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ENERGIZATION OF AMINO ACID TRANSPORT, STUDIED FOR THE EHRLICH ASCITES TUMOR CELL

HALVOR N. CHRISTENSEN, CARLOS DE CESPEDES, MARY E. HANDLOGTEN and GUNNAR RONQUIST

Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Mich. (U.S.A.)

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CONTENTS

Abbreviations: MPA, 4-amino-1-methylpiperidine-4-carboxylic acid; BCH, 2-aminobicyclo [2.2.1]heptane-2-carboxylic acid (2-amino-norbornane-2-carboxylic acid), the levorotatory carboxyl-endo isomer; DMO, 5,5-dimethyl-2,4-oxazolidinedione.

I. DEFINITION OF THE TRANSPORT SYSTEMS CONSIDERED

IA. Neutral amino acids

Na^+-dependent systems: System *A*: Broad specificity in the Ehrlich cells. Strongly concentrative; shows only moderate *trans* stimulation; characteristically tolerates an *N*-methyl group; hence the imino acids are substrates, and α-(methyl amino)-isobutyric acid serves as a model substrate. The iminoglycine system of the kidney and the glycine system of the pigeon red blood cell could be more narrowly specific variants of System *A*.

System *ASC*: Narrower specificity, largely limited to alanine, serine, cysteine and their 4- and 5-carbon homologs. Does not tolerate an *N*-methyl group. Largely limited to exchange in nucleated and reticulated red blood cells, not in the Ehrlich cell.

Na^+-independent system: System *L*: Apolar mass on side-chain intensifies reactivity of amino acids, even if in branched or bicyclic form; hence the use of bicyclic amino acids as model substrates. Shows strong *trans* stimulation. Conspicuous in mature human red blood cell because of absence of other neutral systems.

IB. Basic amino acids

System *Ly^+*: Requires an unambiguous cationic group. 4-Amino-l-guanyl-piperidine-4-carboxylic acid and homoarginine are proposed model substrates.

II. INTRODUCTION

Investigations of this laboratory since 1952, especially on the Ehrlich ascites tumor cell, brought forward two ideas as to how the concentrative uptake of neutral amino acids might be energized, in both cases by an indirect route:

1. For the Na^+-dependent category, by co-transport with Na^+ and possibly also by countertransport with K^+ (refs 1–6).
2. For the Na^+-independent category (System *L*), by countertransport with amino acids endogenously present in the cell[7].

Because many neutral amino acids are transported both by the Na^+-independent system and by the Na^+-dependent systems, the second proposal included the possibility that the movements of alkali-metal ions ultimately predominated in energizing the uphill transport for both categories.

In this examination of progress it will be shown that neither of these sources of energy lies on an obligatory path to uphill amino acid transport. Instead the evidence indicates that the primary process to which coupling occurs is produced in the Ehrlich cell membrane through catalytic ATP cleavage. In other cells and vesicles the primary process may be produced also or instead by electron transport. Observations of the effects of amino acid structure on uphill transport point to a way or ways in which asymmetry between the inward and outward fluxes is generated, other than the two itemized above.

III. PLACE OF CO-TRANSPORT WITH Na^+

IIIA. Must the energy for Na^+-dependent, uphill amino acid transport be channeled through alkali-metal gradients?

The situation in the area of the role of co-transport with Na^+ for organic substrates as of mid-1971 was well represented by the publication of the proceedings of a conference on the subject[8]. Two conclusions may be drawn, that energy stored in the form of the alkali-metal gradients, particularly the Na^+ gradient, is available for amino acid uptake, and also that energy from another source must also contribute to the total uptake of such amino acids as methionine, glycine and α-aminoisobutyric acid by ascites tumor cells.

Nevertheless, results in this area have continued to be inconsistent, particularly when the work on sugar transport is also taken into account. We will summarize here evidence for the basis of this inconsistency, and also seek to determine whether a flow of energy through the form of the Na^+ gradient is essential to uphill amino acid transport.

The decisive question, we felt, was whether energy for uphill amino acid transport must flow through the form of the alkali-metal gradient, or whether co-transport with Na^+ serves only as an incidental source of energy. Referring to Fig. 1, do the two active transports fall in series, as indicated by the sequence of processes pointing in the upwards direction, or do they fall in parallel, as indicated by the placement of amino acid transport at the lower left? In the first case alkali-metal transport is

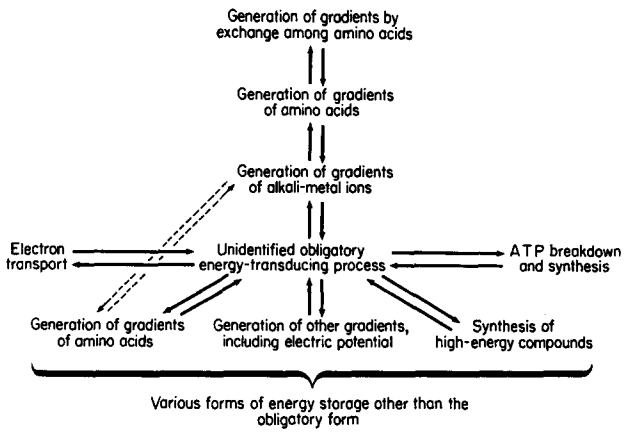


Fig. 1. Proposed relation between primary energy transduction in cellular membranes and the generation of various gradients. The obligatory energy-transducing process may be the formation of a high-energy substance, or the generation of a gradient, either locally within the membrane or across the membrane. The gradient might be of protons, of the chemical potential, and so on. The diagram makes the assumption, not necessary to the presentation in the text, that the process or state intermediating between electron transport and synthesis or cleavage of ATP, and that primarily generating material gradients, are the same. The diagram does not assume for a given cell, organelle or vesicle that the primary energy-transducing state can be established both from electron transport and from ATP breakdown.

primary and amino acid transport is seen as necessarily secondary to it. In the second case the generation of both of the gradients is a primary active transport, even though either gradient can contribute to the other. The interconvertibility of the energy storage of the two gradients might take place by simple reversibility in the formation of each from the underlying activated state of the membrane. In the present case, however, as suggested by the dotted lines, a co-transport not mediated through the reversible contributions to the fundamental activated state probably accounts for the interconvertibility.

Wheeler[9], Koser[10] and Thomas[11,12] observed, in collaboration with the senior author, for another Na^+-dependent transport system, namely System *ASC* of nucleated and reticulated red blood cells, that the flux stoichiometry between the amino acid substrate and Na^+ is not in an integral ratio, and is in fact highly variable depending on amino acid structure. The value of the flux augmentation ratio depends not only on the structure of the co-transported amino acid, but also on the structures of the amino acids available for countertransport in this exchanging system. The stoichiometry between the parallel fluxes of the two co-substrates varied from a ratio of 0.2 to a ratio of 5. That is, in the case of proline as many as five amino acid molecules might move for each Na^+, or in the case of cysteine as many as five Na^+ for each amino acid molecule. Obviously, the value of this ratio does not reveal the

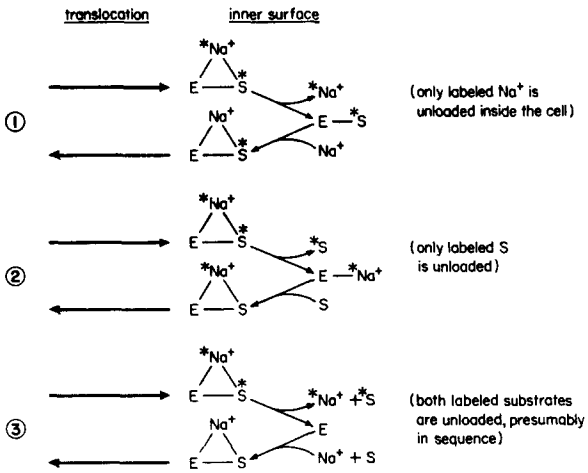


Fig. 2. Model to help explain structure dependence of the stoichiometry of the flux ratios, amino acid and Na^+ entry into pigeon erythrocytes by System *ASC*. Only the dissociation and replacement steps at the inner surface of the membrane are shown. In sequence 1, an external Na^+ exchanges for an internal Na^+. In sequence 2, an external amino acid molecule exchanges for an internal amino acid molecule. In sequence 3, both labeled co-substrates exchange for unlabeled internal equivalents. If *S* is proline, sequence 1 occurs about four times as often as sequence 2. If *S* is cysteine or threonine, sequence 2 occurs about four times as often as sequence 1. Sequence 3 has a relatively low frequency. System *A* appears to show another sequence, as shown at the top of Fig. 5, below, whereby both *S* and Na^+ are unloaded and the site reorientates in an empty state. Only this sequence produces net uphill transport at the expense of the Na^+ gradient. Reproduced from Koser, B. H. and Christensen, H. N. (1971) *Biochim. Biophys. Acta* 241, 9–19.

composition of the transport complex, which by all evidence contains one amino acid molecule and one Na^+ for each receptor site. Fig. 2 shows the explanation proposed for the structure-dependent flux stoichiometry of System *ASC* in the red cells. The diagram illustrates the dissociation step only for the inner membrane surface. For purposes of analysis, it pictures separately three modes of dissociation, one in which the amino acid only is released, one in which Na^+ only is released, and one in which both substrates are replaced. Calculations showed that replacement of both co-substrates occurs only exceptionally, and that structural changes in the amino acids concerned tend to modify which one of the two co-substrates is released and replaced more frequently.

This observation has only limited implications for energy transfer because System *ASC* in the described red cells appears to be locked into exchange, although not a precisely balanced exchange, for both co-substrates. Nevertheless, this observation led Ronquist in our laboratory to study the effects of modifying the cellular amino acid pool on the flux stoichiometry of System *A* in the Ehrlich cell, a system which in contrast to System *ASC* of the red cells engages heavily in net transport.

An informative simplification of the cellular amino acid pattern was obtained by displacing major portions of the endogenous amino acids[13]. This displacement was obtained by incubating the cells four successive times, 5 min each, in a large volume of low-Na^+ medium (6–30 mM Na^+, by choline replacement). During the last two incubations a single substrate of System *A*, usually α-aminoisobutyric acid, was present either at 10 or 0.1 mM. Each 5-min incubation was terminated by a 2.5-min centrifugation. The level of Ca^{2+} was maintained at 0.25 mM throughout the experiments, and 0.5 mM dithiothreitol was included in the media. Subsequent measurements of the initial rates of uptake of ^{14}C-labeled amino acid and $^{22}Na^+$ were made with Na^+ at 120 mM, with K^+ omitted to minimize Na^+ extrusion by the Na^+–K^+ pump. The interval for uptake was terminated by dilution with ice-cold choline-containing Krebs–Ringer bicarbonate medium, followed promptly by brief centrifuging.

This treatment with 10 mM α-aminoisobutyric acid lowered the total endogenous amino acid content of the Ehrlich cell to less than one-third of normal, and for the neutral amino acid content, to about one-fifth of the initial aggregate levels. α-Aminoisobutyric acid was now present at 70–75% of the total pool.

Fig. 3 illustrates the way in which the simultaneous uptake of a test amino acid and Na^+ was compared in such cells. Washed cells not subjected to the four consecutive incubations showed an apparent influx stoichiometry for 1 min of uptake (where $v_{a.a.}$ represents the rate of amino acid influx)

$$\frac{\Delta v_{Na^+} \text{ due to presence of the amino acids}}{\Delta v_{a.a.} \text{ due to presence of } Na^+}$$

of 1.8 to 2 (Fig. 3, left, upper curves), as measured by uptake of the two isotopic labels. For sarcosine (lower curves at left) this ratio was about 1.35. These ratios were approximately independent of the concentration of the amino acid in the range 0.3–6 mM[13].

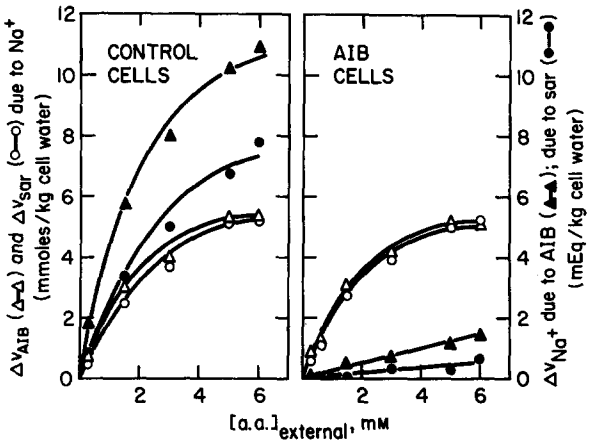


Fig. 3. Dissociation of the inflow of Na^+ from the uphill influx of α-aminoisobutyric acid (AIB) or sarcosine produced by modifying the intracellular amino acid pool. The scale at the left measures the uptake of amino acids, open circles and open triangles; the scale at the right measures the uptake of Na^+ in equivalent units, solid circles and solid triangles. Left the ordinary washed Ehrlich cell in 1 min takes up about 2 equiv of Na^+ (upper curve) for each mole of α-aminoisobutyric acid, or about 1.4 equiv of Na^+ for each mole of sarcosine. Right, the cell modified to contain greatly decreased levels of endogenous amino acids, and a replacing quantity of α-aminoisobutyric acid still takes up α-aminoisobutyric acid or sarcosine in a normal Na^+-dependent manner, but little or no Na^+ accompanies the amino acids. Reproduced with permission from Christensen *et al.*[13]. Table I of that reference records the contents of the cells in free amino acids.

When cells modified as described above ("α-aminoisobutyric acid cells") were used, the further uptake of α-aminoisobutyric acid, now provided in labeled form, continued unchecked (Fig. 3, right, upper curves), although very little $^{22}Na^+$ accompanied the amino acid. That is, the ratio of the flux stoichiometry was very low, 0.0 to 0.3. Nevertheless, in the presence of Na^+ the uptake of α-aminoisobutyric acid was highly concentrative, and continued to levels as high as 95 mM as observed by the amino acid analyser, with relatively tiny simultaneous losses of cellular amino acids[13]. When sarcosine or *a*-(methylamino)-isobutyric acid was used instead of α-aminoisobutyric acid in the preliminary treatment of the cell, the subsequent uptake of α-aminoisobutyric acid also occurred with minimal concurrent uptake of Na^+ (Table I). The dissociation of Na^+ uptake from amino acid accumulation was also seen when one of these methylamino acids was used instead of α-aminoisobutyric acid for the uptake test.

We should emphasize that the uptake of these amino acids by the modified cells remained as Na^+ dependent as ever. Very little uptake took place in the absence of Na^+, and that uptake corresponded in characteristics to the difficultly saturable component of uptake seen under various other conditions.

Study of the opposed outward flux of Na^+ did not modify the above conclusion. The net uptake of Na^+ during α-aminoisobutyric acid accumulation by unmodified cells corresponded to somewhat less than one ion per molecule of the amino acid (Fig. 4). The net uptake of Na^+, like its influx, was small after the α-aminoisobutyric acid treatment.

TABLE I

STRUCTURAL DEPENDENCE OF LINKAGE BETWEEN UPTAKE OF AMINO ACIDS AND Na^+ IN THE EHRLICH CELL[13]

Similar results were obtained in Nos 1, 2, and 4 when [^{14}C]sarcosine or ^{14}C-labeled α-(methylamino)-isobutyric acid was applied externally.

Extracellular amino acid (1–6 mM)	*Intracellular amino acid*	*Na^+ dependence of uptake*	*Stoichiometry of influxes ($\Delta v_{Na^+}/\Delta v_{a.a.}$*
1. [^{14}C]-α-Aminoisobutyric acid	Endogenous, total approx. 25 mM	+	1.8–2.0 (normal linkage)
2. [^{14}C]-α-Aminoisobutyric acid (or sarcosine or α-(methylamino)-isobutyric acid	α-Aminoisobutyric acid, sarcosine, or α-(methylamino)-isobutyric acid replacing 15–18 mM of endogenous	+	0.0–0.3 ("uncoupled")
3. [^{14}C]Ala or [^{14}C]Met	Same as No. 2	+	1–2 (not uncoupled)
4. [^{14}C]-α-Aminoisobutyric α-(methylamino)-isobutyric acid	Norleucine or Pro replacing 15–18 mM of endogenous	+	1.8* (not uncoupled)
5. [^{14}C]-α-Aminoisobutyric acid	Norleucine displacing the replacement α-Aminoisobutyric acid of No. 2	+	1.8 (coupling restored)

* Similar result for cells treated as in No. 2 but with no amino acid added.

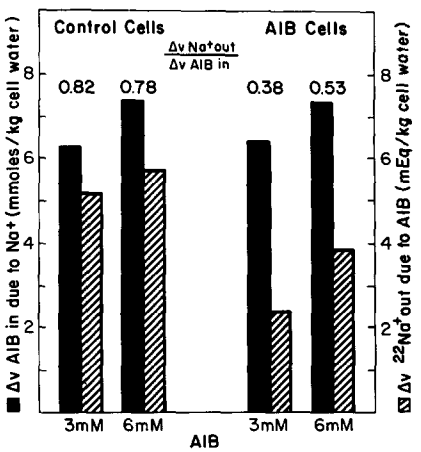


Fig. 4. Comparison of outward flux of Na^+ with influx of α-aminoisobutyric acid (AIB) for Ehrlich cells with a normal content of cellular amino acids (left), and of the same fluxes for cells in which α-aminoisobutyric acid has replaced much of the endogenous amino acids of the cell (right). The solid bar shows the Na^+-dependent uptake of [^{14}C]-α-aminoisobutyric acid during 1 min, scale at left; the hatched bar shows the α-aminoisobutyric acid-dependent exodus of $^{22}Na^+$ during the same time interval, scale at right. The values at the top of the paired bars give the ratio of the two flux augmentations. The amount of Na^+ lost from the control cells is such that the ratio, net Na^+-dependent uptake of α-aminoisobutyric acid/net α-aminoisobutyric acid-dependent uptake of Na^+ (see Table I and Fig. 3) has a value of about 1. In contrast no net uptake but small losses of Na^+ occurred during α-aminoisobutyric acid uptake by the α-aminoisobutyric acid cells.

When the sequence of four incubations was performed without adding any amino acid to the medium, the endogenous amino acids fell below 50% of control values, but the subsequent flux stoichiometry was not modified by the treatment. When α-aminoisobutyric acid was only 0.1 mM during these incubations, cellular α-aminoisobutyric acid rose to only about 0.7 mM, but the loss of endogenous amino acids was nearly as extensive as with 10 mM α-aminoisobutyric acid. Nevertheless, the stoichiometry of the subsequent uptake of ^{14}C-labeled α-aminoisobutyric acid and $^{22}Na^+$ remained unmodified (Table I). This result indicates that the lowering of the endogenous amino acids alone is not a sufficient influence; the high intracellular level of the replacing amino acid is also critical to the effect.

The uptake of L-[^{14}C]methionine or L-[^{14}C]norleucine by the α-aminoisobutyric acid cells showed a normal flux stoichiometry of about two amino acid molecules per one Na^+. When norleucine served as the displacing amino acid, causing the loss of about two-third of the endogenous amino acid, and bringing the cellular level of norleucine to about 15 mM, a normal flux stoichiometry between Na^+ and α-aminoisobutyric acid uptake was also retained (Table I). Hence the structure of the principal amino acids on both sides of the plasma membrane appears to be decisive to the flux stoichiometry.

Furthermore, the α-aminoisobutyric acid cells could be treated with 10 mM norleucine, to remove all but about 2 mM of the cellular α-aminoisobutyric acid and to yield an internal composition rather similar to that obtained by direct norleucine treatment. Again the flux stoichiometry between Na^+ and α-aminoisobutyric acid uptake was normal at about 2 : 1. That is, the norleucine-loaded cells, whether produced directly or by way of an intermediate α-aminoisobutyric acid loading, behaved normally with respect to linkage of the uptake of α-aminoisobutyric acid and the uptake of Na^+ (Table I). This comparison appears to eliminate one possible explanation of the "uncoupling" of the fluxes: If uncoupling had resulted from the leaching of some essential constituent from the cell during the first stage of loading, a second stage of loading with another amino acid would scarcely eliminate the effect of the first. In this way the contrary explanation is supported that the stoichiometry is indeed dependent on the structure of the amino acids outside and inside the cell.

Why does the concentrative uptake of amino acids become largely dissociated from the uptake of Na^+ when the composite pattern of the cellular amino acids is modified in certain ways? The Na^+ flow undoubtedly is responsive to the structure of the amino acid simultaneously bound to the transport carrier because, as we have shown elsewhere, the two co-substrates take side-by-side positions in the ternary complex at least in System *ASC*[14,12], but probably also in System *A*.

Fig. 2 has already suggested a way in which the flux stoichiometry can be changed in System *ASC* in red blood cells. That explanation must be modified somewhat to account for the present behavior, as illustrated in Fig. 5. In the upper cycle we see illustrated the behavior for System *A* of the normal Ehrlich cells. Here both co-substrates are pictured as being dissociated together on a given turn of the cycle. The carrier is pictured as returning entirely empty during many of its oscillations,

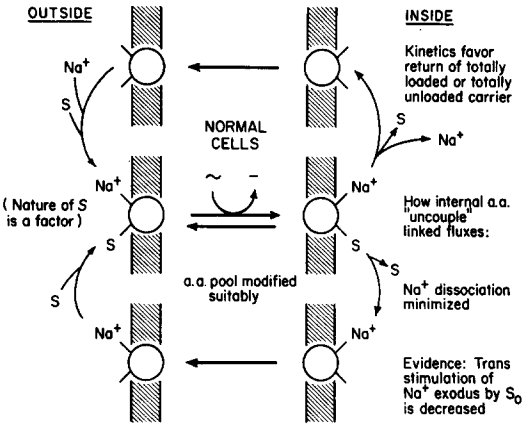


Fig. 5. Hypothesis as to basis for the dissociation of Na^+-dependent amino acid uptake from the influx of Na^+. See text for discussion. This figure adds two more possible sequences for the dissociation of the ternary complex of carrier with the two co-substrates, and for the return of the carrier to an orientation to the external surface of the membrane. Reproduced with permission from Christensen *et al.*[13].

since the ratio of the net fluxes is about 1 : 1. It is not excluded that some oscillations occur in which only one of the co-substates is unloaded, and a roughly equal number occur in which the other co-substrate is unloaded. In that case, however, no energy would be transduced from one flow to the other. Since energy is transferred from Na^+ flow to the uptake of some amino acids, the cycle shown at the top must, however, occur to a considerable extent.

The lower cycle shows only the amino acid, *S*, being unloaded, Na^+ continuing in its position on the carrier until it is again oriented to the outward surface. This sequence is largely followed, we propose, when the structures of the amino acids involved have been appropriately modified, as in the experiments just described.

The proposition of Fig. 5 that the carrier has no vacant place for Na^+ much of the time during its sojourn within the α-aminoisobutyric acid cell is supported by Ronquist's observation that external α-aminoisobutyric acid is only 46–68% as effective in producing *trans* stimulation of Na^+ exodus from α-aminoisobutyric acid cells as from ordinary cells (Fig. 4). Sarcosine was only 56–67% as effective for α-aminoisobutyric acid cells as for ordinary cells. The kinetics must favor the course of events illustrated on the lower part of Fig. 5 when the external amino acid is α-aminoisobutyric acid, sarcosine or α-(methylamino)-isobutyric acid, provided that one of these three amino acids also predominates within the cell.

At the center of the diagram uphill transport is pictured as being driven by an exergonic process other than the flow of Na^+. We conclude that such must be the case from the continued accumulation of the test amino acids after the co-migration of Na^+ has largely ceased.

The results summarized in Table I help us not only to understand the incon-

sistencies observed heretofore as to the necessity of Na^+ flow for uphill amino acid transport into the Ehrlich cell, but also the rather low efficiency of coupling observed by Geck *et al.*[15] between α-aminoisobutyric acid and Na^+ flows. More important, these results establish that the exergonic flow of Na^+ is not an obligatory source of energy, but only an incidental source. We may therefore place this major category of Na^+-dependent amino acid uptake at the lower left of Fig. 1, rather than at the upper center. That is, the generation of the gradients of Na^+ and the amino acids appear to lie in parallel rather than in series, even though these gradients are to a considerable degree interconvertible.

IIIB. Attempts to eliminate experimentally the alkali-metal ion gradients without dissipating other forms of transport-related energy storage

Terry and Vidaver[16] have recently shown that when the Na^+ and K^+ gradients across the pigeon red blood cell membrane are broken down by treatment with gramicidin D, the gradient for glycine is also lost. This finding supported an essential relation between the gradients and fluxes of glycine and Na^+ as already proposed for a glycine-specific transport system of that cell. Since the cellular content of nucleotide polyphosphate was only halved in the meantime, Terry and Vidaver[16] interpreted their result to mean that they had achieved a considerable degree of specificity in dissipating the alkali-metal gradients without a corresponding decrease in ATP.

Subsequently, Christensen *et al.*[13] has shown in this laboratory much the same relationship between the gradients of α-aminoisobutyric acid and the alkali-metal ions

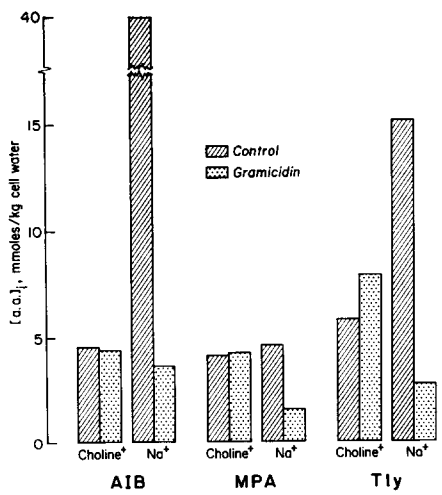


Fig. 6. Elimination by gramicidin of ability of the Ehrlich cell to maintain gradients of amino acids. Gramicidin D was added to a final concentration of $1.8 \cdot 10^{-6}$ M as a solution in 90% ethylene glycol and 10% ethanol, 6 μl of this solution being added per ml of suspending medium. Uptake was then measured for 15 min at 37 °C, pH 7.4, from Krebs–Ringer bicarbonate medium, or the same medium with choline replacing Na^+. Left, uptake of α-aminoisobutyric acid (AIB) middle, of MPA, and right, of thialysine (Tly). Reproduced with permission from Christensen *et al.*[13].

in the Ehrlich cell treated with gramicidin D (Fig. 6). After 15 min of exposure K^+ had largely escaped from the cell, Na^+ had largely entered the cell, and only small residual gradients of α-aminoisobutyric acid could be observed. If the medium contained choline instead of Na^+, the uptake of α-aminoisobutyric acid was of course small. Under these conditions, however, gramicidin caused only moderate cellular losses of K^+, and the small α-aminoisobutyric acid gradients were little affected.

Doubts on our part as to how these results should be interpreted were raised, however, by parallel observations that the largely Na^+-independent, concentrative uptake of another model amino acid, 4-amino-1-methylpiperidine-4-carboxylic acid (MPA) was also largely prevented by gramicidin treatment. Furthermore, this effect also depended on the presence in the medium of Na^+ rather than of choline (Fig. 6, center). That is, even though the uptake of MPA occurs largely by way of the Na^+-independent System *L*, that uptake ceases to be uphill after gramicidin treatment in Na^+-containing media, although it continues to be uphill when the medium contains choline instead of Na^+.

This behavior can be rationalized on the basis of changes in the levels of ATP in the Ehrlich cell produced by the gramicidin treatment. When Na^+ was present, the cellular level of ATP, as measured fluorimetrically by NADP reduction in the presence of hexokinase and glucose-6-phosphate dehydrogenase, was lowered by 75% by the gramicidin treatment. When the medium contained choline in the place of Na^+, the level of ATP was lowered by only 20% (ref. 13). We suppose that in the presence of gramicidin ATP may be depleted by futile alkali-metal-ion pumping, provided that external Na^+ is present. These results, also obtained with other model amino acids, raise the question whether gramicidin can generally be used to break down the alkali-metal gradients without as a secondary consequence depleting other forms of energy storage.

IV. PLACE OF EXCHANGE WITH OTHER AMINO ACIDS IN ENERGIZATION OF THE Na^+-INDEPENDENT SYSTEM *L*

The suspicion that exchange for endogenous amino acids accounts for the Na^+-independent uphill capture of amino acids has arisen because exchange *via* System *L* is so vigorous. As a consequence the net uptake observed may be relatively small. From the first, we had obtained evidence for net uptake even for the substrates showing the smallest Na^+-dependent component of uptake; at the same time the demonstration of a corresponding component of net exodus seemed unequivocal[17].

In the meantime, however, interest has been resurrected in the possibility that all uphill transport of neutral amino acids occurs by a single Na^+-responsive agency, and that the component of uptake seen in the absence of Na^+ represents either (a) the residual activity of the Na^+-stimulated system when for kinetic reasons Na^+ does not enter the ternary complex with the amino acid carrier, *i.e.* under incomplete coupling, or (b) the activity of a process of exchange diffusion incapable of producing net transport. The first view has been applied specifically to intestinal absorption of

alanine by Curran and his associates, as reviewed by Schultz and Curran[18]. The second view has been expressed in two ways: "System *L* is locked into exchange, one molecule of amino acid for another"; or "there is no System *L*, only exchange–diffusion". These two forms of statement are almost equivalent, although the first, if correct, would probably be sounder because it regards exchange–diffusion as a manifestation of a transport system, *i.e.* as a mode of specific chemical transmission of solutes across a biological membrane, even in the extreme case where no net transport can be shown for the exchanging system. An amino acid economy entirely based on such a transport system was pictured by Brock and Moo-Penn[19] for *Streptococcus faecium:* one or more amino acids synthesized from glucose and NH_3 presumably served by exchange to concentrate all the exogenous amino acids needed.

These views may be represented by letting the three processes shown in vertical series in the upper section of Fig. 1 represent the energization of amino acid transport. Arguing against this picture, we had shown earlier by inhibition analysis in several cell types that the characteristics of the Na^+-independent component of uptake are quite different than those for the Na^+-dependent systems; also that this component survives in the maturing rabbit red cell as the Na^+-dependent components disappear; and as mentioned we have shown that this component mediates net transport in both directions. This behavior is particularly conspicuous in the adult red blood cell, which rapidly takes up leucine for example, even though it shows little or no Na^+-dependent transport. In the meantime, evidence has been reported, especially from the laboratory of Guidotti[20–22], for the derepression of System *A*, separate from any apparent changes in System *L*. Nevertheless, sufficient uncertainty has been engendered by these questions to stimulate us to make experiments seeking to minimize experimentally the component of exchange and to maximize the component of net uptake by System *L*, which we shall summarize now.

The problem here has been to minimize the quantity of cellular amino acids available for exchange, without otherwise injuring the transport function under study. Osmotic shock has been applied to that purpose, but so far without the success obtained by the repeated treatment with low-Na^+ isotonic medium containing an amino acid, as already described. The levels of exchangeable amino acids are brought low enough by the latter procedure, so that their subsequent aggregate losses can be shown much lower than the simultaneous uptake of the test amino acid, observed from a Na^+-free suspending medium.

First we should note that unfortunately no model amino acid has yet been discovered whose net uptake is totally insensitive to the replacement of the Na^+ in the medium with choline. A component sensitive to this replacement may be unobservable at concentrations below the K_m for uptake by the Na^+-independent agency. At intermediate concentrations an effect of choline replacement may be made observable by selecting time intervals too long to be appropriate to System *L*. At concentrations ten times as high as the K_m, where over 90% of the relative velocity of Na^+-independent uptake has been lost, a minor Na^+-responsive category of uptake may become observable even after more moderate time intervals.

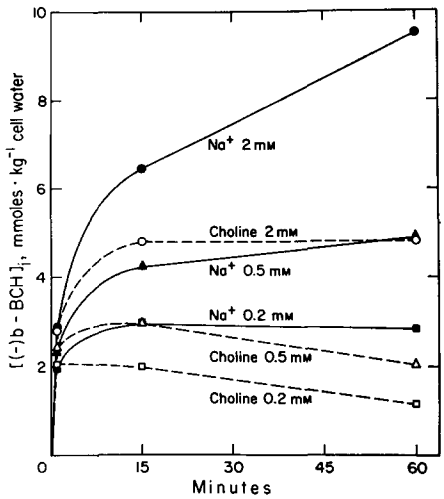


Fig. 7. Effect of concentration of BCH and of extended periods of observation on the apparent Na^+ dependence of its uptake by the Ehrlich cell. The labels on the curves show first whether Na^+ or choline was the principal cation; second, whether BCH was at 0.2, 0.5 or 2 mM. For 1-min intervals, uptake of the levorotatory b isomer was indistinguishable whether Na^+ was present in the medium (Krebs–Ringer bicarbonate solution) or whether Na^+ was replaced by choline. At 15- and 60-min intervals, however, net uptake was smaller from the Na^+-free, choline-containing medium, a result that shows exodus is faster into the latter medium. Indeed net loss of BCH from the cells is apparent in the two lowest curves. Under these conditions the role of Na^+ may well be to sustain the level of cellular amino acids with which BCH was able to exchange. Only at 2 mM levels, about ten times its K_m for Na^+-independent System *L*, did BCH show clear evidence of a gradual net accumulation sustained by the presence of Na^+.

These effects of elevated concentrations and prolonged intervals are illustrated in Fig. 7 for the uptake of the levorotatory "b" isomer of the norbornane amino acid[23–25]. Similar results have been recorded for MPA[13]. Obviously by using conditions inappropriate to the study of the transport entity in question, *i.e.* high concentrations and long time intervals, one can confound its properties with those of a minor, low-affinity Na^+-dependent mode of uptake, or even (Fig. 7) with what appears to be a choline-stimulated mode of exodus.

The other side of the technical dilemma we face is that unless substantial concentrations of the test amino acid are used, and a correspondingly extended interval of time is allowed, it becomes likely that the absolute amount of the test amino acid taken up against a gradient will scarcely be larger than the simultaneous even if unrelated exodus of endogenous amino acids. In the experiments now to be described we believe we have avoided this dilemma by the substitution of choline for the Na^+ of Krebs–Ringer bicarbonate medium, and by making the experiments on Ehrlich cells first depleted as to their endogenous amino acids by incubation in 0.1 mM α-aminoisobutyric acid, as already described.

Fig. 8 shows that the uptake of MPA (left) and that of 2-aminonorbornane-2-carboxylic acid (BCH, right) from 3 mM solutions under these conditions produced substantial gradients, which in fact exceeded by 13-fold and 3.4-fold, respectively, the

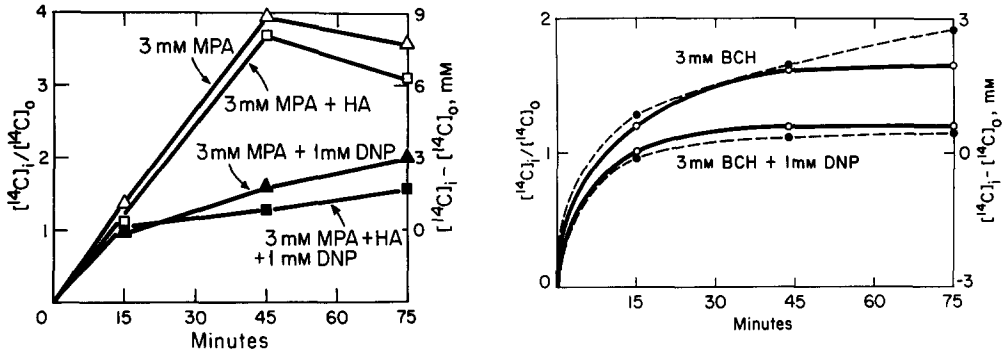


Fig. 8. Concentrative nature of Na^+-independent amino acid uptake. Uptake of the two amino acids, MPA (left) and BCH (right), was studied from 3 mM solutions in Na^+-free, choline-containing Krebs-Ringer bicarbonate medium, pH 7.4, except Krebs–Ringer phosphate medium, pH 5.0, was used for the dashed lines at the right. For the curves marked +HA at the left, 10 mM homoarginine was present to minimize migration by the cationic amino acid transport system. The Ehrlich cells had first been treated as described above with 0.1 mM α-aminoisobutyric acid to diminish their content of exchangeable amino acids. Because the prior treatment with α-aminoisobutyric acid had about halved the ATP level, the somewhat smaller gradients observed here were not unexpected. During the 75-min experiment, their losses of residual endogenous alanine, valine, leucine, isoleucine, methionine, phenylalanine and tyrosine in the aggregate were, in mmoles per liter of cell water, in the experiment at left, 0.59, in the experiment at the right at pH 7.4, 0.58, and in the experiment at the right at pH 5.0 (dashed curves) 1.25. These losses are far smaller than the gradients established by uptake of the amino acids (ordinate scale at right, in each case). Previously unpublished experiments of Handlogten, M. E. and Christensen, H. N.

concurrent exodus of seven endogenous amino acids (alanine,valine, leucine, isoleucine, methionine, phenylalanine and tyrosine) from Ehrlich cell. Even if losses of other neutral amino acids less likely to exchange *via* the Na^+-independent route of System *L* are also taken into account, net uphill transport of the test amino acids is apparent. The uptake of α,α-diethylglycine by similarly depleted cells during 45 min was 84% as large as that by untreated cells, leading to a gradient of 6 mM.

These experiments show that the uphill action of System *L* can be energized in another mode than by the down-gradient exodus of endogenous amino acids from the cell. The results further indicate that the energization of active transport of amino acids is inadequately described by the three processes placed in series in the upper center of Fig. 1. Although the interchanges of gradients among amino acids, as well as that between amino acids and Na^+, are of undoubted functional importance, they leave undescribed a presumably more fundamental source of the concentration gradients of these metabolites.

V. NATURE OF THE UNDERLYING MODE OF ENERGIZATION OF AMINO ACID TRANSPORT

VA. Evidence that the cellular ATP level is critical

These demonstrations left unanswered then the question of the input of energy for the accumulation of amino acids when little or no linked flow of Na^+ can be shown, and when exchange for other amino acids can be only a minor factor.

During the generation by the Ehrlich cell of substantial gradients of MPA, α,α-diethylglycine or BCH, each provided in Na^+-free media, we have found uptake to be much more sensitive to inhibition by 2,4-dinitrophenol or oligomycin than is the case for the Na^+-independent uptake of the ordinary more weakly concentrated amino acids. This response to dinitrophenol has already been recorded without comment in Fig. 8; results with oligomycin are shown in Fig. 9. In the absence of O_2 and in the presence of glucose at 10 mM, oligomycin inhibits the uptake of MPA, but only if the antibiotic is provided at a 10 mg/liter level, 100 times as much as is required under aerobic conditions[26]. In the presence of O_2, oligomycin needs perhaps only to shut off the mitochondrial synthesis of ATP in the Ehrlich cell to block concentrative uptake. Under the anaerobic condition, however, glycolysis is presumably the sole source of ATP. For example, after 1 h of incubation in O_2, oligomycin at 10 mg/ml lowered the level of ATP (in μmoles/g of dry cells) in Krebs–Ringer phosphate medium from 2.5 to 0.16; for the same suspension held in N_2, however, the ATP was lowered only from 3.1 to 2.8.

In the absence of O_2, oligomycin may be able to act only by inhibiting an ATPase of the plasma membrane. High levels of oligomycin are required for obstruction of ATPase action in known instances. Accordingly, these results are consistent with a dependence under aerobic conditions on mitochondrial ATP

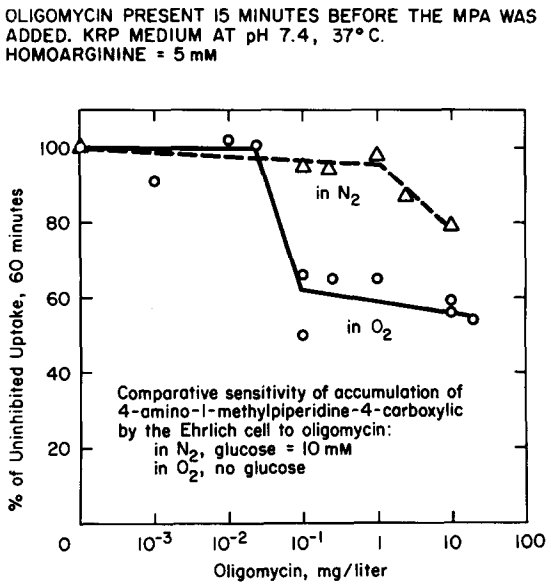


Fig. 9. Inhibition of the accumulation of MPA into the Ehrlich cell by oligomycin. ○——○, high sensitivity in the presence of O_2; △——△, much higher tolerance when N_2 replaced the O_2 atmosphere, glucose being added to 10 mM. The oligomycin was present 15 min before the ^{14}C-labeled MPA (3 mM) was added. Uptake was observed during 1 h from Krebs–Ringer phosphate (KRP) medium at 37 °C, pH = 7.4, homoarginine present at 5 mM to restrict migration by System Ly^+. Reproduced with permission from Christensen, H. N., *Proc. IV Int. Congr.*, Moscow, in the press.

production, and under anaerobiosis on ATP of glycolytic origin. These results indicate the need of Na^+-independent System *L* for an external energy source, most likely a coupled cleavage of ATP.

VB. Amino acid stimulation of ATP cleavage by membrane-bound enzyme systems

As for all endergonic processes driven by energy made available from the cleavage of ATP, certain uphill transport processes may be expected to act as ATPases. The hydrolytic activity of such systems presumably will depend on the presence of the amino acid substrate, as well as of any co-substrate for transport. Allfrey *et al.*[27] noted some years ago that the uptake of amino acids by calf thymus nuclei was readily blocked by agents that interrupt nuclear ATP synthesis. Klein *et al.*[28] described a Mg^{2+}-dependent ATPase preparation from embryonic chick-heart nuclei which could be stimulated by either Na^+ or K^+. The further addition of any of a number of amino acids approximately doubled the ATPase activity due to Na^+. Note that the actions of Na^+ and K^+ were not synergistic, although ouabain was able to abolish the increment in activity due to an amino acid in the presence of Mg^{2+} and Na^+. Karjaleinen[29] has also provided evidence on the contribution of the ATPase of thymus nuclei to amino acid transport.

Skou[30] noted in 1964 that the use of a histidine buffer enhances the activating effect of Na^+ on the Na^+- and K^+-dependent ATPase of crab nerve. Amino acid stimulation of the corresponding Na^+- and K^+-dependent enzymes of the rabbit renal medulla[31], of the chick brain[32], and of the Ehrlich cell[33] have subsequently been observed. In all of these three cases, such chelators as EDTA also proved effective as stimulants, and at much lower concentrations than the natural amino acids. The authors of the first two of these three papers[31,32] tended to attribute the stimulation of ATP cleavage to a complexing of an otherwise inhibitory metal ion present in the preparation. In contrast Forte *et al.*[33] regard this stimulation of ATP cleavage as significant for the Na^+-dependent transport of amino acids. The latter investigators showed that the presence of EDTA at earlier stages of the preparation of the membrane fraction did not eliminate the subsequent response to EDTA or to amino acids; hence they question the idea that these effects are due to an unphysiological chelation of an inhibitory metal. D-Amino acids proved as active as L-amino acids in stimulating ATP cleavage by this membrane preparation.

All enhancing effects of amino acids on ATPase action discussed so far have depended on the presence of at least one alkali-metal ion and have been subject to ready inhibition by ouabain. Several obvious factors deter the acceptance of a physiologic role for these phenomena. These factors include the strong action of EDTA, not transported as an amino acid, and not immediately inhibitory to amino acid transport in the Ehrlich cell. They also include the gradualness of the onset of ouabain inhibition of Na^+-dependent amino acid uptake[34,35]. These deterrents are not present for a new kind of ATPase observed in Ehrlich cell membranes by Ronquist in this laboratory. This new membrane-bound hydrolytic activity is stimulated by diamino acids, independent of the presence of Na^+ or K^+ and is not sensitive to

TABLE II

ALKALI METAL-INDEPENDENT ATPase ACTIVITY PRODUCED BY THE PRESENCE OF VARIOUS AMINO ACIDS

Upper section, conditions similar to those of Fig. 10, experiments of Ronquist and Christensen[63]. Under line at lower right, by analysis of orthophosphate ion release from ATP in HEPES buffer, pH, unpublished experiments by Sportes. No ionophores added in either case.

No significant effect		*Small or equivocal effects*	*Substantial stimulation*
Alanine	*p*-Aminophenyl-alanine	Diethylglycine	MPA
Methionine	Tyrosine	1-Phenethylpiperidine 4-aminocarboxylic acid	1,4-Diaminocyclohexane-carboxylic acid (both *cis* and *trans* forms)
Glycine	2-Aminonorbornane-2-carboxylic acid	4-Amino-1-carbamyl-piperidine-4-carboxylic acid	DL-1-Guanylpiperidine-4-glycine
L-Norleucine	2,4-L-Diamino-butyric acid		1-Guanyl-4-aminopiperidine-4-carboxylic acid
Sarcosine	Glutamine		
α-Aminoiso-butyric acid	α-Methyl-δ-*N*-diethylornithine		
α-(Methylamino)-isobutyric acid	2,3-L-Diaminopropionic acid	Arginine	
Threonine		Ornithine	DL-4-Zzaleucine
Phenylalanine		Lysine	L-Thialysine
m-Aminophenylglycine		Homoarginine	

the presence of ouabain. The effective amino acids include MPA and 1,4-diaminocyclohexanecarboxylic acid, as well as thialysine and azaleucine (Table II). The three most common basic amino acids, along with homoarginine, showed equivocal effects under one set of conditions, and small but (for arginine and ornithine) definite effects on chemical analysis for orthophosphate ion release (Table II). Fig. 10 documents the lack of effects of alkali metals and ouabain. A determination of the apparent K_m of MPA at about 5 mM is shown in Fig. 11.

The effective diamino acids retained their action in the presence of the norbornane amino scid. Stimulation was observed whether they are transported largely by the cationic amino acid system or by System *L*, alternatives which we will consider later in this paper. Furthermore, their stimulatory activity appears not to be related to the stability of the chelates formed with transition metals, nor to the type of chelate formed, whether it involves both of the amino groups or the α-amino and the α-carboxyl groups[36]. EDTA produces no enhancement of the ATPase activity under the same conditions.

It may be noted that in the attributes studied so far, this amino acid-stimulated Na^+-independent ATPase activity meets plausible criteria for physiological involvement in Na^+-independent transport of amino acids rather better than the previously observed Na^+-dependent activity meets criteria for involvement in Na^+-dependent amino acid uptake. It is not yet clear, however, whether the energy hypothetically made available from ATP cleavage by the presence of a diamino acid can be directed to both System *L* and System Ly^+, or is physiologically available to only one of these,

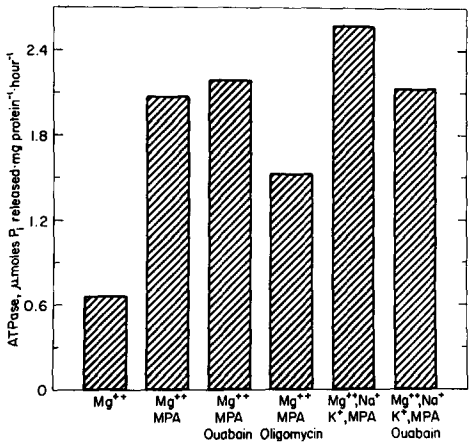


Fig. 10. Amino acid-dependent ATPase activity, not dependent on the presence of Na^+ or K^+ and not inhibited by ouabain at $5 \cdot 10^{-4}$ mM. The observations were made in 30 mM Tris buffer, pH 7.4, using 1.2 mg of membrane protein/ml medium. The amino acid (MPA) was present at 8 mM. Mg^{2+} was at 7 mM, Na^+, when present, at 100 mM, and K^+, when present, at 20 mM. The fourth bar shows a degree of inhibition of the amino acid-stimulated activity by oligomycin at 10 μg/ml. Ronquist and Christensen.[63]

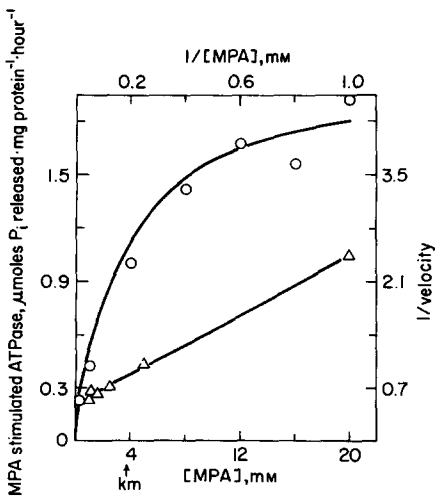


Fig. 11. Determination of apparent K_m of about 4 mM for MPA in stimulating membrane-bound ATPase activity in the presence of Mg^{2+} and the absence of Na^+ and K^+. The upper curve relates to the scales at the bottom and left, the straight line is a Lineweaver–Burk plot relating to the scales at the top and the right. Ronquist and Christensen.[63]

or indeed to neither. The following section will bring out that the structure of the diamino acid determines which of these systems predominates in its transport.

VI. EVIDENCE THAT MODIFICATIONS IN THEIR STRUCTURES MAY PERMIT AMINO ACIDS TO RESPOND MORE STRONGLY TO MEMBRANE FORCES DRIVING TRANSPORT

Although the foregoing section points to a role for ATP cleavage in energizing the transport of neutral amino acids, it leaves us with no proposal as to how the driving force must be applied to the amino acid molecule. The remaining section will enquire into clues on this difficult question.

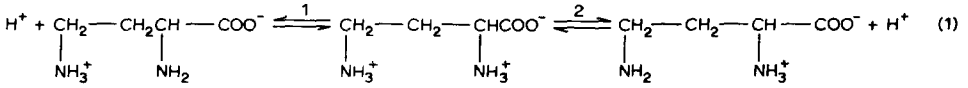
Beginning in 1952 we had encountered changes in the structure of the amino acids which sharply intensified asymmetries between their influx and efflux[37,38]. Such intensifications of asymmetry have persisted in more recent experiments when both influx and efflux were restricted experimentally either to the Na^+-dependent or to the Na^+-independent component. These results have suggested to us the possibility of exaggerating by structural changes the ability of amino acids to respond to the driving force of uphill transport, thus perhaps permitting its identification and localization.

VIA. Anomalous transport of diamino acids: background

The first of these structural features observed[37,38] was the presence of a second amino group in 2,4-diaminobutyric acid and 2,3-diaminopropionic acid. These compounds differ from their higher homologs, ornithine and lysine, in that the dissociation of both amino groups falls under the strong inductive influence of the carboxylate group. For the four-carbon amino acid the macroscopic pK_{a2}' is 8.4, and for the 3-carbon acid, 6.7, whereas when the amino groups are more widely separated, pK_{a2}' tends to have values above 9.5. At one time we proposed that their intense uptake, only minimally inhibitable by lysine, or more informatively by the une quivocally cationic homoarginine, might occur by a special system differing from the ordinary transport system for basic amino acids[39]. This special system we supposed might be one that would accept either neutral or cationic amino acids. To our surprise it ultimately became clear that the exceptional component of the uptake of diaminobutyric acid and diaminopropionic acid is instead mediated by System *A* for neutral amino acids[40]. This circumstance itself, especially when evaluated in the light of the pH dependence of their transport, indicates that uptake occurs for the dipolar form of these amino acids. Furthermore, the reactive form must be the α-zwitterion, not the γ-zwitterion, since γ-aminobutyric acid, β-alanine and isoserine show little inhibitory action on System *A*.

We can use diaminobutyric acid to illustrate the two courses that may be taken

alternatively by the second stage of the dissociation of diamino acids:



(1)

The dissociation pathway (No. 1) to the left produces the γ-zwitterion, that to the right, the α-zwitterion. The latter product is closely analogous to homoserine, and accordingly its transport by System *A* is not unexpected. The unexpected features are the extremely, almost immeasureably, high *V* of diaminobutyric acid for that transport, and the high asymmetry between influx and efflux, leading to very high gradients. In free solution, the α-amino group of diamino acids is believed to contribute more heavily than the distal amino group to the value of pK_2, with the result that only a minor amount of the α-zwitterion should be present in free solution. This circumstance, however, can not at the same time explain the slow exodus and the intense uptake of diaminobutyric acid. The implication is that the transport receptor site itself stabilizes the substrate in the reacting form. Furthermore, this stabilization must apply to entry much more strongly than to exodus.

The role of the transport system in determining in which way the transported molecules of these diamino acids dissociate is brought out by a further feature of diaminobutyric acid transport. Homoserine is an excellent substrate of another Na^+-dependent transport system, System *ASC*, not only in the Ehrlich cell, but also in the rabbit reticulocyte and in the pigeon red blood cell. This mode of uptake is measured separately as the Na^+-dependent component of uptake retained on introducing an excess of an *N*-methyl amino acid, usually α-(methylamino)-isobutyric acid, to block System *A*. By analogy with homoserine, to which it is approximately isosteric, the α-zwitterion of diaminobutyric acid should also be an excellent substrate for System *ASC*. Hence if this species existed in any large proportion in free solution, it should inhibit System *ASC* and be transported by it. Neither effect is appreciable in any of the three cell types[41]. The implication is that the α-zwitterionic species may as expected be a very minor one in free solution, and that System *ASC* fails to cause its formation at the receptor site.

Actually, this behavior of System *ASC* is not surprising, if we take into account the microenvironment we have already demonstrated for the vicinity of a γ-hydroxyl group of an amino acid at the receptor site for System *ASC*[12,14]. A hydroxyl group at that position of the amino acid substrate, if it is able to assume a *trans* orientation with respect to the carboxyl group, greatly increases the interaction of the amino acid with Na^+, and strongly accelerates the dissociation of Na^+ from the ternary complex, as already illustrated above with reference to Fig. 2[10,12,14]. The most likely interpretation is that Na^+ itself occupies a position adjacent to C-4 at the receptor site, lying *trans* to the carboxyl group. The behavior reveals in any case that the environment near C-4 has an electrostatic character unlikely to destabilize the γ-zwitterion. We believe on the basis of studies of structural effects on the Na^+/Li^+

selectivity of System *A*, that the alkali-metal ion occupies quite a different although adjacent location at the receptor site of that system.

We should emphasize another implication of the above illustrative formulation for the two courses for the dissociation of these diamino acids: Once the dissociation has been directed to either the α-amino group or to the distal amino group, deprotonation of the remaining charged amino group becomes substantially more difficult. The dissociation of the diamino acids whose behavior we are discussing may be said to be more or less delicately poised between the course shown toward the left and that shown to the right. Under these conditions as we shall discuss below, we believe the substrate can serve as a sensor of the microenvironment provided for each of these amino groups at the receptor site. More important, it can report a difference between the microenvironments provided by the site for entry and that provided by the site for exodus. We will return to this subject after considering another type of structural change leading to high flux asymmetry.

VIB. Anomalous transport of neutral amino acids with two apolar-side chains

Another different structural basis has been found to produce high asymmetry between Na^+-independent influx and efflux, namely the presence of two alkyl groups on the α-carbon as illustrated by α,α-diethylglycine and its higher homologs[42]. The consequence is a relatively slow, low-affinity uptake, but an even slower exodus so that steep gradients are gradually established, not a typical feature of Na^+-independent transport of amino acids into various animal cells. Furthermore, exchange has proved virtually indetectible between previously accumulated diethylglycine and typical substrates of System *L* when the latter are presented externally. These two features led us at first to doubt that diethylglycine uptake occurs by System *L*, whereas manifestly it does not occur to any considerable extent by one of the Na^+-dependent systems. At the same time diethylglycine uptake by the Ehrlich cell showed the apparently unique feature that it is stimulated by lowering the pH of the suspending medium. These peculiarities led us to suppose for a time that its uptake might occur by an otherwise undescribed transport system[42].

When countertransport was tested in the opposite direction, however, diethylglycine was found to stimulate BCH exodus, and internal BCH, to stimulate diethylglycine uptake. In the meantime, furthermore, we have encountered numerous other instances of one-sided countertransport attributable to the Na^+-independent mediation. For a given pair of substrates, *A* and *B*, if *A* is placed inside the cell and *B* is placed outside, both the outward flux of *A* and the inward flux of *B* will be stimulated. But if their positions are reversed, no flux enhancements may be seen. This behavior can arise as a logical kinetic consequence of a very high flux asymmetry for *A*, associated in the case selected for illustration with very low efflux of that substrate only[43].

Furthermore, we then found that the peculiar nature of the pH dependence of diethylycine uptake is a regular feature of the Na^+-independent uptake of ordinary amino acids, although in less exaggerated form (Table III). The stimulation of their

TABLE III

ACCELERATION OF THE UPTAKE OF VARIOUS AMINO ACIDS BY LOWERING THE pH FROM 7.4 TO 5.0.

Uptake 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 diethylglycine was 0.5 mM. Typical values.

Uptake of:	*Velocity (mmoles/kg cell water per min)*		*Ratio of acceleration*
	pH 7.4	*pH 5.0*	
Diethylglycine	0.19	0.54	2.78
α,α-Dipropyl-glycine	0.16	0.39	2.41
Ile	2.41	3.04	1.26
Norleucine	1.52	2.02	1.33
(−)b-2-Aminonorbornane-2-carboxylic acid	1.25	1.56	1.25
Val	1.25	2.18	1.74
Met	1.44	2.68	1.86
Phe	1.20	1.80	1.50

uptake on lowering of the pH is usually observable, however, only from Na^+-free media, containing choline as the chief cation[43].

The foregoing interpretation of the anomalies in the transport of diethylglycine indicate that this amino acid and its homologs are atypical substrates of System *L*, barely acceptable to the receptor site for entry, but largely unacceptable to the exodus site. Its exaggerated response to an intensification of the pH gradient we shall interpret as representing a second type of structural modification permitting amino acids to report on the presence and perhaps transport-driving nature of forces in the plasma membrane.

Fig. 12 illustrates the converse effect of an intensified pH gradient, namely the slowing of the exodus of System *L* substrates when the external pH is lowered. The origin of these effects of the H^+ distribution is indicated by the results of Fig. 13. During the uptake of diethylglycine, a progressive acidification occurs for the cell interior, much greater than that seen during a control incubation, or during incubation with α,α-dicyclopropylglycine, an amino acid selected as a control substance because it undergoes almost no mediated transport into the Ehrlich cell[17]. Similar but weaker effects are seen for normal substrates of System *L*. Eddy and Nowacki[44] had earlier reported clear evidence that H^+ may be co-transported with neutral amino acids when these are taken up by yeast, cellular K^+ being released in exchange for the H^+. The uptake of glutamate by the Ehrlich cell was earlier observed to be stimulated by acidification (Heinz *et al.*[45]), an effect apparently not caused by titration of glutamate to its zwitterion form.

It may have been noticed in Fig. 6 that gramicidin stimulated rather than inhibited the uptake of thialysine from the Na^+-free, choline-containing medium. The same relation proved to hold for replicate experiments with MPA (mean increase

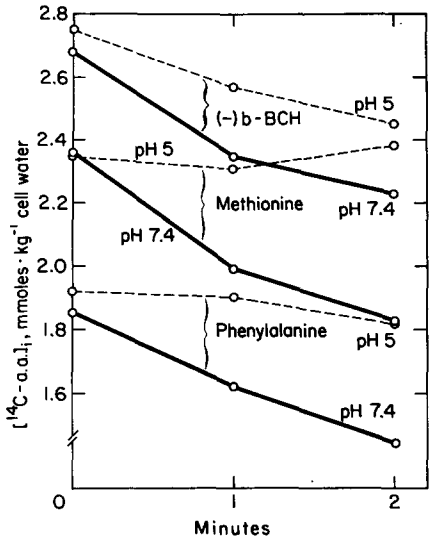


Fig. 12. Slowing of exodus of three test amino acids on lowering the external pH from 7.4 to 5.0. Ehrlich cells were first loaded to the indicated levels of the ^{14}C-labeled amino acid by incubating 5 min at 37°C in a 1 mM solution in Na^+-free, choline-containing Krebs–Ringer phosphate medium, pH 7.4. Exodus was then measured by the decline in cellular content during incubation at 37°C, in 300 vol. of the same medium, initially free of any amino acid, the pH being as indicated. Reproduced with permission from Christensen *et al.*[13].

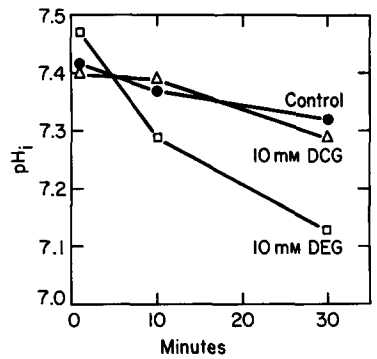


Fig. 13. Acidification of interior of the Ehrlich cell during the uptake of α,α-diethylglycine. The cells were incubated at pH 7.4 and 37°C in 100 vol. of Krebs–Ringer phosphate medium containing either no amino acid (line marked control), 10 mM α,α-dicyclopropylglycine (DLG; middle curve) or 10 mM diethylglycine (DEG). Changes in cellular pH were observed by changes in distribution of labeled DMO according to the technique of Poole *et al.*[55]. Reproduced with permission, from Christensen *et al.*[13].

10%), even though the effect is very small in the case of Fig. 6. It is known that gramicidin is able to stimulate a cellular uptake of H^+ at the expense of the K^+ gradient[46] and we have confirmed such an effect for the Ehrlich cell. This result suggests that the inward flow of H^+ induced by gramicidin under these conditions

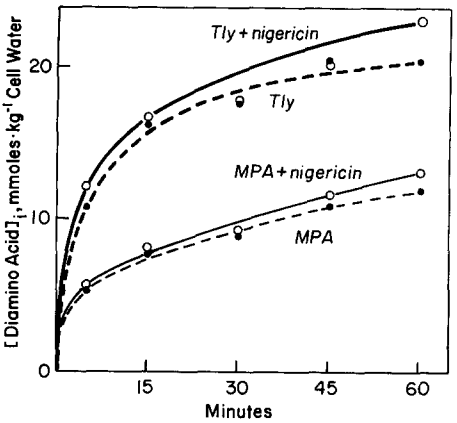


Fig. 14. Action of nigericin, 0.3 mg/l, on uptake of MPA (lower curves) and thialysine (Tly) (upper curves). Uptake of the labeled amino acid was measured at 37°C and pH 7.4 from 0.8–1 mM solutions in Krebs–Ringer phosphate medium under an N_2 atmosphere. Glucose was present at 10 mM. In the presence of O_2, nigericin exerted instead a 30% inhibitory effect on MPA uptake during 60 min. The solid circles and dashed lines represent uptake of the amino acid present alone, the open circles and solid lines, uptake in the presence of the ionophore.

can stimulate the net uptake of these amino acids. Experiments with nigericin in normal Krebs–Ringer phosphate medium under glycolytic conditions showed small but consistent stimulation of the uptake of MPA (Fig. 14). Small stimulating effects on the uptake of thialysine (Fig. 14) and diethylglycine were obtained at 45 and 60 min, but not consistently at shorter time intervals.

VIC. Diamino acid transport by System L. Experimental restriction of transport to that system

So far we have examined two apparently unrelated phenomena: an especially asymmetric transport of short-chained diamino acids by System *A*; an especially asymmetric transport of amino acids with two side-chains by Na^+-independent System *L*.

We have found it possible to introduce enough apolar mass into the diamino acid molecule to make it highly reactive with System *L*, without obtaining the high values for pK_{a2} characteristic of ornithine and lysine. We have obtained this effect with thialysine[13],

$$^+H_3N \cdot CH_2 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_3^+) \cdot COO^-$$

in which the sulfur atom in the chain permits pK_2 to be low[47] despite the wide separation of the two amino groups. As a predictable consequence of the linearity of the thialysine chain, however, this amino acid also serves as a good substrate for System *A*, just as norleucine[7] and ε-hydroxynorleucine do. That is, it shows a substantial Na^+-dependent as well as a large Na^+-independent component of uptake[42].

Branching of the chain in two dimensions seemed desirable to decrease as much

as possible the reactivity of diamino acids with System *A*, thus to try to restrict their migration to System *L*. We had already initiated the use of the piperidine ring in the design of substrates for the cationic amino acid system. Some of these turned out unexpectedly to be much better substrates for System *L* than for the cationic system[43,48]. The simplest of this series, 4-aminopiperidine-4-carboxylic acid, is an analog of 2,4-diaminobutyric acid as to the separation of the two amino groups; rather than only one ethyl, however, two parallel ethyl groups separate the α-carbon and the distal amino group. In another sense we may see it as an analog of α,α-diethylglycine; it differs only in bearing a second amino group attached to both ethyl groups in such a way as to close a ring structure.

The l-methyl derivative of this piperidine amino acid has already been introduced in Figs 6 and 8 under the abbreviation MPA. Like other members of the series, its pK'_{a2} has a low value of 7.2, not an unexpected circumstance by analogy to 2,4-diaminobutyric acid. A related analog has also proved very interesting, namely 1,4-diaminocyclohexanecarboxylic acid, especially in view of its existence as a *cis* and as a *trans* isomer. These amino acids and others of the same series are vigorously accumulated by the Ehrlich cell in the absence of Na^+, even if we include homoarginine in the medium to minimize uptake by the cationic amino acid transport system.

In 1964 we observed that β-(dimethylamino)-alanine, which has come to be called azaleucine[49], is an effective inhibitor of phenylalanine transport, although this activity decreased as the pH was lowered, with a midpoint for the effect at about pH 6. The pK_{a2} of this diamino acid was shown, however, to be 6.8 (ref. 50). This discrepancy in the midpoint of the titration in free solution and the midpoint in the variation of the K_i value also points to a stabilization of the deprotonated form of the amino acid at the receptor site. In agreement we now calculate that the L isomer of this amino acid at an external concentration of 2.5 mM is accumulated to an apparent internal concentration of 22 mM in the Ehrlich cell in 75 min by a largely Na^+-independent, BCH-inhibitable process, *i.e.* mainly by System *L*. When the DL form of this ^{14}C-labeled amino acid has been resolved into the optical antipodes, it may prove one of the more useful substrates in reporting vectorial forces encountered along the pathways of transport.

In contrast to these basic amino acids, the arginine analogs obtained by guanylating the distal N of several of these diamino acids are true model substrates for System Ly^+ and do not react appreciably with the transport systems for neutral amino acids[43,48]. No doubt the energetic difficulty of removing the proton from the guanidinium group assures their unequivocal cationic nature for transport.

We have seen that the uptake of diethylglycine and other more typical substrates of System *L* can be rendered much more asymmetric by acidifying the medium and by other devices that stimulate an inward proton flow. For obvious reasons, lowering the pH of the suspending medium slows the uptake of MPA and *cis*-1,4-diaminocyclohexanecarboxylic acid, their K_m values rising rather as predicted from their titration curves[43]. This behavior shows as expected that a zwitterionic rather than a cationic species is the true substrate for transport. This effect might well mask,

TABLE IV

RATE OF UPTAKE AND DEGREE OF ACCUMULATION BY THE EHRLICH CELL OF SOME OF THE SUBSTRATES DISCUSSED

Uptake was measured at pH 7.4 and 37 °C from Krebs–Ringer bicarbonate medium. The initial rates shown were essentially unchanged by replacing Na^+ with choline, except in the case of thialysine.

Amino acid	*V for uptake (mmoles/kg cell water per min*	*System to which attributed*	*Distribution ratio at 60 min at stated external level*
α,α-Diethylglycine	2.4	*L*	8.3 (1 mM)
4-Amino-1-methyl-piperidine-4-carboxylic acid	4	*L*	25 (0.2 mM)
4-Amino-1-guanyl-piperidine-4-carboxylic acid	0.9	Ly^+	12.7 (0.2 mM)
1,4-Diaminocyclo-hexane-4-carboxylic acid			
Cis	4	*L*	10 (0.2 mM)
Trans	1.7	*L*	2.4 (0.2 mM)
L-Thialysine	7	*A*	30 (0.2 mM)
	10	*L*	
L-Azaleucine*	6	*L*	24 (0.2 mM, 5 min)

* Unpublished result (1973), Handlogten, M. E. and Christensen, H. N. Other results from refs 13, 41, 44.

however, an effect of the kind shown for diethylglycine and the other amino acids listed in Table IV. To look at the matter in another way, the cationic form of MPA may itself to some degree supply the H^+ apparently needed for co-transport; furthermore, the zwitterion may reaccept this proton on reemergence from the membrane, on the cytoplasmic side.

Let us explore further the effect of the second amino group on transport by neutral amino acid systems. Had the only consequence of a rather low pK_a for the distal amino group of these compounds been to allow them to behave in transport like neutral amino acids, we could account in a simple way for their behavior. All that might be needed would be a highly apolar microenvironment at the part of the receptor site occupied by the distal amino group, to stabilize the group in its unprotonated form. We need to explain also, however, why the same effect does not apply for exodus, which for these analogs is very slow. We propose, therefore, that the behavior of the external receptor site that permits the distal amino group to be tolerated scarcely occurs at the inward receptor site. Superficially examined, this circumstance is not remarkable: at typical values for the cytoplasmic pH, diaminobutyric acid and thialysine should be perhaps 95% in their cationic forms, MPA about 50% in its cationic form. And, of course, the wrong γ-zwitterionic form probably represents much of the non-cationic portion of each. The remarkable feature is the differential ability of the external receptor site to see these amino acids as the α-zwitterion, or stated in another way, the apparent change in the nature of the receptor site in the

passage from the external to the internal orientation. Because this feature can permit the generation of steep amino acid gradients, it obviously has a cost in energy external to the system.

Table IV shows that characteristically these diamino acids are accumulated from 10- to 30-fold by the Ehrlich cell, the maximum velocities for entry being exceptionally high. The Na^+-dependent uptake of diaminobutyric acid shows a V of over 60 mmoles/kg cell water per min, a value over ten times the usual ones for System A[40]. Thialysine shows maximum velocities about three times the usual values, both for the Na^+-dependent and the Na^+-independent component. MPA shows a V about three times the usual values for its Na^+-independent uptake readily inhibitable by the norbornane amino acid. Similarly high rates are observed even when we minimize first the contribution that can be made by exchange for endogenous amino acids, by prior treatment with 0.1 M α-aminoisobutyric acid in low-Na^+ media, as already described.

VID. Interpretation of special transport asymmetry shown by modified amino acids. Do they act as gradient-sensing probes?

On comparing the extent to which amino acids of these two classes are concentrated by the Ehrlich cell, often ten times the extent shown by the ordinary substrates, we came to the conclusion that they probably encounter energetic gradients in their transit of the membrane[13,26,43]. Perhaps then, their chief value will be as probes for heretofore undetected gradients.

Some totally different kinds of mobile probes for membrane gradients are familiar to the field of membrane research. Such probes are generally selected for chemical inertness with respect to components of the membrane. The transmembrane potential may be estimated from the distribution assumed by the Cl^- or the dibenzyldimethylammonium ion, for example, only if its movements in the membrane respond to that force and to no other force. The indicator 5,5-dimethyl-2,4-oxazolidinedione (DMO) will report the pH gradient across the membrane only if its movements respond to the pH gradient[51] and to no other gradient. If any probes of these types were to encounter specific uphill transport in crossing the membrane, their distributions would yield invalid measures of the desired gradient.

Furthermore, probes of the types just illustrated yield only statistical measures of an energetic gradient, that is, all the way across the membrane and averaged for the entire available area. They cannot at the same time give information about local gradients through energy-transferring reaction with components of the membrane, without destroying the validity of the statistical measure.

The possibility has emerged from the present experiments of using selected transport substrates as quite a different kind of probe. Such probes should traverse the membrane only through the events of chemical bonding and release inherent in active transport. Their passage by diffusion through the lipoid substance is rendered negligible by their hydrophilic, dipolar or tripolar structure. These transport substrates appear to be offering us information as to gradients specific to an interval

between successive topographic points lying on the transport pathway. The selected diamino acids bear a potentially charged, dissociating structure not present in the ordinary substrates for Systems *A* and *L*. Because it is not ordinarily present, we should not expect this structure to be engaged by any complimentary structure on the receptor site or carrier. Hence we might expect its effect in responding to energetic gradients to be felt by the substrate–carrier complex. We may be able through the use of such substrates to obtain measures of gradients restricted to selected although unmapped portions of the thickness and area of the membrane.

Our results so far have provisionally persuaded us that local, microscopic gradients are present within the membrane, and that these gradients are much larger than the statistical ones ordinarily reported. Let us examine the available information about the gradients existing all the way across the membrane of the Ehrlich cell which might account for these results.

Because diaminobutyric acid and thialysine are mainly cationic in free solution, these amino acids as a first possibility might well be expected to respond to a potential gradient across the plasma membrane. Because MPA is present less extensively in the charged form, its response should be smaller. Hempling[52] estimated the potential gradient across the plasma membrane of the Ehrlich cell, using the distribution of the Cl^-. A distribution ratio of 1.6 to 2.5 was obtained, in favor of the extracellular fluid[52]. Similar ratios have been observed in this laboratory. If the migration of the Cl^- has a sufficient electrogenic component to impose a transmembrane potential on the system or to respond to a transmembrane potential, this distribution ratio implies that the interior aspect of the membrane is at –12 to –24 mV, if we set the exterior aspect at 0 mV. De Cespedes in this laboratory has attempted to eliminate or reverse this transmembrane potential by substituting the Cl^- of the experimental suspending medium with SO_4^{2-}, preserving isotonicity. Only very small effects were obtained on the time course of the uptake of MPA or thialysine[13].

Ehrlich ascites tumor cells were impaled with microelectrodes by Lassen *et al.*[53] in order to estimate the potential difference. The earliest readings were –24 mV for the cytoplasm. De Cespedes* has investigated the transmembrane potential by measuring the distribution of tritiated dibenzyldimethylammonium ion. This lipid-soluble cation, presented at a concentration of 0.1 mM, reaches a distribution ratio of 1.9 to 2.3, in favor of the cell interior, corresponding to a potential difference of –17 to – 22 mV.

Gibb and Eddy[54] suggest that the transmembrane potential for metabolizing ascites tumor cells may rise to values higher than those just cited, through an electrogenic effect of Na^+ pump. Such an electrogenic effect might arise from a deficiency in the number of K^+ that are pumped inward, in relation to the number of Na^+ that are pumped outward. These authors studied the distribution of methionine and other amino acids for a mouse ascites cell different from the Ehrlich cell, under conditions such that only the Na^+-dependent transport activity was presumed to be included.

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* De Cespedes, C. and Christensen, H. N., *Biochim. Biophys. Acta*, submitted for publication.

They further propose that this transmembrane potential difference accounts for the accumulation of amino acids. Treatment of their cell with valinomycin, depleted or not depleted as to ATP, caused intensification of the peak gradient achieved by methionine and other amino acids, presumably because the potential gradient was increased at the expense of the K^+ gradient.

De Cespedes has tested the effect of increasing the transmembrane potential difference at the expense of the ambient K^+ gradient characteristic of the Ehrlich cell through the use of valinomycin. The distribution ratio attained by dibenzyldimethylammonium ion under the same conditions but in the absence of tetraphenylboron was now increased to 3.2 to 4.5, corresponding to a transmembrane potential of 34 to 40 mV, the interior as usual being negative in relative charge. It is significant that incubation with valinomycin under these conditions caused no increase, only a considerable decrease, in the extent to which *a*-(methylamino)-isobutyric acid, thialysine, MPA or homoarginine was accumulated during 90 min (Table V). Replacement of the Cl^- in the suspending medium with SO_4^{2-} -did not greatly affect the distribution of dibenzyldimethylammonium ion, a result that raises doubt that Cl^- passage across the plasma membrane of the Ehrlich cell has a substantial electrogenic component.

The foregoing results reveal a transmembrane potential of a magnitude that seems quite insufficient to represent the gradient to which our modified substrates appear to respond. They also fail to show effects of modifying the transmembrane potential on the transport asymmetry of these amino acids.

As a second possibility, if a pH gradient were maintained across the plasma membrane, the amino acids under study would be protonated to different extents

TABLE V

EFFECT OF VALINOMYCIN ON THE ACCUMULATION OF SEVERAL MODEL AMINO ACIDS BY THE EHRLICH CELL

Uptake was observed during 60 min α-(methylamino)-isobutyric acid and thialysine) or 90 min in Krebs–Ringer bicarbonate medium under $O_2 - CO_2$ (95:5, v/v), the amino acids being added at 1 mM α-(methylamino)-isobutyric acid, thialysine and homoarginine) or 0.8 mM (MPA). Valinomycin was added as 0.0001 vol. of an ethanolic solution to a final concentration of $5 \cdot 10^{-7}$ M. The effects became significant after 30 min of incubation. Cellular ATP was brought to very low levels under these conditions. Typical results are shown. de Cespedes and Christensen, *Biochim. Biophys Acta*, submitted for publication.

Amino acid	*Cellular uptake (mmoles/kg cell water)*
α-(Methylamino)-isobutyric acid alone	20.3
α-(Methylamino)-isobutyric acid + valinomycin	7.4
Thialysine alone	22.6
Thialysine + valinomycin	12.7
Homoarginine alone	5.8
Homoarginine + valinomycin	4.0
MPA alone	14.1
MPA + valinomycin	7.4

in the cytoplasm and in the suspending medium. Hence their distribution might well respond to a pH gradient that would not by sensed by a typical neutral amino acid. The distribution of DMO has indicated, however, both in the prior work of Poole *et al.*[55] and in our recent tests, that the cytoplasmic pH under the conditions of our experiments may be only about one-tenth unit lower than the pH maintained in the suspending solution. This gradient likewise does not assist us in accounting for the special transport responsivity of the substrates under study.

Thus far we recognize no other effective gradients across the plasma membrane. Accordingly if we conclude that these selected transport substrates owe the especially high asymmetry of their transport to a sensing of energetic gradients, then these gradients are larger than those estimated to exist across the whole membrane. We have therefore proposed that gradients of a larger magnitude are being generated within the plasma membrane, presumably in this case through the action of a membrane-bound ATPase. Presumably the structure that senses these gradients is a complex of the amino acid with the carrier site. We may think of such an amino acid as MPA or thialysine as reacting with the external receptor site as though the immediate environment had a chemical potential of the H^+ corresponding to a pH

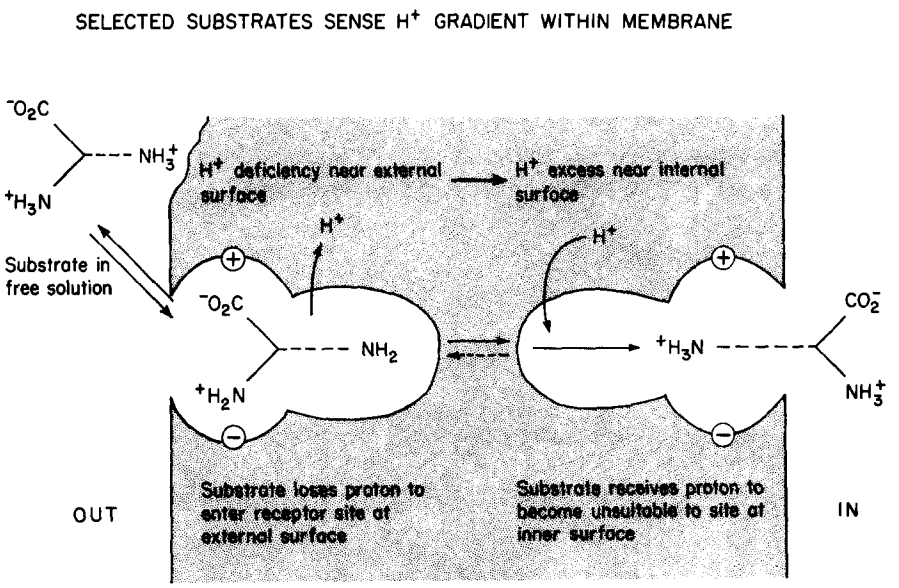

Fig. 15. Receptor site for transport by System *L* visualized in two orientations, toward the inside and toward the outside of the membrane. Hypothetical imposition of asymmetry by a gradient of H^+ or its equivalent. At the left the receptor site is shown stabilizing the sidechain amino group in its unprotonated form, even though the protonated form may predominate in free solution. A deficiency in the chemical potential of H^+ in the immediately adjoining region of the membrane may contribute. The internally oriented receptor site does not, however, tend to stabilize the side-chain amino group in the unprotonated form. Instead it sees the amino acid more largely in the unacceptable cationic state. This difference leads to asymmetry in the fluxes and by the investment of energy in the maintenance of the H^+ gradient (or in a polarization of chemical structures) produces particularly strong uphill transport of the selected diamino acids.

perhaps 2 units lower than that of the external solution (Fig. 15). Whether this H^+ poverty is maintained steadily or is generated *ad hoc* through the stimulus of the entry of the substrate becomes a matter for speculation. A logical extension of this concept is pictured at the right of Fig. 15: the portion of the membrane adjacent to the cytoplasm may conceivably be correspondingly enriched, relatively speaking, in H^+. Hence the distal amino group of the bound substrate may tend to become protonated, and the amino acid expelled into the cytoplasm. Here it remains relatively unacceptable to the site for exodus because the internally oriented site "sees" the amino acid in its cationic form.

Agents that provoke the breakdown of transmembrane gradients of the H^+ might be expected to break down local gradients also. In this connection de Cespedes has observed that the uptake during 1 h of thialysine in an N_2 atmosphere from Krebs–Ringer bicarbonate medium containing 10% glucose is inhibited 30% by the presence of 0.5 mM carbonyl cyanide *p*-trifluoronomethoxyphenylhydrazone. This effect is under study.

VIE. A general theory for the molecular basis of the energization of amino acid transport?

The observations that imposed H^+ gradients can steepen the asymmetry between the transport fluxes even for amino acids not bearing a third dissociable group (Fig. 12, Table II), and that the uptake of neutral amino acids from Na^+-free medium is accompanied by H^+ uptake (Fig. 13) also support the role of H^+ gradients in driving uphill amino acid transport. For these cases, however, two deficiencies emerge in the hypothesis of Fig. 15 as so far described:

1. For the neutral amino acid molecule, we have as yet not proposed for the substrate a chemical group whose protonation can be varied to explain the generation of asymmetry. Although the special structure of the selected diamino acids may be valuable in reporting the presence of intramembrane gradients underlying uphill transport, they give us so far no clue to the position in which the proton is held during its hypothetical work-performing flow *via* System *L*, since the ordinary substrates of System *L* provide no corresponding position.

2. We have not accounted for what may be a special or variable degree of access of H^+ from bulk aqueous phases to the interior of the membrane in these experiments.

It would be easy of course to propose a protonatable structure on the receptor site itself, and to suppose that a second alkyl side-chain on the α-carbon of the substrate might take a position at the receptor site facilitating access of H^+ between this structure and the aqueous phases. Let us instead examine whether a broader explanation of the energization of uphill amino acid transport may be compatible with these results. In this effort we may bear in mind that our need is for an explanation for the uphill transport of both the Na^+-dependent and the Na^+-independent categories. We should emphasize, furthermore, that Na^+-independent transport is strongly uphill in many organisms. We probably do well to keep in mind therefore the possibility that these systems evolved originally as strongly uphill processes, and that

only in the higher animal have they been restrained to the point that their concentrative character is often comparatively inconspicuous, a view that should encourage comparisons among species. The results with diethylglycine and its higher homologs tell us, furthermore, that System *L* can be strongly concentrative in the higher animal even when the substrate had only the usual two functional groups, namely the α-amino and α-carboxyl groups.

Our synthetic efforts have brought forward another unexpected finding that also tends to suggest an extension of the hypothesis of Fig. 15. The arginine analogs, 4-amino-l-guanylpiperidine-4-carboxylic acid and *cis*-l-guanidino-4-aminocyclohexane-4-carboxylic acid, are concentrated by 10 to 20 times by the Ehrlich cell[43], whereas arginine, homoarginine and lysine appear to be accumulated to only the degree predicted from the transmembrane electrical gradient, i.e. about 2- or 3-fold[40]. The most conspicuous special chemical trait of these arginine analogs is their relatively low values for pK_{a2}, for example 8.0 for 4-amino-1-guanylpiperidine-4-carboxylic acid. For such analogs, pK_2 must apply almost exclusively to the α-amino group.

Conceivably, as proposed in the upper line of Fig. 16, the internal receptor site sees the α-amino group of these analogs mainly in its unacceptable uncharged form, with the result that the amino acid is "trapped" as the α,ω-dipolar ion in the cell. This provisional suggestion holds that the removal of a proton from the α-amino group near the inner surface of the membrane, to the degree that it is possible, handicaps the outward transport of the amino acid.

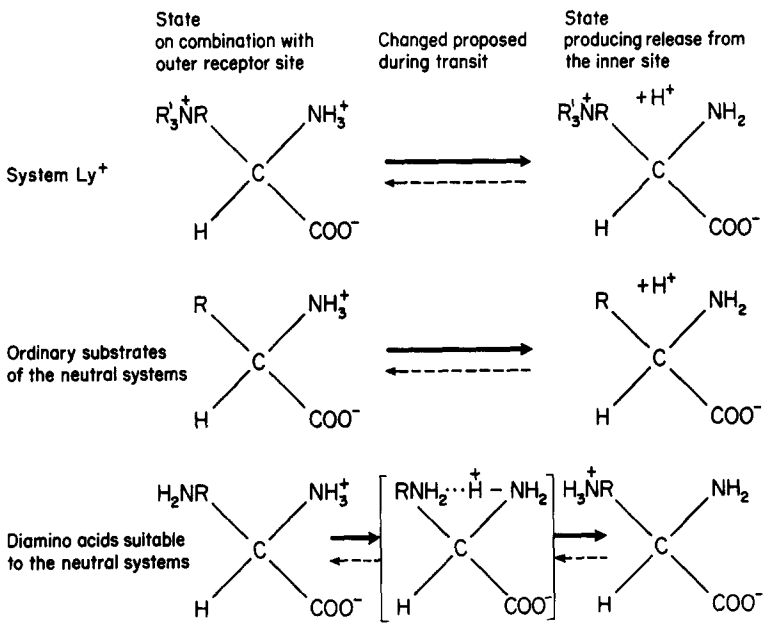

Fig. 16 transfers attention to a possible change in the state of dissociation of the α-amino group, a concomitant possibility disregarded by the scheme of Fig. 15. The driving force for the uphill transport may be applied to the released proton, across an interval of the membrane through which only the deprotonated form of the amino acid can pass.

For the ordinary basic amino acids, the strength of such a proton-displacing influence may well be too weak in the animal organism to permit appreciable concentration of arginine, lysine and the like. In single-celled organisms higher degrees of accumulation of these basic amino acids may mean either that the transmembrane potential is larger or that such proton-displacing activity is stronger. When pK_{a2} is relatively low, however, this influence could be greatly enhanced to account for strong accumulation in the Ehrlich cell. A special participation by the guanidinium group is further suggested by the finding that the quaternary N atom of the 1,1-dimethyl analog of MPA does not serve in place of a guanidinium group. This quaternary amino acid shows essentially no mediated uptake by the Ehrlich cell, even though pK_{2a} is 7.0 and even though we have no evidence for a lack of space at the receptor site for the second *N*-methyl group.

Fig. 16 then proceeds to explore the possibility of extending the same general concept to the transport systems for neutral amino acids, treating the α,α-dipolar ion as the effective form of substrate, which we suppose tends if anything to be stabilized by the receptor site for entry. Perhaps, however, it may be destabilized by the receptor site for exodus, as illustrated at the right. In the middle line of Fig. 16 no dissociable group is present on the side-chain: in the lower line a suitable distal amino group is present, this amino group being able to take an orientation such that it can approach and receive a proton from the α-amino group. This model transfers our attention from the hypothetical role first proposed for the distal amino group in adding a proton, thus permitting the diamino acid to respond to an intramembrane gradient (Fig. 15), to a further possible role for it in assisting the withdrawal of a proton from the α-amino group to generate a fundamental asymmetry in transport. The distal amino group may then alternatively retain the added proton until the amino acid is released into the cytoplasm to permit a special response to an electromotive gradient; or it may pass the proton onto an acceptor structure within the membrane. Such an action by the distal amino group is indeed supported by the much more rapid uptake and the much higher accumulation of the *cis* than the *trans* isomer of 1,4-diaminocyclohexanecarboxylic acid[43].

This proposal reminds us of an effort of many years to understand the stronger accumulation of amino acids that bear an electron-attracting structure, not necessarily an amino group, on the side-chain[56,57]. Indeed we noted as early as 1955[56] that the consequence of such side-chain structures tended to be not so much a higher influx as a higher level of steady-state accumulation, hence a relatively lower efflux. A test of β-fluoro-α-aminoisobutyri cacid, $pK_{a2} = 8.6$, showed a slower initial rate of uptake than for α-aminoisobutyric acid, $pK_{a2} = 10.2$ (ref. 57). We were accordingly persuaded that the acceptable form for entry carries the protonated amino group, but we did not adequately consider at the time the possibility that a feature more significant to steep uphill transport than the apparent value of pK_2 in free solution might be the ability of this pK to rise and fall in a pattern responding suitably to changes in the environment inherent in the passage through the transport system. In retrospect, we now suppose we obtained this feature fortuitously in α,γ-diaminobutyric acid and its analogs.

Recall also that the D isomers of the neutral amino acid are taken up more slowly by System *A* and *L* than are the L isomers, but that ultimately the D isomer tends to reach just as high a cellular accumulation as the L isomer[58]. This effect of placement of the side chain in the "wrong" positions deserves comparison with the effect of placing a side-chain in both positions, as in α,α-diethylglycine. Indeed we have observed that the uptake of D-valine and D-isoleucine is more readily stimulated by lowering the pH than that of their L isomers. We may speculate that the side-chain in the D-amino acid falls close to an important protonatable structure at the transport receptor site.

The amino acids on entering the cytoplasm at the right-hand side of Fig. 16 will tend to recapture the proton hypothetically extracted from the α-amino group. Since the amino acid is thus returned approximately to its original state of protonation, little net flow of H^+ across the membrane is implicit in the schemes of Fig. 16. Instead the flow of H^+ will be restricted to the membrane. An outward pumping of H^+ may underlie the deprotonation hypothetically preceding release of the amino acid into the cell interior. The experimental provision of an inward gradient of H^+ (as in Fig. 12) may then add to the gradient produced by the outward pumping.

We may note that only in the case of the cationic amino acid system (line 1 of Fig. 16) do we yet have important evidence for the origin of flux asymmetry in amino acid transport proposed in Fig. 16. Perhaps, in view of the difference in Na^+ dependence and H^+ sensitivity of the systems, it may be too much to expect that a single principle would serve for the linkage of all uphill amino acid movements to a fundamental activated state of the membrane. We judge from the recognized H^+-linked uptake of simple sugars by *Escherichia coli*[59–62] that the substrate probably does not universally need to participate by proton donation and acceptance in the linkage to H^+ flow, if H^+ flow is indeed a general driving force for "primary" active transport. The results summarized above are highly suggestive however, that nature has taken extensive advantage of the dissociable groups of the amino acids in forging the linkage of their transport to the hypothetical energy-supplying proton flows and potential gradients of membrane.

VII. SUMMARY STATEMENT

Although the uptake of amino acids by cells receives energy from linkage to the inflow of Na^+ and by linkage to the outflow of cellular amino acids, the results summarized above show that these processes do not account for the energization of uptake in any obligatory or general way. A number of indications support the importance of cellular ATP in bringing energy to the plasma membrane. A membrane-bound, amino acid-dependent ATPase not dependent on the presence of alkali-metal ions may play a role in the utilization of ATP. As to the mode in which the vectorial effects are produced, the influence of H^+ distribution on amino acid fluxes, and of amino acid fluxes on the H^+ distribution, point to a role of the H^+ movement in neutral amino acid transport. At the same time the presence of a suitably protonat-

able structure on the amino acid side-chain causes marked intensification of the flux asymmetry, in a manner consistent with the sensing of an intensified vectorial influence by the amino acid molecule, apparently permitted by a changing state of its protonation within the membrane. It is even conceivable that a temporary deprotonation of the α-amino group of the substrate occurs inherent to the operation of one or more of the transport systems.

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