*Na^+-FACILITATED REACTIONS OF NEUTRAL AMINO ACIDS WITH A CATIONIC AMINO ACID TRANSPORT SYSTEM**

By Halvor N. Christensen, Mary E. Handlogten, and Edwin L. Thomas

DEPARTMENT OF BIOLOGICAL CHEMISTRY, THE UNIVERSITY OF MICHIGAN, ANN ARBOR

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Abstract.—The predominant basis for transport interactions between neutral and cationic amino acids in the Ehrlich ascites-tumor cell and the rabbit reticulocyte has been identified as a reaction of the neutral amino acid plus Na^+ with the cationic amino acid transport system. This reaction is revealed both by a Na^+-dependent transport inhibition by the neutral amino acid, and by mutual flux accelerations whereby the neutral amino acid and Na^+ exchange for the cationic amino acid.

Although a distinct transport system appears to serve for the cationic amino acids in the cells and tissues studied,[1–9] mutual inhibitory actions between cationic and neutral amino acids have frequently been recognized.[4–7] In the case of the 3- and 4-carbon diamino acids, such interactions at first suggested the participation of a second transport system for diamino acids,[4] but subsequently these were largely explained by the circumstance that the α-zwitterionic forms of these short-chain diamino acids make conspicuous or even major contributions to their total migration. In the Ehrlich cell the α-zwitterionic species without net charge serves as a substrate for the principal (A) neutral system.

Transport of the biologically ubiquitous 5- or 6-carbon basic amino acids shows, however, a heretofore unexplained sensitivity to inhibition by neutral amino acids with large apolar side chains, typical substrates of the L system,[10] and in turn they inhibit the uptake of those amino acids. We have now been able to exclude two attractive explanations for these apparently reciprocal interactions, as discussed and diagrammed elsewhere.[5, 11] We can find no evidence for a transport system that does not differentiate between amino acids with uncharged and positively charged side chains; and when highly specific substrates are used, only a very small interaction can be detected between substrates of system L for neutral amino acids and system Ly^+ for cationic amino acids. Instead, the combination *Na^+ plus neutral amino acid* appears to serve as a substrate for the Ly^+ system, occasioning the migration in opposite directions of (1) Na^+ plus the neutral amino acid, and (2) the diamino acid.

Methods.—Procedures for handling the Ehrlich cell and the red blood cells and for studying uptake by them and exodus from them have been described in several recent papers.[12–16] Suspending solutions were based on Krebs-Ringer bicarbonate (KRB) medium (maintained under 5% CO_2 and 95% oxygen) for the Ehrlich cell, and Krebs-Ringer phosphate medium (maintained under 100% O_2) or a similar tris-(hydroxymethyl-)aminomethane-buffered medium[14] for erythrocytes. Comparisons were made at uniform Na^+ concentrations, with this ion replaced as desired by choline and various amino acids in an isoosmotic manner. To obtain the Na^+-free medium, $NaHCO_3$ was usually replaced by choline bicarbonate, but in indicated cases by $KHCO_3$.[15]

The origins of the substances used have also been described,[12–16] except for the case of homoarginine which we obtained both in unlabeled and ^{14}C-guanidine-labeled form from

Calbiochem. The latter was repurified by elution from the cation-exchange resin column of the amino acid analyzer, by using a sodium or potassium citrate buffer, pH 5.25. This treatment eliminated a minor, rapidly eluted component from the preparation. After 1 min of incubation we recovered 92% of the ^{14}C-homoarginine from the extracts of the Ehrlich cell as a single spot on paper chromatograms (70:15:15, butanol:acetic acid: water). After 15 min of incubation the recovery was 88%, 3–8% of a more slowly eluting radioactive substance being also observed. Observations of migration were continued for 1 min; where cells were first loaded with an amino acid, 15 min. were used.

Results.—Exclusion of participation of certain transport systems in the interaction: Figure 1 illustrates the inhibitory action of diamino acids on neutral amino acid uptake. We noted previously[4, 5] that the amino acids with large hydrocarbon side chains (phenylalanine, leucine, and methionine) are the ones that encounter the largest inhibitions by lysine, a factor leading us to associate the action with system *L*.[5, 11] Our recent finding of a substrate apparently specific to the *L* system, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid[12] (BCH[17]), permitted us to show, however, that cationic amino acids in actuality have exceedingly little effect on uptake by the *L* system, as illustrated by the action of homoarginine on the uptake of BCH (Fig. 1).

First, however, we had to come to understand that lysine itself serves as a substrate for system *L*, presumably in the form of its α-zwitterion. Figure 1 shows that lysine at high concentrations extensively inhibits the uptake of BCH; the results of Figure 1 led to a Dixon plot[18] describing a K_i of 44 mM. This degree of reactivity might be expected if 1 per cent or somewhat less of the lysine present were in the form of the α-zwitterion, $NH_2(CH_2)_4CH(NH_3^+)COO^-$, an analog of ϵ-hydroxynorleucine[4] and a plausible substrate for system L[10, 19]. We found in agreement that BCH inhibits an increasing proportion of the uptake of lysine as the lysine concentration is raised. The BCH-inhibitable component of lysine uptake appeared to have K_m of about 40 mM, in agreement with the K_i shown for lysine in its inhibition of BCH uptake (Fig. 1).

To avoid this complication, we have provisionally selected (on the basis of an inhibition analysis) homoarginine as our model substrate for system Ly^+. Its ϵ-guanidinium group, in contrast to the ϵ-amino group of lysine, should undergo

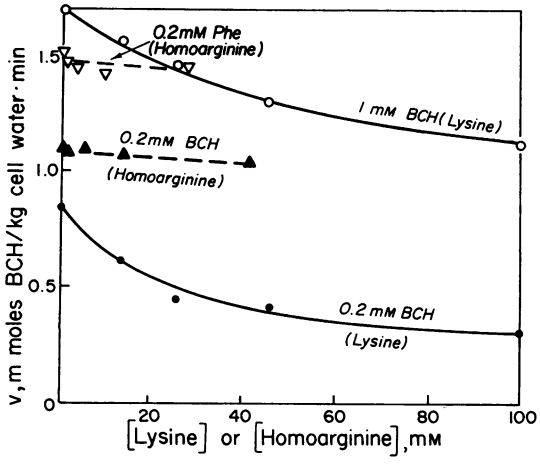

FIG. 1.—Contrasting inhibitory actions of lysine (*solid lines*) and homoarginine (*dashed lines*) on the uptake of BCH or phenylalanine by the Ehrlich cell. Uptake observed during 30 sec at 37° from Na^+-free, choline-containing KRB medium, pH 7.4. The ordinate records the uptake rate for either BCH or phenylalanine. The data for lysine correspond to a value for K_i of about 40 mM; The K_i value for homoarginine, if any, obviously is too high to be measurable.

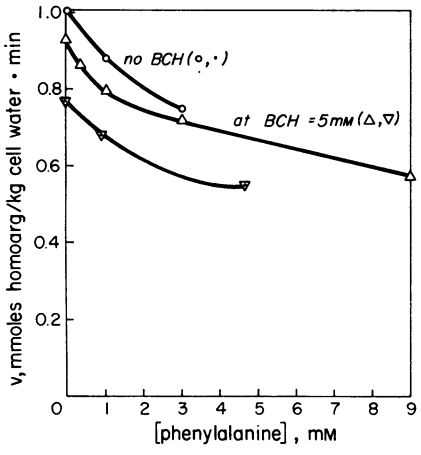

FIG. 2.—Persistence of the phenylalanine-sensitive component of homoarginine uptake in the presence of BCH. Uptake of homoarginine-^{14}C observed during 1 min at 37° from a 0.2 mM solution in KRB medium, BCH = 5 mM, [Na^+] = 138 mN. The small difference in the rate at zero phenylalanine concentration shown in the upper two curves is not typical (cf. with two lowest lines of Fig. 3, *left*).

no significant deprotonation at pH 7.4; that difference accounts, we believe, for the contrasting effectiveness of lysine and homoarginine in inhibiting BCH uptake (Fig. 1). Homoarginine proved an equally weak inhibitor of phenylalanine uptake, the slowing being usually not more than 3 to 8 per cent, although most of this effect was already apparent at 3 mM homoarginine (Fig. 1). Therefore we concluded that the action of system Ly^+ substrates on the L system is probably too small for further profitable study and is unrelated to the converse action.

The opposite aspect of the interaction under discussion—that of neutral amino acids on cationic amino acid uptake—is, however, a substantial one, as illustrated in Figure 2 for phenylalanine. As Figure 2 also illustrates, the presence of excess BCH has a negligible effect on the interaction; hence, the inhibition cannot be exerted through occupation of the L site by phenylalanine. In agreement with the selection of homoarginine as a better model substrate than the lysine for system Ly^+, Figure 3 (*left*) shows that BCH inhibition of its uptake is negligible at low homoarginine concentrations, and still much smaller than the inhibition of lysine uptake at high substrate concentrations. Figure 3 (*right*) shows that MeAIB[17] likewise has very little effect on homoarginine uptake. This substance also inhibits the uptake of lysine only very weakly, except at high lysine concentrations, thus showing that lysine serves as a substrate for system A to a much smaller extent than for system L. Possibly the much higher V_{max} of lysine for system L (12mM) than for system A arises from a protonation of the α-zwitterion after it binds to site L, as suggested for α,γ-diaminobutyric acid.[5]

The significant participation of certain other neutral amino acid systems (beside L and A) in the interaction appeared to be excluded by the smallness of the effects of taurine, α,α-diethylglycine, and proline on lysine transport (data not shown). Although alanine produces a small incremental inhibition of the uptake of cationic amino acids when superimposed on the near maximal action of phenylalanine,[5] no indications could be obtained that this action concerned any substantial component of lysine uptake by a distinct route.

Stimulation of counterflow: We were thus left without any significant component of their uptake that could be associated with the inhibitory action of neutral amino acids on cationic amino acid transport. If such a component really exists, we thought that it ought to be susceptible to exaggeration by the setting up of an exchange process. We had previously found that the external presence of either lysine or leucine stimulates the exodus of lysine.[5]

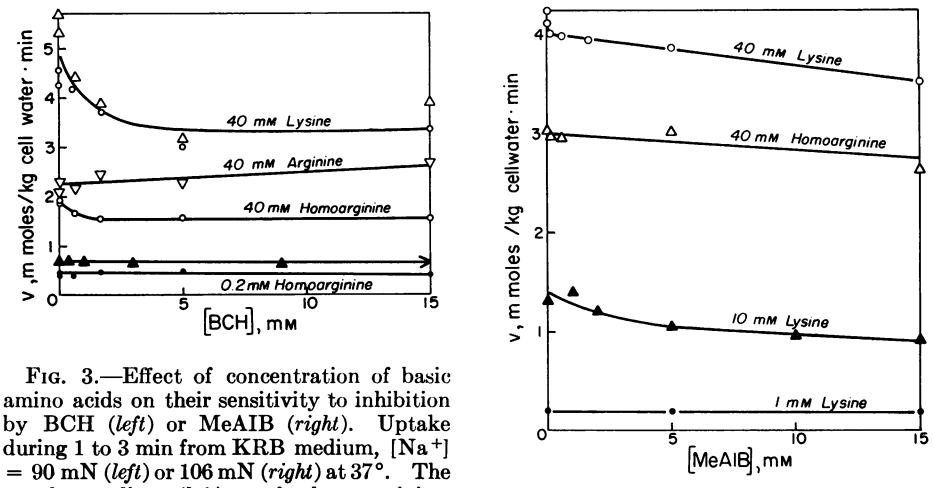

FIG. 3.—Effect of concentration of basic amino acids on their sensitivity to inhibition by BCH (*left*) or MeAIB (*right*). Uptake during 1 to 3 min from KRB medium, $[Na^+]$ = 90 mN (*left*) or 106 mN (*right*) at 37°. The two lowest lines (*left*) are for homoarginine, with the arrow pointing to a result obtained at 27 mM BCH. Only at 40 mM lysine does one see components of its uptake sensitive to the inhibitors at concentrations close to their K_m values for systems *A* and *L*, respectively.

Other neutral amino acids were shown to have this effect in correspondence with their inhibitory effectiveness on cationic amino acid uptake. The cationic amino acids in turn stimulated the exodus of neutral amino acids. We could detect a reciprocity in this action only when we used 5 mM (or higher) concentrations of the neutral amino acid; in the cases of leucine and glutamine the stimulation of uptake of the neutral amino acid (15–40%) was then approximately 1 mole/mole of exodus augmentation for the cationic amino acid. External homoarginine at a concentration of 0.06 to 0.09 mM showed a half-maximal rate of uptake, both in the presence and the absence of phenylalanine (Fig. 4), essentially the same level being required to provoke half-maximal rates of exit of either leucine or homoarginine (Fig. 5).

Na^+ dependence of interaction: Both the inhibition of lysine or homoarginine uptake (Fig. 6) and the stimulation of homoarginine exodus (Fig. 7) by neutral amino acids showed strong dependencies on the external Na^+ level. Although several amino acids showed very little action in the absence of Na^+, others with large hydrocarbon components produced substantial effects when Na^+ was absent, although again the effect was not correlated with their reactivity with system *L*. Furthermore, the transport model BCH had no effect either in the presence or absence of Na^+. Kinetic indications were obtained that the combination of neutral amino acid plus Na^+ was able to inhibit essentially all the mediated uptake of homoarginine in an approximately homogeneous way. That appeared to be the case for such amino acids as homoserine, in which the actions were most powerfully potentiated by Na^+ (Fig. 7), and also for those with large apolar side chains such as phenylalanine (data not shown).

These dependencies change Na^+ from a weak inhibitor of uptake of cationic amino acids by the Ehrlich cell and a moderate stimulator of their exodus, to a potent agent in both respects when a neutral amino acid is present. The neutral amino acid and Na^+ enhanced each other's reactivity. Li^+ shared both the

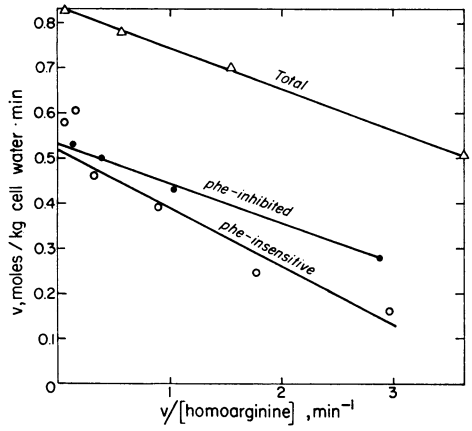

FIG. 4.—Concentration dependence of homoarginine uptake in the presence and in the absence of 50 mM phenylalanine. Uptake observed during 1 min. From KRB, $[Na^+]$ = 105 mN, pH 7.4 at 37°. Uptake corrected for a nonsaturable component assumed[5] to occur at 0.03 min^{-1}. The total uptake (*upper line*) corresponded to V_{max} = 0.83 mmole/kg cell water·min, K_m = 0.09 mM. The results for the total and phenylalanine-insensitive components derive from the same experiment; those for the phenylalanine-inhibited component were, however, selected as typical from a different experiment. Phenylalanine eliminated about half the uptake, the K_m for the inhibited and uninhibited portions not being clearly different from each other or that for total uptake.

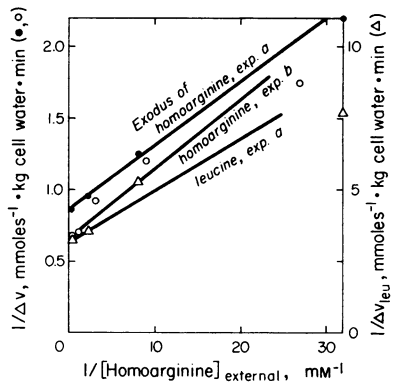

FIG. 5.—Lineweaver-Burk plot of concentration dependence of stimulation of amino acid exodus by external homoarginine. Ehrlich cells loaded to apparent levels of 2.8–3.5 mmoles homoarginine or leucine per kilogram of cell water. Exodus was then observed during 1 min at 37° into 160 vol of KRB containing various concentrations of homoarginine. Δv, acceleration of exodus produced by the presence of the external amino acid. Note that the scale on the right applies only to the lowest line, data represented by (Δ). The lines correspond to the following values for K_e, the external level of homoarginine required to produce a half maximal acceleration of exodus: homoarginine exodus, 0.05 mM (*a*) and 0.07 mM (*b*); leucine exodus, 0.06 mM.

actions of Na^+ (e.g., Fig. 6); K^+ did not. The most effective amino acids encountered are analogs of lysine, but without net charge. They have linear chains of four to six carbon atoms; in the ideal cases the apolarity of the side chains is interrupted or terminated by the presence of oxygen or sulfur. The reactivity of various amino acids with respect to inhibition of homoarginine uptake and that with respect to stimulation of its exodus are approximately correlated; also well correlated is the inhibition of homoarginine uptake in the rabbit reticulocyte[20] with that in the Ehrlich cell. Neither the sequence of the reactivity nor the concentrations of neutral amino acid required to produce half-maximal actions (Table 1) suggest that system *ASC* is the site at which the neutral amino acid component reacts. In addition, the inhibitory action of homoserine on homoarginine uptake is also seen in the mature rabbit erythrocyte, even though the *ASC* system can not be detected in that cell (data not shown).

Figure 8 illustrates that the presence of Na^+ accelerated homoarginine exodus fivefold, efflux being very slow in its absence (note points for zero external homoarginine). Separate experiments (not tabulated) of ten-minute duration failed to show evidence of saturation of this effect with respect to Na^+ at levels up to 144 mM. Nevertheless, the presence of Na^+ decreased the maximal efflux pro-

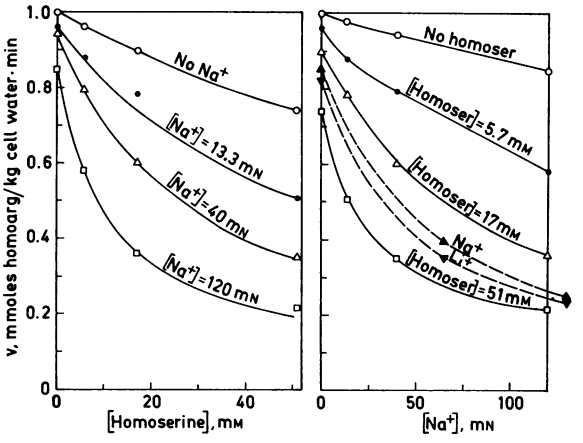

FIG. 6.—Inhibitory effect of increasing concentrations of homoserine and Na^+ on homoarginine uptake by the Ehrlich cell. Uptake measured during 1 min of 0.2 mM homoarginine-^{14}C in KRB or the same solution in which choline replaced Na^+, at 37°, pH 7.4. (*Left*) Effect of varying the homoserine concentration at constant $[Na^+]$. The curves are drawn to correspond to the Michaelis-Menten equation, after determination of the magnitude of the inhibitable component by the plot of Inui and Christensen.[15] This component was as follows, (in mmoles/kg cell water·min: no Na^+, 1.00; 13.3 mN Na^+, 0.96; 40 mN Na^+, 0.93; 120 mN Na^+, 0.80. These rates correspond to nearly all the saturable uptake in each case. (*Right, solid lines*) Effect of varying the Na^+ concentration at constant [homoserine] (same data as *left*). The curves, drawn by inspection, suggest that most of the uptake of homoarginine would be inhibited if sufficiently high Na^+ concentrations could be attained without causing other effects. (*Right, dashed lines*) Comparison of inhibition by Na^+ and Li^+ in the presence of 25 mM homoserine. The rates have been divided by 1.44 to permit them also to be shown on this plot.

duced by external homoarginine (Fig. 8) as if the two cations homoarginine and Na^+ were competing for the same site, the amino acid being more effective than Na^+ in stimulating homoarginine exodus.

Na^+ migration: The preceding results, particularly those shown in Figure 8, raised the expectation that Na^+ may enter into an exchange process with homoarginine and other cationic amino acids. Table 2 shows that $^{22}Na^+$ entry into the Ehrlich cell is stimulated when the combination of neutral amino acid and Na^+ stimulates homoarginine exodus. The circumstance that the most effective

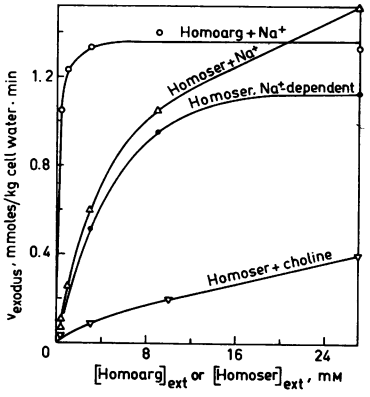

FIG. 7.—Concentration dependence of stimulation of homoarginine exodus from the Ehrlich cell by external homoarginine, and by external homoserine at $[Na^+]$ = 0 and 116 mM. The cells were loaded to about 3 mM in homoarginine-^{14}C and washed twice; exodus was then observed by the decrease in cellular ^{14}C during 1 min at 37° in about 100 vol of KRB medium, pH 7.4, containing 116 mN Na^+ and the indicated amino acid, or an isoosmotic quantity of choline chloride. For the lowest curve, the NaCl of the medium was all replaced by choline chloride, and the $NaHCO_3$ by choline bicarbonate. The next to the lowest, a *difference* curve, shows the homoserine concentration dependence of the Na^+ augmentation of exodus. It corresponds to a maximal velocity of exodus augmentation of about 1.4 mmoles homoarginine/kg cell water·min, and indicates that a half-maximal augmentation occurred at 5 mM homoserine. These values apply for 116 mN Na^+. The upper curve corresponds also to a V_{max} of 1.4 mmoles exodus augmentation per kilogram of cell water·min, and indicates that a concentration of 0.1 mM produced half that augmentation.

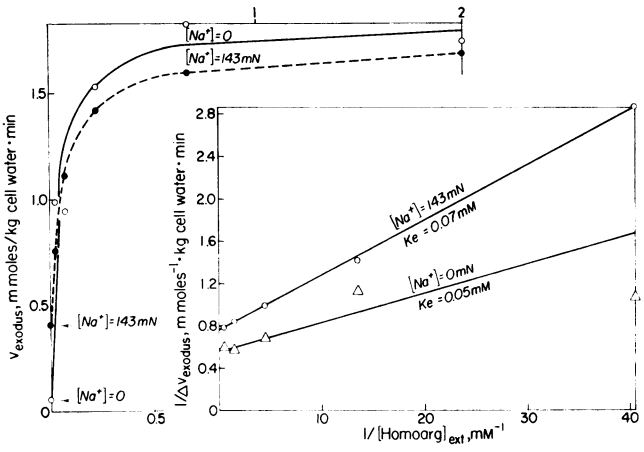

FIG. 8.—Diminished stimulation of exodus of homoarginine-^{14}C by external homoargininein the presence of Na^+. (*Left*) The directly observed rates as functions of homoarginine concentration (in mM units). The arrows call attention to the stimulation produced by Na^+ when no homoarginine had been added. (*Right*) These rates for zero external homoarginine levels have been subtracted from all other rates observed, and the resulting values for Δv plotted according to Lineweaver and Burk. The lines correspond to maximal augmentations of exodus of 1.8 mmoles/kg cell water·min in the absence of Na^+, and 1.3 mmoles/kg cell water·min in its presence. Homoarginine produced a half-maximal stimulation at 0.05 mM in the absence of Na^+, at 0.07 mM in its presence, a difference of doubtful significance.

TABLE 1. *Concentrations of three amino acids required to produce half-maximal effects on transport in the Ehrlich cell.*

Amino acid	K_m or K_i for system *ASC*	K_i, on homoarginine uptake	K_e, on homoarginine exodus
Homoserine	0.2	5	4–5
Serine	1.0[16]	90	40
Phenylalanine	12[16]	5–6	2

The results (mmoles/liter) have been obtained by plots of one of the linear transformations of the Michaelis-Menten equation, the data in each case supporting the applicability of that equation. Transport by system *ASC* was measured by the difference in uptake of a substrate at $[Na^+]$ = 128 mN and at $[Na^+]$ = 0, in the presence of 25 mM MeAIB.[16] The K_i determinations for inhibition of homoarginine uptake were made at 120 mN Na^+, the K_e determinations for the stimulation of homoarginine exodus at 130 mN Na^+. Serine and homoserine react with the *ASC* system to yield both K_i and K_m values in a consistent fashion; phenylalanine shows no measurable uptake by system *ASC;* the value shown is the K_i for the inhibition of serine uptake under the conditions specified for system *ASC*. Note that homoserine has only very small effects on homoarginine movements (Figs. 6 and 7) at a concentration that half-saturates system *ASC*.

TABLE 2. *Stimulation by internal homoarginine of the uptake by the Ehrlich cell of $^{22}Na^+$ from homoserine-containing medium.*

Experiments (no.)	External (homoserine) (mM)	External Na^+ (mN)	Δv_{entry} due to internal homoarginine (mmoles/kg cell water·min)
3	10	75	0.9 ± 0.2
3	25	75	0.9 ± 0.2
3	25	107	0.6 ± 0.2
12	10–25	75–107	0.7 ± 0.2

Cells were incubated for 15 min in 20 mM homoarginine-^{14}C in KRB medium, or the same medium without homoarginine. The cells were then washed twice with homoarginine-free medium. Estimated cellular homoarginine (after loading), 11 mM. $^{22}Na^+$ uptake was then observed during 1 min at 37° from KRB medium, with choline chloride and homoserine replacing NaCl to obtain the indicated Na^+ levels. The mean rates of $^{22}Na^+$ uptake (in meq/kg cell water·min) were as follows: no homoserine in the medium, no homoarginine in the cells, 13.8; homoserine in the medium, no homoarginine in the cells, 19.5; homoserine in the medium, homoarginine in the cells, 20.2. Hence a mean increase of Na^+ entry of 4% was under measure. The stimulated flux of homoarginine was of the order of 1 mmole/kg cell water/min.

amino acids also stimulate Na^+ uptake by the *A* and *ASC* systems caused this increase in Na^+ uptake to be just within the limits of measurability, even though it is about the amount required for a mole-for-mole exchange with homoarginine.

Discussion.—We believe that these results show that neutral amino acids exert their effects on cationic amino acid movements at the Ly^+ transport site in two cell types. If no Na^+ or Li^+ is available to occupy the position normally taken by the distal amino group of the basic amino acid, apolar forces may suffice to fix the neutral side chain. We do not yet know what cation, if any, may join with neutral amino acids to fill the Ly^+ site from within the cell when their exodus is stimulated by an external amino acid. Although the presence of neutral amino acids is recognized in certain other cases to generate a binding site for Na^+ transport,[21–25] the present results point for the first time to the position taken by Na^+ at a transport site, within 1 or 2 Ångstrom units.

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