ^{14}C -L-methionine or ^{14}C -L-norleucine to test uptake by the AIB cells, we observed normal stoichiometry between the inflow of Na^+ and the test amino acid (FIGURE 5). Hence the structure of the principal amino acids on both sides of the plasma membrane appears to be decisive for the flux stoichiometry.

When, however, norleucine was used as the displacing amino acid during the pretreatment of the cells, even though extensive displacement and extensive accumulation of norleucine took place (TABLE 1), the subsequent flux stoichiometry ratio remained unaltered at a value of about 2 (FIGURE 6a). Further-

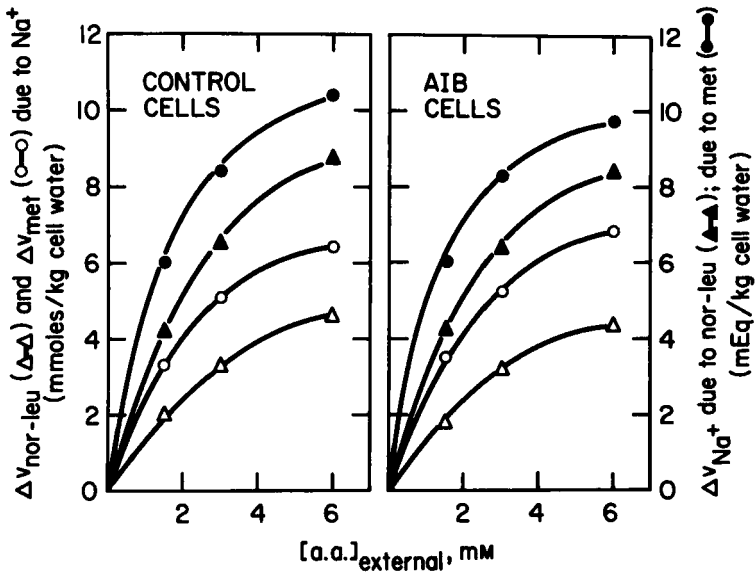


FIGURE 5. Uptake by Ehrlich cells in which AIB has replaced about three-quarters of the endogenous free amino acids. They show a normal flux stoichiometry ratio in their uptake of L-norleucine or L-methionine and Na^+ .

more, when the AIB cells were further treated with 6–10 mM norleucine (for two 5-min intervals), 90% of the AIB was displaced, half of the total pool now being attributable to norleucine. Such cells no longer behave as AIB cells, but as “norleucine cells;” that is, the flux stoichiometry is normal (FIGURE 6b; see also the figures on norleucine-restored cells in TABLE 1).

The reader might suspect that the subsequent uptake of AIB by the AIB cells occurs by exchange with the previously accumulated AIB. This is by no means the case (TABLE 2), since large net quantities of AIB are taken up, to produce apparent gradients as high as 89 mmole/kg water, with comparatively little further loss of cellular amino acids.

Similarly, FIGURE 7 shows the mean result of three experiments in which the AIB taken up during the preparation of the so-called AIB cells was tritium-

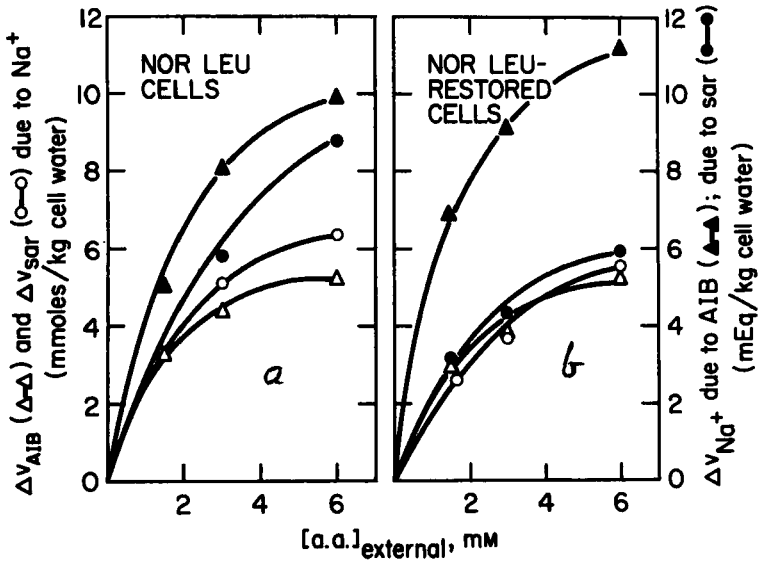


FIGURE 6. Uptake by Ehrlich cells in which norleucine has displaced substantial portions of the endogenous amino acids, either directly (a) or after preliminary AIB enrichment (b). They retain high ratios for the flux of stoichiometry of Na^+ and AIB or sarcosine.

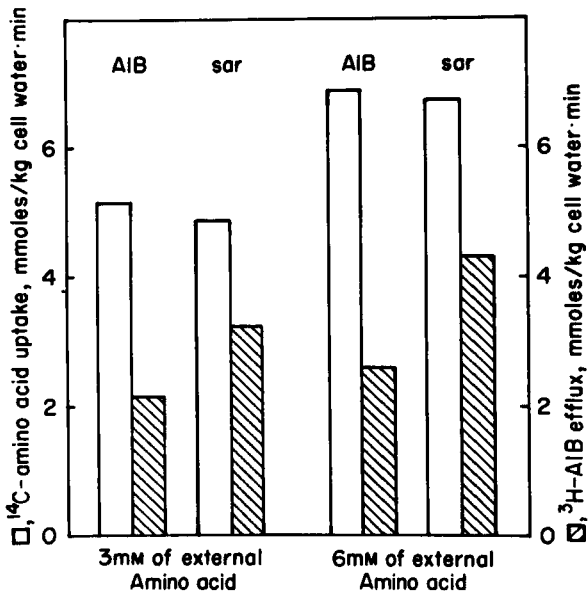


FIGURE 7. Balance between uptake of ^{14}C -labeled AIB (\square) and exodus of ^3H -labeled AIB (\boxtimes) from "AIB cells." The difference in height of the clear and the striated bar indicates that net uptake took place in each case.

labeled, and that taken up during a following incubation of 1 min was ^{14}C -labeled. A rapid net uptake of AIB obviously occurred under these conditions.

Simultaneous measurements of Na^+ exodus have been made, and the net Na^+ flux during amino acid uptake by these several types of cells is under study. These results, which will be reported subsequently, confirm the conclusion that the net uptake of Na^+ ordinarily associated with the uptake of System *A* substrates can be largely abolished by alterations of the internal amino acid pool, as described above.

Why does a dissociation of amino acid and Na^+ uptake occur when the amino acid structure of the internal pool is changed from the composite pattern

TABLE 2
AMINO ACID CONTENT OF "AIB CELLS"
AFTER FURTHER INCUBATION WITH AIB *

Amino Acid	Cell Content mmole/kg cell water
Aspartic acid	0.16
Threonine	0.54
Serine	0.41
Glutamic acid	0.10
Proline	0.64
Glycine	3.98
Alanine	0.21
Valine	0.20
Methionine	0.07
Isoleucine	0.20
Leucine	0.44
Tyrosine	0.15
Phenylalanine	0.16
AIB	98.7
Total	106.0

* Subsequent uptake of AIB by AIB cells is concentrative, despite the small accompanying uptake of Na^+ that they show. The Ehrlich cells were first incubated for four intervals of 5 min each in a concentration of 30 mN Na^+ , the last two also in levels of 10 mM AIB. These cells were then subsequently incubated for three more intervals of 40 min each in 10 mM AIB and 120 mN Na^+ .

shown by the endogenous amino acids to that in which AIB is the dominant amino acid? Responses of the Na^+ flow to amino acid structure undoubtedly arise because Na^+ and the amino acids take a side-by-side position in the ternary complex formed at the site, as has been discussed elsewhere.¹ These responses in System *A* cannot, however, be quite the same as those discussed for System *ASC* in one of our papers. (Koser and Christensen,¹³ page 18). FIGURE 8 shows our provisional explanation. The upper cycle shows both Na^+ and the amino acid substrate *S* being unloaded inside the cell, the kinetics favoring the return of the carrier in either the fully loaded or the totally unloaded state. The lower cycle shows only *S*, not Na^+ , being unloaded inside the cell. This view that the carrier has no vacant place for Na^+ during much of its sojourn within the cell is

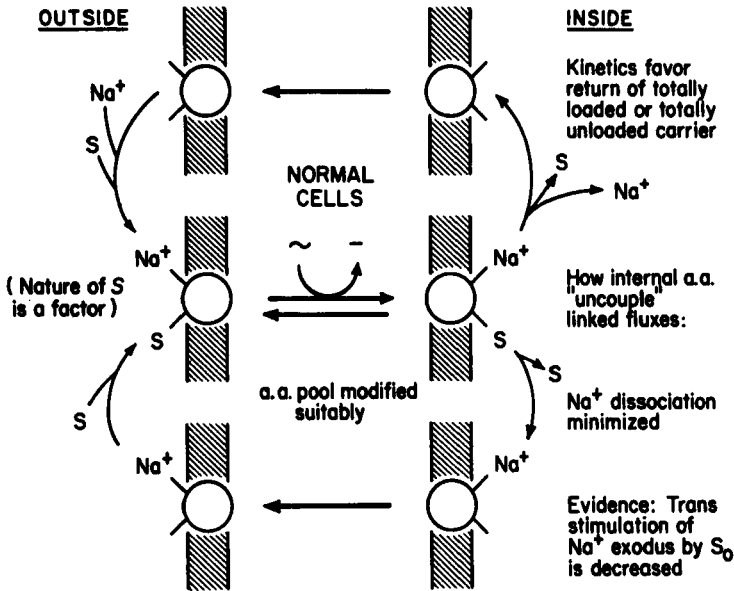


FIGURE 8. Provisional explanation of the "uncoupling" of Na^+ flux from the uptake of certain amino acids when the composition of the cellular pool of amino acids is suitably modified. See text for explanation.

supported by our observation that external AIB is only about one-half as effective in the *trans* stimulation of Na^+ efflux from AIB cells as from normal cells.

The above results provide plausible rationalizations for the inconsistencies observed heretofore by various investigators as to whether Na^+ flow is needed if amino acids are to flow uphill into ascites cells. Furthermore, they show that the flow of Na^+ is not an obligatory source of energy, and must be regarded therefore as an incidental source. The Na^+ -dependent amino acid uptake appears to be placed in position III rather than in position II in the scheme shown in FIGURE 1.

Breakdown of the Alkali-Metal Gradients and Dissipation of Other Forms of Energy Storage in the Membrane; The Need to Identify the Obligatory Energy Flow to Na^+ -Independent Transport

The conclusion that there is an essential connection between alkali-metal ion gradients and glycine gradients in the pigeon red blood cell has been supported by a recent observation by Terry and Vidaver.¹⁴ As the cation gradients broke down under the influence of gramicidin D, the ability of the cells to maintain the glycine gradient was also lost. Since the level of nucleotide polyphosphate was concurrently decreased by only 50%, these authors interpreted the result to mean that the alkali-metal gradients had been dissipated rather specifically.

We have subsequently observed an essentially similar relation among these gradients in the Ehrlich ascites tumor cell, noting (FIGURE 9) that only very

small gradients of AIB were retained after a 15-min exposure to gramicidin; in the meantime Na^+ had entered the cell and K^+ had largely escaped from the cell. If the medium contained the cation choline in place of Na^+ , however, the loss of K^+ in the presence of gramicidin was small. This result suggested that under the conditions followed, gramicidin does not occasion much exchange between H^+ and K^+ across the plasma membrane; this behavior is in partial contrast to results reported by Chappell for the red blood cell.¹⁵

Our doubts whether treatment with gramicidin had left other forms of energy storage of the membrane largely undiminished, as was suggested by Terry and Vidaver for the pigeon red blood cell, were raised by the discovery that in the Ehrlich cell the Na^+ -independent uptake of a substrate of System *L*, namely 4-amino-1-methylpiperidine-4-carboxylic acid (MPA), was also largely terminated under the action of gramicidin. Furthermore, this effect depended on the presence of Na^+ , rather than of choline, in the medium (FIGURE 9, center). Hence we see a Na^+ -dependent inhibition of a Na^+ -independent transport, a

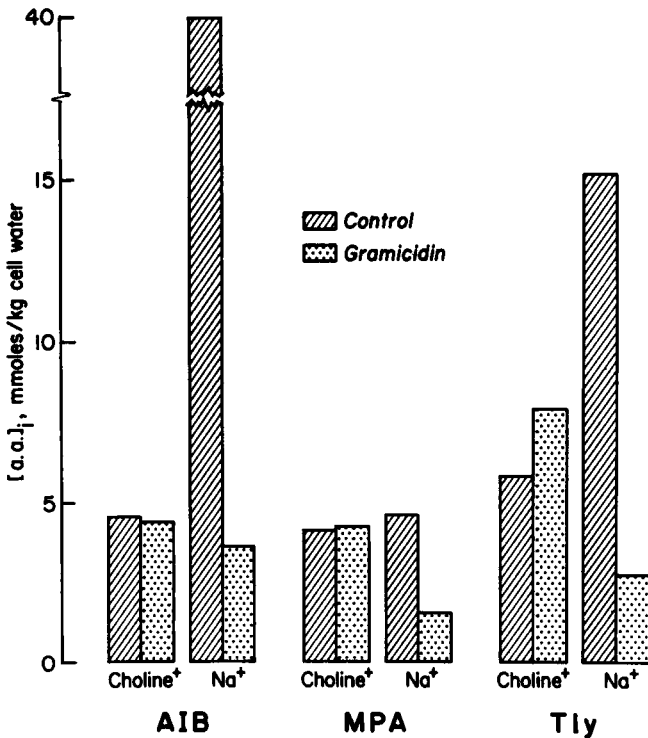


FIGURE 9. Effect of gramicidin on retention of the ability of the Ehrlich cell to accumulate amino acids. Gramicidin D was added, to a final concentration of 1.8 μmol , in a solution of 90% ethyleneglycol and 10% ethanol; 6 μl of this solution was added per ml medium. Uptake was then observed for 15 min at 37° C, pH 7.4, in a KRB medium, or in the same medium with choline instead of Na^+ . Left, effect on AIB uptake in choline- and in Na^+ -containing medium. Center, the same for MPA. Right, the same for thialysine.

TABLE 3
EFFECTS OF GRAMICIDIN ON ATP LEVELS * IN THE EHRlich CELL

Medium	Gramicidin	ATP nmole/mg dry cell
Choline	—	0.98
	+	0.75
Sodium	—	1.29
	+	0.22

* ATP was assayed fluorometrically, using the hexokinase-glucose-6-phosphate dehydrogenase coupled reaction. See the text for details on the treatment of the samples analyzed.

phenomenon observed earlier in another context.¹⁶ Similar results were obtained with thialysine, another of our gradient-sensing substrates (FIGURE 9, right). Only when the medium was based on Na⁺ rather than on choline did gramicidin prevent accumulation of the amino acid. Assays for the simultaneous levels of ATP (TABLE 3) showed that its level fell by 75% or more when Na⁺ was present but by only about 20% when choline had replaced Na⁺. Our provisional interpretation is that ATP is depleted by futile pumping of Na⁺ when that cation is available, and that the depletion of ATP or equivalent forms of energy storage, rather than the breakdown of the alkali-metal ion gradients, accounts for the failure to accumulate MPA in the Ehrlich cell.

The results summarized in TABLE 1 and FIGURES 2–6 show that we still need to identify the primary, obligatory coupling state or process that drives uphill, Na⁺-dependent amino transport in the Ehrlich cell. Furthermore, the same need applies to the Na⁺-independent category of neutral amino acid uptake. In trying to establish the net uphill operation of Na⁺-independent transport of ordinary amino acids, the problem has been that their uptake is accompanied by losses of endogenous amino acids. These losses occur partly by exchange for the test substrate, and it can be argued that exchange drives all the concentrative uptake observed in a given experiment. Osmotic shock and other extractive treatments for first diminishing the endogenous pool have been tried, although such methods carry a risk of otherwise injuring the function under study. Some investigators have taken advantage of these inherent difficulties to raise doubts about either the reality or the net operation of System L.

FIGURE 10 illustrates this problem in the case of MPA, and shows one pathway to its solution. So long as the time interval was not over 15 min, the Na⁺-dependent component was relatively small, and at 1 min it was not measurable. But during an hour of uptake, especially at 3 mM, the Na⁺-dependent component became substantial. During the first minute, 0.2 mM MPA was concentrated about 40-fold, 1 mM MPA about 20-fold. With the external MPA at 3 mM, the Na⁺-independent gradient formed was substantially larger than the simultaneous loss of endogenous amino acids. The same hazard has been observed with the norbornane amino acid: by working at high substrate levels and over extended intervals, one can exaggerate the relative contribution of an otherwise very small Na⁺-dependent component of the uptake, and the suspicion can be raised that the net uphill transport may be attributable to that agency.

FIGURE 11 shows that under carefully selected conditions, the Na^+ -independent uptake of these two amino acids considerably exceeds the concurrent losses of amino acids that might well exchange for them. This conclusion is not changed if we include all neutral endogenous amino acids in the calculated loss.

During the formation of substantial gradients of MPA or 2-aminonorbornane-2-carboxylic acid (BCH) in a Na^+ -free medium, uptake is strongly inhibited by 2,4-dinitrophenol, much more than is the case for the Na^+ -independent uptake of the ordinary neutral amino acids.² Uptake is also sensitive to oligomycin. Under anaerobic conditions, with glucose present at 10 mM, the uptake of MPA can be inhibited by oligomycin, but only at 10 $\mu\text{g}/\text{ml}$, 100 times more than is required under aerobic conditions.² These results are consistent with a dependence on mitochondrial ATP production under aerobic conditions, and on the access of ATP of glycolytic origin, presumably to a plasma membrane ATPase, under anaerobic conditions. The levels of ATP measured in the Ehrlich cells during these experiments are also consistent with that interpretation (TABLE 4). These results indicate that the Na^+ -independent transport ac-

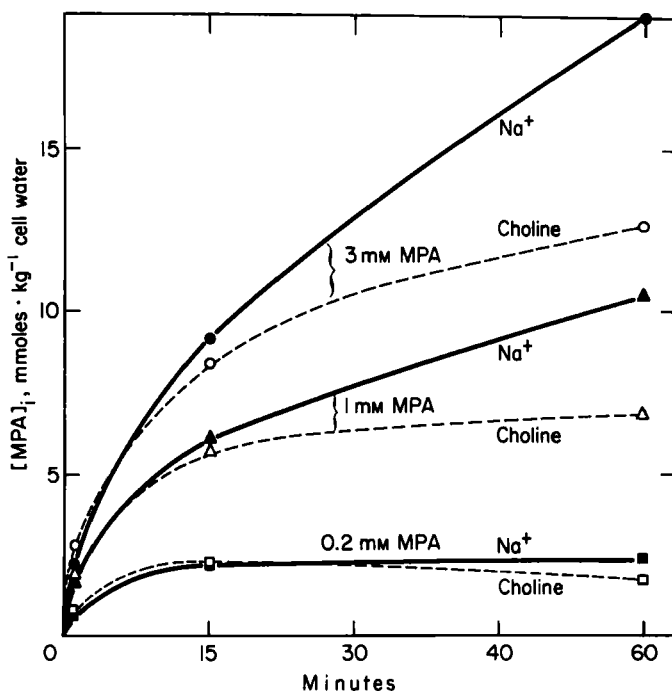


FIGURE 10. Effect of MPA concentration and of interval of observation on the Na^+ -dependence of MPA uptake by the Ehrlich cell. Uptake was observed at 37° C, pH 7.4, from KRB medium, with $\text{Na}^+=128$ mN and choline=15 mN, or from the corresponding medium in which choline completely replaced Na^+ . The uptake during 1 min was not Na^+ -dependent; that during 15 min became slightly so only when the MPA level was raised to 1 mM or more. At 60 min the extra uptake observed in the presence of Na^+ became appreciable at both of the higher MPA levels.

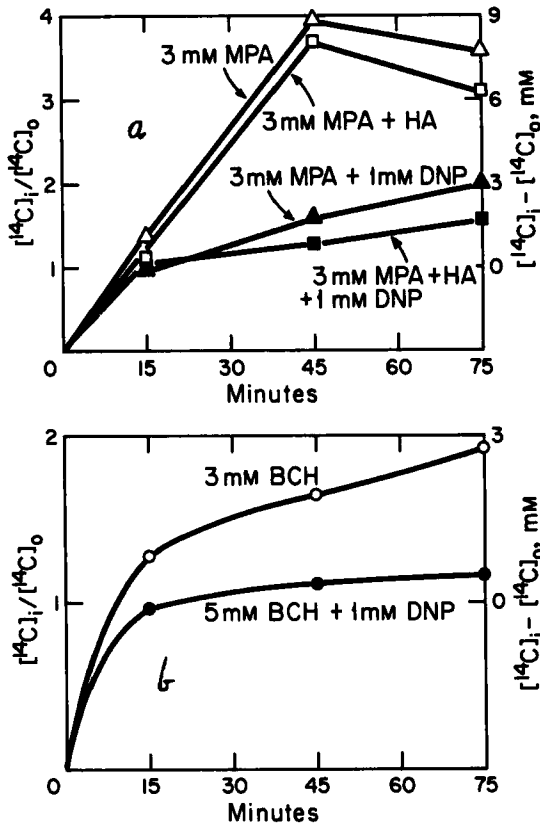


FIGURE 11. Concentrative nature of Na^+ -independent amino acid uptake. Above, uptake of 3 mM MPA at 37°C from Na^+ -free, choline-containing KRB medium, pH 7.4, which for the next-to-highest curve also contained 10 mM homoarginine to minimize migration by the cationic amino acid transport system. Below, uptake of 3 mM BCH [(−)b isomer] from Na^+ -free Krebs-Ringer phosphate medium, pH 5.0. The Ehrlich cells had first been treated as in TABLE 1 with 0.1 mM AIB to diminish their content of exchangeable amino acids. Their loss of residual endogenous alanine, valine, leucine, isoleucine, methionine, phenylalanine and tyrosine in the aggregate, associated with the uptake shown, was 0.59 mmole/kg cell water for Experiment a, and 1.25 for Experiment b. A similar experiment with BCH at pH 7.4 showed results very similar to those in Figure 11b, with a maximal apparent gradient of 2.0 mM; the associated loss of the same seven endogenous amino acids was 0.58 mM. These losses were accordingly far below the gradients established by uptake, in mmole/kg cell water. In the meantime the ATP level had been approximately halved by the AIB treatment, so that the somewhat smaller gradients observed here are not unexpected.

tivity does require an external energy source, and hence is another worthy target for our testing of gradient-sensing by substrates. Indeed, the significance of the results to be described below does not depend greatly on the interpretation one may wish to place on the dichotomy between the Na⁺-dependent and the Na⁺-independent categories of uphill amino acid transport. Of course, gradient-sensing substrates for a facilitated diffusion should also be informative, provided only that we have proved that it is really a facilitated diffusion.

What Structural Features Permit Special Sensing of Membrane Gradients?

The structural features of the amino acid side chain that lead to gradient-sensing properties have so far been encountered in the first instance fortuitously, and in subsequent instances by imitation. The side chain should first of all be acceptable to the transport system in question through the presence of a suitably disposed apolar mass, as follows:

TABLE 4
ATP LEVELS OBSERVED IN THE EHRLICH CELL AFTER INCUBATION
IN PRESENCE OF OLIGOMYCIN OR DINITROPHENOL

	ATP Levels		
	Experiment 1		Experiment 2
	nmol/mg dry cells		
	in O ₂	in N ₂	in O ₂
Control	2.48	3.09	0.94
+ oligomycin	0.16	2.78	(+DNP) 0.11

* Experiment 1 was made in Krebs-Ringer phosphate medium with or without oligomycin (10 μg/ml in 10 μl ethanol); Experiment 2 in Krebs-Ringer bicarbonate medium with or without 2,4-dinitrophenol, added in NaCl solution to a 1 mM concentration.

1. For System *A*, the side chain should be a linear chain,⁸ as illustrated by α,γ-diaminobutyric acid and *S*-(2-aminoethyl)-cysteine or thialysine. The latter is acceptable also to System *L*.

2. For System *L*, the side chain should optimally be branched or cyclic, not only to increase the apolar mass, but also to exclude the substrate as completely as possible from System *A*.⁸ The best-studied examples are MPA, *cis* 1,4-diaminocyclohexanecarboxylic acid (DCH), and α,α-diethylglycine (DEG).

A gradient-sensing property is introduced then by either of two totally different features: (1) for either System *A* or System *L*, a distal amino group with a p*K'*_{a2} in a range at least as wide as 7–8.4 (this amino group may be primary, secondary, or tertiary, the last as illustrated by MPA); or (2) for System *L*, a feature contributed by DEG, presumably a crowding of a particular part of the receptor site, leading to marginal accommodation by the site for entry and almost complete exclusion from the site for exodus. As a result, this analogue is gradually accumulated to high gradients.¹⁷ Although this and other features of the uptake of DEG are unusual for System *L*, we have now convinced ourselves,

contrary to an earlier view, that its uptake probably occurs largely by that system.

The Phenomenon

The gradient-sensing behavior in the second case is of a more mysterious nature, namely, a greater than usual intensification of uptake caused by lowering of the external pH, exodus being slowed at the same time. This effect was first reported for DEG.¹⁷ Consistently with this behavior, the uptake of DEG is conspicuously accompanied by the uptake of H⁺ (FIGURE 12). An analogue of DEG, α,α -dicyclopropylglycine, the mediated entry of which into the Ehrlich cell is extremely slow,¹⁹ serves here as a control: it does not stimulate any significant migration of H⁺. Uptake of H⁺ along with BCH has not yet been observed, even though this amino acid is a typical substrate of System L. When its uptake, or that of such ordinary amino acids as leucine, isoleucine, or phenylalanine, is measured from a medium in which choline replaces Na⁺, a stimulation of uptake is, however, also observed on lowering of the pH from 7.4 to 5 (TABLE 5). In the presence of Na⁺ this characteristic is presumably masked by the simultaneous inhibitory action of H⁺ on the uptake of these amino acids by

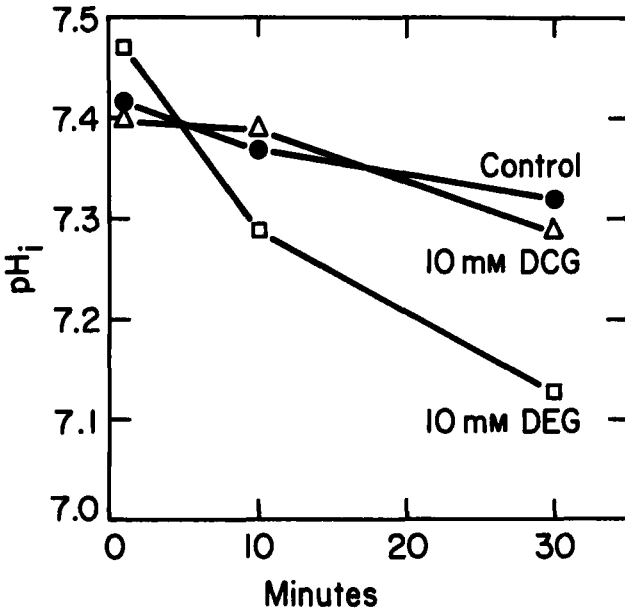


FIGURE 12. Acidification of the interior of the Ehrlich cell during the uptake of DEG. The cells were incubated at pH 7.4 and 37° C in 100 volumes of Krebs-Ringer phosphate medium containing 10 mM DEG or α,α -dicyclopropylglycine (DCG). The latter serves as a control substance, since its mediated uptake by the Ehrlich cell is extremely slow. Changes in cellular pH were observed through changes in the distribution of labeled DMO, according to the technique used by Poole *et al.*¹⁸

TABLE 5
EFFECT OF pH ON UPTAKE OF SEVERAL ¹⁴C-LABELED AMINO ACIDS *

Amino Acid	Velocity of Uptake		Velocity at pH 5
	pH 7.4	pH 5.0	Velocity at pH 7.4
	mmol/kg cell water · min		
DEG	0.194	0.540	2.78 (2.73)
Isoleucine	2.41	3.04	1.26
Norleucine	1.52	2.02	1.33 (1.55)
<i>b</i> -BCH isomer	1.25	1.56	1.25 (1.12)
Valine	1.25	2.18	1.74 (1.34)
Methionine	1.44	2.68	1.86
Phenylalanine	1.20	1.80	1.50
DPG	0.162	0.390	2.41

* Uptake was measured during 1 min at 37° C from Krebs-Ringer phosphate medium in which choline replaced Na⁺. Each amino acid was initially 0.2 mM in the suspending medium, except DPG, which was 0.5 mM. The parenthetic values are for separate experiments made in the same way. The BCH isomer used was the levorotatory *b* isomer.

System *A*. Whether the coupling of amino acid flows to H⁺ flows in System *L* is variable and is a determinant of the extent to which the substrate is concentrated, or whether it is the communication of the H⁺ gradients to the surfaces of the membrane that is variable, we do not yet know. In any event, the Na⁺-independent uptake of all tested System *L* substrates is distinctly stimulated when the external pH is lowered (TABLE 5), and exodus into Na⁺-free medium is distinctly inhibited (FIGURE 13). Accordingly the substrates are accumulated much more strongly at pH 5, this effect being exaggerated for DEG. Evidence of inward cotransport between H⁺ and neutral amino acids has already been seen in yeast; cellular K⁺ is released in that case in exchange for the hydrogen ion.²⁰

In contrast, lowering of the pH slows the uptake of MPA and DCH, but in these cases the effect can be rationalized as a logical consequence of a more complete conversion of these compounds from their zwitterionic to their cationic forms, one of the latter clearly being the substrate of System *L* (see Figure 6 in Reference 1). So far we have not been able to segregate from this inevitable effect of the lowering of pH another possible component that is not dependent on the titration of the distal cationic group.

Let us turn then to the intense effect of a distal amino group with a suitably low pK_a on the flux asymmetry and maximal rates of uptake of amino acids. Here also we use for the standard of reference ordinary substrates that do not bear a second nitrogen atom. We first observed an effect of this kind two decades ago with α,γ -diaminobutyric acid,^{5, 21} although only much more recently have we been able to show that this high V_{max} uptake is effected by neutral amino acid system *A*.²² The lower homologue, α,β -diaminopropionic acid ($pK'_{a2} = 6.7$) shows a milder form of the same behavior.^{5, 21, 22}

It was while we were trying to design metabolism-resisting model substrates for the transport system for cationic amino acids²³ that we discovered that many synthetic diamino acids are much poorer substrates for that system than

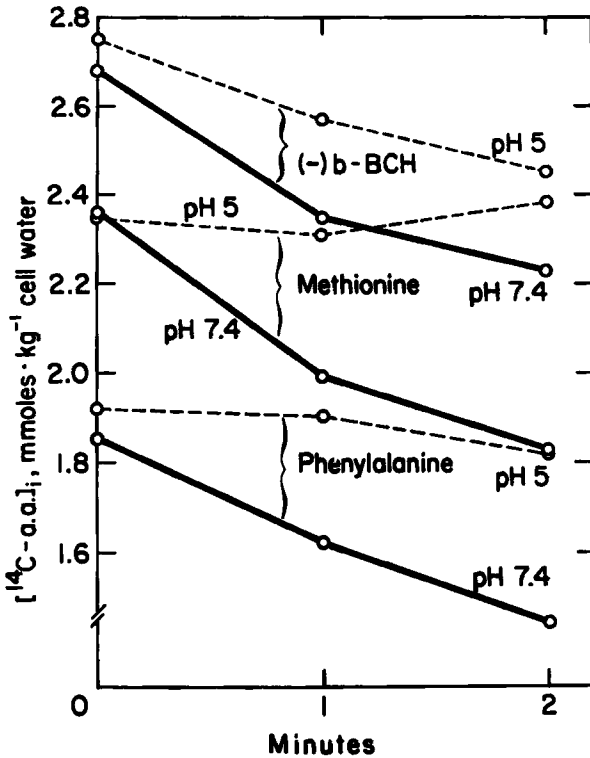


FIGURE 13. Slowing of the exodus of several amino acids on lowering of the external pH to 5. Ehrlich cells were loaded with the selected ^{14}C -amino acid to the indicated level by incubation for 5 min at 37°C in a 1 mM solution in Na^+ -free, choline-containing Krebs-Ringer phosphate medium, pH 7.4. Exodus was then permitted from the cells into 300 volumes of an initially amino-acid-free medium that was otherwise of the same composition, except that where dashed lines are shown the pH of the medium was 5.

we should expect from their degree of cationicity, and much better substrates for System *L* than we should have predicted on the same basis. The critical factor in securing this effect appears to be that the pK'_a should be rather low. Lysine and ornithine are transported largely as cationic amino acids, and only to a very minor degree as neutral amino acids. When an inductive effect can be transmitted to the distal amino group, however, either by bringing it near to the α -amino group, as in α,γ -diaminobutyric or α,β -diaminopropionic acid, or by the insertion of a sulfur atom in place of a methylene group midway between the two amino groups, as in thialysine, then transport tends to be transferred to the neutral system. The criterion for this shift is a shift in the inhibitory action from a principal one on the uptake of homoarginine, a type substrate for the cationic amino acid system, to a principal action on the uptake of norbornane amino acid, a model substrate for System *L* (TABLE 6), and also the parallel effects seen when the roles of substrate and inhibitor are reversed in these tests.

Had the only effect of a distal amino group with a rather low pK_a value been to permit a diamino acid to act like a neutral amino acid in transport, these effects could be accounted for by the presence of an apolar microenvironment at that part of the receptor site where this amino group falls, stabilizing the group in the unprotonated form. It becomes necessary also, however, to explain why the same effect does not apply to the exodus, which for these analogues is very slow. We have to propose that the effect that occurs at the outer receptor site, permitting the distal basic group to be tolerated, does not occur at the inner receptor site. This circumstance by itself is not remarkable: after all, at pH 7.4 thialysine should be 91% cationic and only 9% zwitterionic in free solution, and perhaps it may be the wrong zwitterion (the α,ω zwitterion) at that. The remarkable aspect of this phenomenon, aside from the ability of the external receptor to "see" these amino acids largely as the rarer α,α zwitterion, is the apparent change in the nature of the receptor site from the external to the internal surface, so that this ability is lost at the inside. From this change, we suppose, arises the extraordinary accumulation of amino acids of this kind.

TABLE 6 shows that these diamino acids are characteristically accumulated from 10- to 25-fold by the Ehrlich cell. At the same time, the maximal velocities for uptake are exceptional. For diaminobutyric acid, the V_{max} for the Na^+ -dependent uptake is over 60 mmole/kg cell water·min.²² For thialysine, both the Na^+ -dependent and the Na^+ -independent components show V_{max} values that are three times the usual values. MPA shows a correspondingly high V_{max} for its Na^+ -independent uptake, which can be inhibited by the norbornane amino acid. During the uptake of MPA, as we have shown, the exodus of exogenous amino acids can be proportionally negligible, so that exchange cannot play a significant role.

These effects are what we mean by the term "gradient-sensing": structural features have caused an unusual degree of asymmetry between entry and exodus rates, and unusual maximal rates of uptake. These effects require an energy

TABLE 6
SOME TRANSPORT PROPERTIES OF SELECTED DIAMINO ACIDS
IN THE EHRlich ASCITES-TUMOR CELL

Diamino Acid	K_i on Uptake of Homoarginine	K_i on Uptake of Norbornane Amino Acid	V_{max} for Uptake	Distribution Ratio Observed at 60 Min (Na^+ Present) *
	mM	mM	mmol/kg cell water·min	
MPA	90	1	4 (Na^+ -independent)	25
DCH	30	2.7	4 (Na^+ -independent)	10
L-Thialysine	1.2	0.7	7 (Na^+ -independent) 10 (Na^+ -dependent)	30

* Although the listed distribution ratios were obtained with test amino acid at 0.2 mM, exceptionally high ratios were also obtaining at higher concentrations.

input, and may be expected to cause uptake to be highly dependent on the delivery of energy. The high sensitivity of the uptake of MPA to the presence of dinitrophenol (as already illustrated in Figure 2 of Reference 2) is of course consistent with that expectation.

Interpretation

What then is the nature of the gradient to which these amino acids are exposed through their special structure, so that they can be concentrated 10- to 25-fold? Thialysine and α,γ -diaminobutyric acid, being largely cationic in free solution, should respond to the transmembrane potential difference. MPA, being more extensively without net charge, should feel only a minor effect of that gradient, but all of the members of this class could well be expected to respond to pH differences across the membrane, depending on the degree to which their protonation changes from one aqueous phase to the other.

At the same time, the study of DEG has brought to our attention the generality with which the hydrogen ion is to some degree involved in System *L*, quite as clearly for substrates that lack a protonatable group on the side chain as for those that have such a group. We first discussed in 1958⁶ how the movement of neutral amino acids might generate and be responsive to transmembrane gradients of the hydrogen ion. Even though comigration of H^+ with the amino acid has not always been shown to occur, and appears to be a quantitatively variable feature, nevertheless pH sensitivity is a general feature of uptake. These findings suggest that gradients of H^+ may perhaps be the most significant aspect of the energized state of the membrane, at least for this transport system.

TABLE 7 includes Hempling's estimates of the transmembrane potential gradient for the plasma membrane of the Ehrlich cell; he used the chloride ion as a

TABLE 7
TRANSMEMBRANE GRADIENTS COMPARED WITH AMINO ACID CONCENTRATION
IN THE EHRlich CELL

	Equivalent Distribution Ratio for a Univalent Ion
Potential difference	
by Cl^- distribution (Hempling ²⁴): -12 to -24 mV	1.6-2.5
by impaling with microelectrodes (Lassen, et al. ²⁵): -24 mV	2.5
pH difference by DMO distribution	
after 30 min, aerobic, in pH 7.4 Krebs-Ringer phosphate (Poole <i>et al.</i> ¹⁸): $\Delta pH = -0.14$	1.4
present work, with or without 2 to 10 mM MPA: $\Delta pH = 0.1$	1.3
correcting for prediction that if potential difference is -24 mV, ΔpH should be 0.4, given passive H^+ distribution	0.5
Observed degree of concentration of 3 mM MPA (possible contribution of amino acid exchange negligible)	4-14

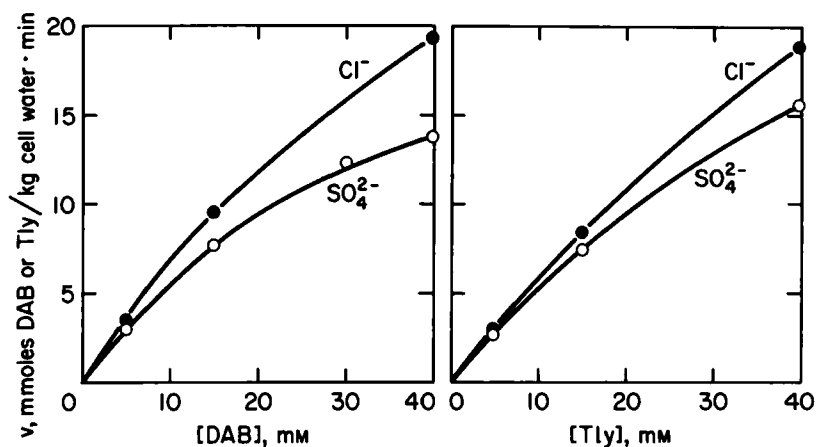


FIGURE 14. Retention of the high uptake of L- α,γ -diaminobutyric acid and L-thialysine when sulfate replaced Cl⁻ isotonically. Uptake of the labeled amino acid was observed during 1 min from KRB medium at 37° C, pH 7.4, or from the same medium in which sulfate replaced chloride at a concentration to minimize swelling or shrinkage of the cells.

measure.²⁴ Our chloride analyses have in general been in agreement with his. If the migration of chloride is to a significant degree electrogenic, so that it responds to the transmembrane potential gradient (statistically, of course, for the whole thickness of the membrane), then the gradient available from that source is not sufficient to account for the additional gradient that MPA can develop, relative to ordinary neutral substrates of the same transport system. Similar values for the transmembrane potential have been obtained by impaling the Ehrlich cell with a microelectrode (TABLE 6).²⁵

The conclusion that the potential difference expressed all the way across the membrane is insufficient is supported by our inability to decrease greatly the accumulation of α,γ -diaminobutyric acid or thialysine by isotonically replacing the chloride ion of the medium with sulfate ion, which penetrates the membrane only very slowly (FIGURE 14). Under these conditions the transmembrane potential should be decreased or even reversed; yet the initial rate of uptake is scarcely slowed.

TABLE 7 also provides an estimate of the pH gradient across the plasma membrane of the metabolizing Ehrlich cell, as calculated from the distribution of DMO. The potential difference recorded in the same table would lead one to expect a pH 0.4 units lower inside the cell than outside at a uniform electrochemical potential of H⁺ across the membrane. Hence a weak overall pumping of H⁺ outward is suggested by the gradient listed in TABLE 7. Although the recorded pH gradient is subject to experimental increase, as we have seen in FIGURE 10 and as is known from published work, it can scarcely have become large enough during our experiment to cause an amino acid to be concentrated 10- to 25-fold through linked transport.

Finally, we have added to TABLE 7 the partition coefficients to which one of the selected substrates can be concentrated by Ehrlich cell. Here we have a

problem in selecting the data. As has already been illustrated in Figure 2 of Reference 2, external MPA at between 2 and 3 mM can be concentrated at least 14-fold, with no possible significant contribution of energy from the concurrent exodus of endogenous substrates of the same transport system. By using lower levels of external MPA, we can obtain distribution ratios two or three times higher; but here we encounter the problem already referred to, which has heretofore handicapped demonstrations of the active character of System *L*. By using an external pH of 5, we can also get much higher gradients. In any event, the range provisionally supplied in TABLE 7 for the gradient of MPA that can be attained without significant assistance from exchange for Na^+ may be considered a conservative figure. This effect may be seen both with substrates that themselves appear to supply the proton apparently needed for an energizing cotransport, and with substrates that do not.

Accordingly our conclusion is that these special transport substrates are able to sense gradients larger than those estimated across the whole membrane. Therefore we need to suppose that gradients of a larger magnitude are being generated within the membrane, presumably in this case through the action of a membrane ATPase. Presumably it is the complex of the amino acid with the carrier site that senses these gradients. We may think of these gradients as gradients of pH. MPA may be thought of as reacting with the external receptor site as though the environment of the site had a pH perhaps two units lower than that of the external solution. At the internal surface this paradox is not seen; the receptor site there scarcely sees the diamino acid in an acceptable form. I suppose it is also possible, as was discussed by Green and Ji,²⁶ that the gradient maintained within the membrane are gradients of structural polarization, which would cause equivalent groups near one surface of the membrane to be much less highly protonated than those near the other surface.

We have dealt in some detail elsewhere¹ with the question, Which of the two nitrogen atoms of MPA and its analogues releases its proton first on the lowering of the hydrogen ion concentration in aqueous solution? Our experiments indicate that it is principally the α -amino group that does so, to generate the α,ω zwitterion. This result again emphasizes the special environment that is necessary to stabilize the amino acid in the rarer α,α zwitterionic form, which available evidence indicates is required for transport by the neutral amino acid systems under discussion.

Summary Exposition of the Gradient-Sensing Property

FIGURE 15 summarizes in a diagrammatic way the provisional proposals we offer on the nature and location of the intramembrane gradients that can intensify the vectorial character of the movements of suitably designed substrates for transport, using substrates of our second class. Part I of this figure shows an ordinary substrate of System *L*, in this case leucine, introducing its apolar side chain into a portion of the receptor site that is quite adequate to receive it and to form apolar bonding with it. Introduction of a stable cationic structure into the side chain (shown here is 4-amino-1,1-dimethylpiperidine-4-carboxylic acid, center section) leads to an unacceptable substrate, one that is unequivocally a cationic amino acid.²³ But if the distal cationic structure (right) has a low pK_a , the proton can be displaced at the site, and the gradient-sensing property may be observed.

I. SUBSTRATE STRUCTURES FOR TRANSPORT SYSTEM L

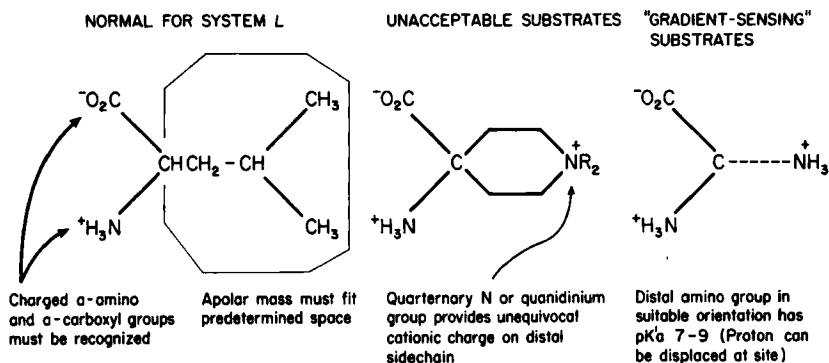
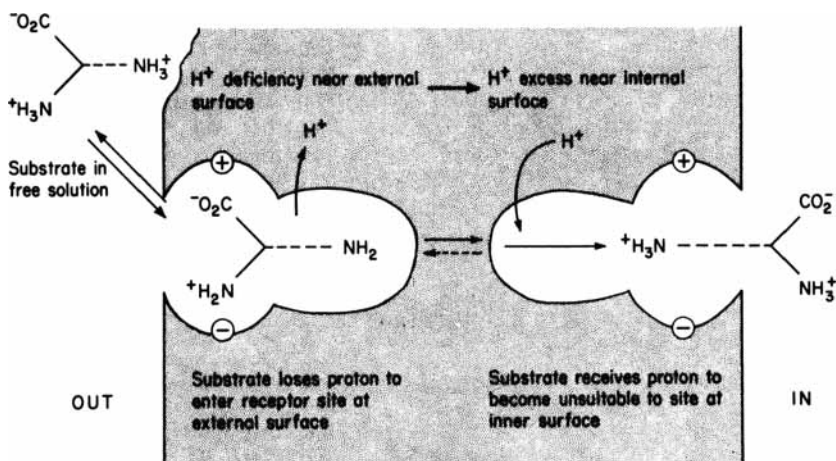
II SELECTED SUBSTRATES SENSE H^+ GRADIENT WITHIN MEMBRANE

FIGURE 15. Hypothesis on how selected substrates for transport may sense gradients otherwise largely confined to the membrane. In (I), at the left an ordinary substrate for System L, leucine, presents an apolar side chain that must be accommodated in a rather generous space at the site. Other structures not shown recognize the α -amino and the α -carboxyl group. In the center a substrate is shown that is unacceptable to System L because it has an unequivocally cationic quaternary N. At the right is shown an analogue that contains a distal N atom with a pK'_a of 7 to 9. The proton can in this case be displaced at a suitable receptor site.

In II, the receptor site is shown in more detail in two orientations within the membrane, perhaps in an oligomeric protein molecule that extends almost from one surface of the membrane to the other. At the left the substrate, protonated in free solution, is shown donating its proton to adjoining membrane structures, perhaps because of a proton-poor environment, either as a preexisting condition or stimulated by the entry of the substrate into the site. At the right the substrate is shown regaining a proton because of a relatively proton-rich environment. Therefore the substrate is released almost irreversibly, because the protonated substrate is unacceptable to the receptor site in this orientation.

In Part II of FIGURE 15 the receptor site is shown as belonging to a membrane structure, possibly a protein oligomer, that can generate or maintain a gradient of H^+ internal to the membrane structure. The energy required is pictured as being due to the catalysis of a cleavage of ATP at an ATPase site on the inner membrane surface, although in other membranes electron transport may serve as an alternative source. The receptor site for the amino acid is shown as entering, near the external surface of the membrane, a region of relative proton poverty, or one from which its presence stimulates proton pumping. The site is then pictured as reorientating so as to come nearer to the inner surface, into a relatively proton-rich environment, so that the distal amino group tends to be reprotonated and the substrate discharged more or less irreversibly from the site.

The stimulation by H^+ of the uptake of ordinary substrates of the Na^+ -independent transport systems, and the corresponding comigration of H^+ (so far shown only for some neutral amino acids), indicate that the relation of transport asymmetry to H^+ is not restricted to abnormal substrates with protonatable side chains, even though it can be exaggerated experimentally with the selected substrates. Our proposal obviously is closely related to that of Williams²⁷ regarding the establishment of intramembrane gradients within the inner mitochondrial membrane, to serve in the transduction of energy between electron transport and the phosphorylation of ATP.

Some of the aspects of this model are certainly conjectural and may, we hope, provoke suggestions for challenges to it. Whether transport of the ordinary substrates of System *L* is coupled more loosely to the flows occasioned by the proposed gradients, or whether such flows are determined by the structure of the amino acid, so that less ATP or ATP precursor is consumed by structures that may occasion little or no uphill transport, remains to be discovered.

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