THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 8, Issue of April 25, pp. 4443-4449, 1982
Printed in U.S.A.

Cationic Amino Acid Transport into Cultured Animal Cells

I. INFLUX INTO CULTURED HUMAN FIBROBLASTS*

(Received for publication, September 16, 1981)

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Cationic amino acid transport into human skin fibroblasts occurs by a saturable mediation which we designate System y^+, which may prove identical with the earlier $L\overset{+}{y}$. This plasma membrane agency is kinetically distinguishable from systems transporting neutral and acidic amino acids and serves to catalyze the flows of ω-guanidino amino acids and α,ω-diamino acids. Uptake of cationic substrates by System y^+ is Na^+-independent, pH-insensitive, stereoselective, and inhibitable by neutral amino acids in the presence of Na^+. Uptake and exodus of System y^+ substrates are strongly stimulated by cationic amino acids inside and outside the cell, respectively. Arginine and homoarginine accumulate in human fibroblasts reaching distribution ratios of more than 20 at external amino acid concentrations in the physiological range.

Arginine and its homologs have been selected as test transport substrates because they presumably present unambiguous cationic amino acid structures to plasma membrane transport receptor sites at physiological pH. We expect the flux of these substrates to be imperceptible by the neutral (1) and anionic (2) transport systems but to occur rapidly by one or more discrete routes that tolerate or require a net positively charged substrate (3). Transport activity specific to arginine and its homologs, excluding diamino acids, has not yet been reported for mammalian tissues, although the basis for the specificity of a familiar system of this type in bacteria has been described (4). Members of this laboratory showed earlier the presence of a general transport system for cationic amino acids, the so-called System $L\overset{+}{y}$, in Ehrlich cells (5) and rabbit reticulocytes (6). Subsequently, a corresponding component has been seen in various tissues. The plus symbol serves to designate that a positive charge on the side chain is necessary for recognition by the transport system. We now propose, at least for the present cases, abbreviation of this designation to y^+, avoiding the unfortunate implication of the symbol $L\overset{+}{y}$ that the system is specifically related to lysine.

Characteristics of System y^+ that can be explored to advantage in comparing tissues, beyond the level of transport activity, are substrate selectivity, asymmetry of operation, participation in exchange through *trans*-stimulation, and response to the combined action of selected neutral amino acids and Na^+ as surrogate substrates. The present paper reports such studies for the human skin fibroblast in monolayer culture, and the following paper (7) for the rat hepatocyte and transformed cell lines derived from it.

EXPERIMENTAL PROCEDURES

Cell Culture—Human skin fragments obtained from a healthy 5-year-old boy during skin transplantation in the Burn Center at the University of Michigan Hospital were grown in Medium 199 (GIBCO) containing Earle's salts (8). Many of the results were shown also for fibroblasts from a separate subject at Parma. The culture medium was buffered with 25 mM $NaHCO_3$ at pH 7.4, supplemented with 10% fetal calf serum (Flow Laboratories, Inc.), 60 μg/ml of penicillin, 6 μg/ml of streptomycin, and 30 μg/ml of gentamycin. To prepare the fibroblasts for a transport assay, stock cultures of confluent cells were released from 75-cm^2 flasks (Corning No. 25110) with 0.25% trypsin solution and suspended in 125 ml of fresh culture medium. One ml of this cell suspension was distributed into each of the 24 wells of 4 Costar cluster trays (Costar No. 3524). The remaining suspension was transferred into a new flask to continue the cell line. The fibroblasts were allowed to grow to confluence for 5 to 7 days.

Transport Assay—All washes of the monolayers were made and uptakes were measured in Earle's balanced salt solution modified to contain 0.2 mM $CaCl_2$, 2.5 mM $KHCO_3$ replacing $NaHCO_3$, 25 mM Na_2HPO_4, and 0.01 mg/liter of phenol red adjusted to pH 7.4. Other components were unchanged (9) except in experiments where choline chloride and choline hydrogen phosphate[1] replaced sodium chloride and sodium hydrogen phosphate, respectively. Details of the transport assay and the equipment used have been published (10). The uptake interval was short enough so that the rate was linear and extrapolated to the origin. Specific incubations and washes indicated by individual experimental designs are described in the appropriate figure or table legend.

The steady state distribution of 3-*O*-methyl-D-glucose was used to estimate the apparent intracellular water volume as described by Kletzien *et al.* (11). The value measured for fibroblasts after a 30-min incubation of the monolayers in EBS[2] with 1 mM [U-^{14}C]3-*O*-methyl-D-glucose (New England Nuclear) at 37 °C was 4.0 $\mu l \cdot mg^{-1}$ of protein.

Amino Acid Analysis—Amino acid analyses were obtained with a Beckman model 120C amino acid analyzer equipped with a microbore column (60 cm × 6 mm inside diameter), packed with 30 g of W-2 Beckman ion exchange resin, and operated at 54 °C. Sodium citrate (Fisher) buffers were used at pH 3.25 (35 min), 4.12 (18 min), and 6.40 (113 min) for the indicated time intervals (12). Cellular samples for amino acid analysis were prepared by extraction of the monolayers maintained in 75-cm^2 Corning flasks with 75% ethanol for 12 h at

* This work has been supported by Grant HD01233 from the Institute for Child Health and Human Development, National Institutes of Health, United States Public Health Service or (in Italy) by the Consiglio Nazionale delle Ricerche Gruppo Nazionale Struttura e Funzione di Macromolecole Biologiche and by the Ministero della Pubblica Istruzione, Roma, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The experiments reported here have been taken from a dissertation submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The University of Michigan.

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[1] Prepared by stoichiometric titration of 250 mM choline bicarbonate with phosphoric acid followed by boiling to remove CO_2.

[2] The abbreviations used are: EBS, modified Earle's balanced salt solution; GPA, 4-amino-1-guanylpiperidine-4-carboxylic acid; MeAIB, 2-(methylamino)isobutyric acid; BCH, the racemic 2-aminoendobicyclo[2.2.1]heptane-2-carboxylic acid.

room temperature, evaporated to dryness, and dissolved in 0.5 to 1.0 ml of sodium citrate buffer, pH 2.2.

Analysis of Data—Amino acid uptake expressed as nmol·mg^{-1} of protein·min^{-1} was calculated from the primary data with a BASIC computer program on a Radio Shack TRS-80 Model I microcomputer. Secondary analysis of initial velocity kinetic curves was carried out using FORTRAN programs (13) applying the Gauss-Newton nonlinear least squares method to data described by the following equations:

$$\log v = \log\left(\frac{V_{max}\cdot S}{K_m + S} + K_d\cdot S\right) \quad (1)$$

$$v = \frac{V_{max}\cdot S}{K_m(1 + [I]/K_i) + S} \quad (2)$$

$$v = \text{constant}\cdot\left(\frac{1 + [I]/K_{in}}{1 + [I]/K_{id}}\right) \quad (3)$$

The input and output statements were modified before the computer programs were compiled with the IBM FORTRAN G compiler and run on an Amdahl 470 V/6 computer operated at the University of Michigan.

Materials—[U-^{14}C]3-*O*-Methyl-D-glucose, 300 mCi/mmol, L-[2,3-^{14}C]arginine, 333 mCi/mmol, L-[U-^{14}C]lysine, 343 mCi/mmol, and L-[2,3-^{3}H]ornithine, 15.4 Ci/mmol, were purchased from New England Nuclear. L-[*Guanidino*-^{14}C]arginine, 45 mCi/mmol, and L-[3,4-^{3}H] arginine, 20 mCi/mmol, were obtained from Research Products International Corp. [1-^{14}C]4-Amino-1-guanylpiperidine-4-carboxylic acid (14), 0.5 mCi/mmol, 2-(methylamino)isobutyric acid, 2-aminoendobicyclo(2.2.1)heptane-2-carboxylic acid, L-[2,3-^{14}C]homoarginine (15), 0.5 mCi/mmol, and L-[2,3-^{3}H]homoarginine, 2 mCi/mmol, were synthesized in this laboratory. Naturally occurring L-amino acids were obtained from Sigma or Calbiochem-Behring. Antibiotics, choline chloride, choline bicarbonate, and trypsin were obtained from Sigma. Costar 24-well tissue culture cluster dishes were purchased from Rochester Scientific Co.

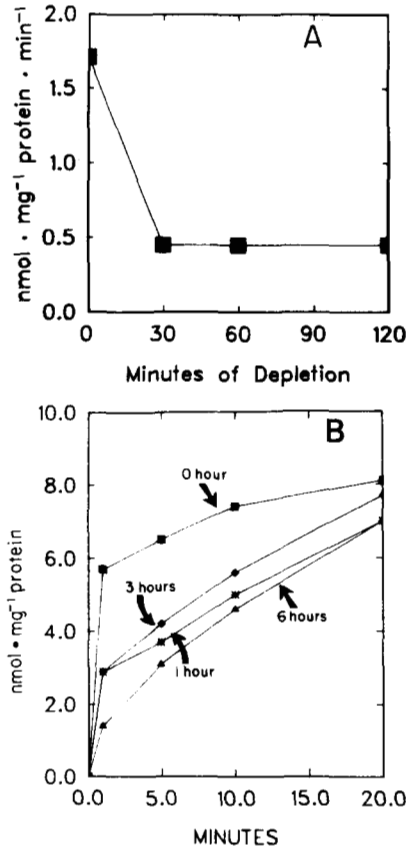

FIG. 1. **Effect of amino acid starvation on the uptake of arginine and homoarginine.** Fibroblasts (*A*) were washed twice with EBS and incubated in this amino acid-free medium for the specified time intervals. Following this treatment, 1-min uptake of 0.1 mM [^{3}H]arginine was determined in triplicate. The time course (*B*) of 0.1 mM [^{14}C]homoarginine uptake was obtained after 0 h (■), 1 h (*), 3 h (◆), and 6 h (▲) of amino acid depletion in EBS supplemented with 10% dialyzed fetal calf serum. Each *point* represents an average of three determinations.

RESULTS AND DISCUSSION

Prior Amino Acid Depletion Decreases Influx of Arginine and Homoarginine—Internal amino acid pools influence neutral amino acid influx into human fibroblasts (1), a phenomenon also observed for cationic amino acid transport in Ehrlich cells (3, 5, 6) and kidney slices (16). Fig. 1*A* shows the rate of arginine uptake after prior incubation of fibroblasts in amino acid-free medium for various time intervals. The decrease of the uptake velocity during 2 h to a constant value represents a substantial release from *trans*-stimulation which we arbitrarily assume to be complete. This result is attributed to the time-dependent depletion of endogenous amino acids from the cultured fibroblasts during incubation in amino acid-free medium as shown in Table I. The decreased influx of arginine is related to the loss of cationic amino acids as is to be shown later. In a similar experiment described in Fig. 1*B*, the time course was obtained for homoarginine uptake following amino acid depletion for 0, 1, 3, and 6 h in EBS supplemented with 10% dialyzed fetal calf serum. No adaptive increases were produced by amino acid starvation even though the depletion of cellular amino acids produced concurrently should serve to minimize possible masking effects of *trans*-stimulation. This observation appears to add one more unresponsive amino acid transport system for contrast with systems known to be adaptively influenced by the amino acid environment, namely System A in human fibroblasts (1, 17) and HTC cells (18), and Systems A and N in ordinary hepatocytes (19, 20). Guided by these initial studies, we routinely incubated fibroblasts in serum-free EBS for 90 min prior to the selected tests.

Uphill Transport of Cationic Amino Acids—Arginine and lysine attain apparent distribution ratios 20- to 30-fold greater than unity in suspensions of human fibroblasts (21). A distribution ratio of about 20 was approached for homoarginine (Fig. 2*A*) after 40 min whether our fibroblasts were previously incubated in amino acid-free medium or not. The complete replacement of Na^+ in EBS with choline had a small effect on the accumulation of this substrate as shown in Fig. 2*B*. This

TABLE I

Intracellular amino acid pools

Human fibroblasts were incubated in fresh Medium 199 containing 10% fetal calf serum for 24 h. The monolayers were then washed and incubated in EBS for the indicated time intervals, extracted with 5 ml of 75% ethanol, and applied to the amino acid analyzer. ND indicates that no metabolite was detected.

Metabolite	0 h	0.25 h	0.5 h	1 h	2 h	4 h	6 h	8 h
	nmol amino acid·mg^{-1} protein							
Asp	70.9	71.1	75.8	69.4	64.5	30.6	17.9	25.6
Thr	42.6	32.1	22.2	15.3	11.7	2.0	0.4	0.4
Ser	36.7	25.7	19.1	12.0	7.1	2.2	0.9	0.7
Glu	135	126	88.3	72.1	70.4	20.3	12.0	21.3
Gly	69.6	47.7	26.0	10.0	4.4	2.0	1.1	1.5
Ala	49.5	31.6	13.8	6.3	3.9	1.5	0.7	0.7
Cys	1.3	0.5	0.2	ND	ND	ND	ND	ND
Val	6.6	3.0	3.0	2.5	2.5	0.7	ND	ND
Met	4.9	1.2	0.2	0.2	ND	ND	ND	ND
Ile	2.8	2.0	1.2	1.2	0.5	ND	ND	ND
Leu	12.8	5.3	1.8	1.5	1.3	ND	ND	ND
Tyr	9.7	5.4	3.6	2.6	2.1	0.9	ND	ND
Phe	10.5	6.7	4.6	4.1	3.0	3.3	2.0	2.0
His	4.6	2.0	0.8	0.8	0.3	0.4	ND	ND
Lys	6.6	2.3	1.8	1.5	1.5	0.7	ND	ND
NH_3	6.7	5.9	5.1	6.6	4.4	5.9	5.5	3.7
Arg	3.8	1.5	1.3	0.8	1.3	0.2	ND	ND

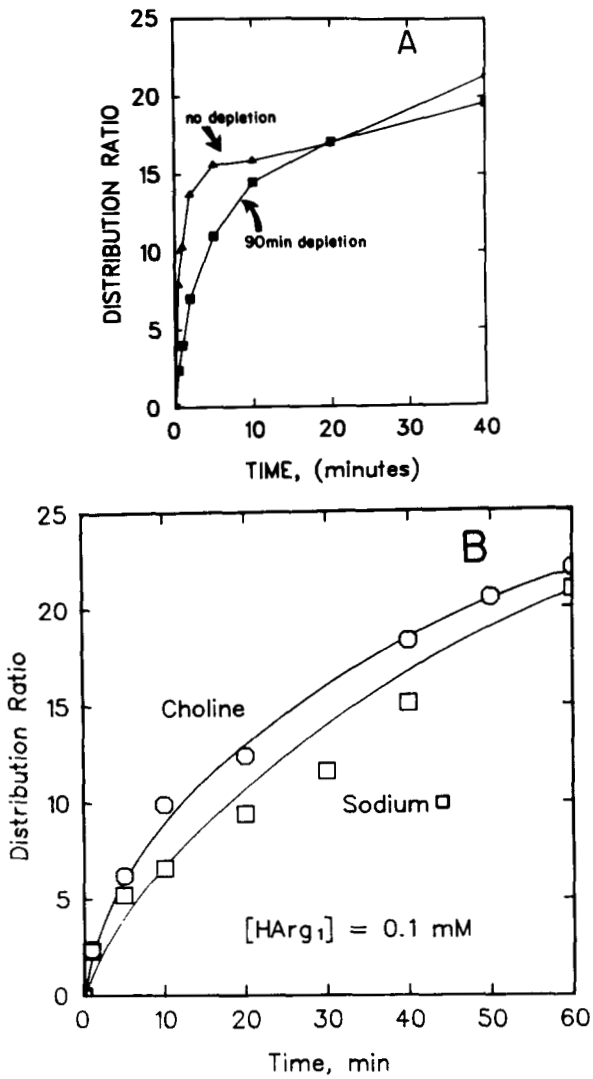

FIG. 2. **Steady state distribution ratio reached by homoarginine.** Fibroblasts (*A*) were washed with EBS and then incubated in a fresh portion of this medium for 90 min (■) or immediately assayed for the uptake (▲) of 0.1 mM [^{14}C]homoarginine for the indicated time intervals. In a similar experiment (*B*), the monolayers were incubated in EBS for 90 min and then changed to Na^+-free EBS for 30 min prior to the uptake experiment. Then [^{3}H]homoarginine accumulation was assayed in Na^+-containing (□) or Na^+-free (○) EBS. The distribution ratio for the amino acid was approximated at each time interval by assuming an internal water volume of 4 $\mu l \cdot mg^{-1}$ of protein, ignoring some inevitable compartmentation of cellular solvent water.

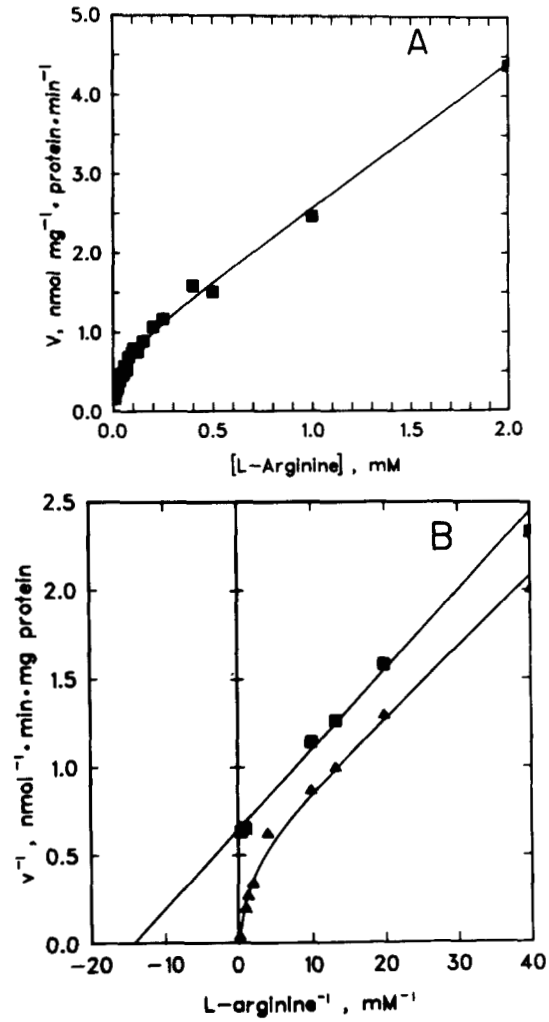

FIG. 3. **Concentration dependence of arginine influx.** The initial velocity of [^{3}H]arginine uptake was approximated by 30-s incubations with varying extracellular concentrations. The data obtained between 0 and 2.0 mM are shown in *A*. The double reciprocal plot (*B*) shows arginine influx before (▲) and after subtraction (■) of the first order uptake component. The kinetic parameters obtained by fitting the data to Equation 1 between 0.005 and 20 mM are K_m = 0.043 mM, V_{max} = 0.77 nmol·mg^{-1} of protein·min^{-1}, and K_d = 1.8 nmol·mg^{-1} of protein·min^{-1}·mM^{-1}.

effect is consistent with a weak competition of Na^+ with the organic cationic group for binding.

After a 40-min incubation period with [^{14}C]arginine (labeled in positions 2 and 3 or in the guanidino group) or with [^{3}H] homoarginine, chromatograms were prepared on thin layers of cellulose. All the radioactivity extracted from the monolayers migrated with the amino acid standards (results not shown), indicating that no significant metabolism occurred during that time interval.

The source of energy for uphill transport of arginine and homoarginine has not been determined, but is clearly not a Na^+ gradient. Villereal and Cook (22, 23) measured values for the plasma membrane potentials in growing and confluent human fibroblasts of −47 to −24 mV, respectively. From the Nernst equation at 37 °C, the range of distribution ratios for a freely permeating cation corresponding to these membrane potentials should be 6 to 2.5, respectively. It is therefore questionable whether the 20-fold gradient of homoarginine (Fig. 2) can be due solely to the electrical gradient. To produce a distribution ratio of 20 electrophoretically would require the

TABLE II

Kinetic constants for cationic amino acid influx

Fibroblasts were incubated in amino acid-free EBS for 90 min to deplete endogenous amino acids. Initial velocities were estimated from 30-s (Arg), 1-min (HArg, Lys, Orn), or 2 min (GPA) incubations over a concentration interval of 0.005 to 20.0 mM. K_m and V_{max} have their usual meanings and K_d is the slope describing the nonsaturable component. Parameters ± S.E. were determined by fitting data to Equation 1.

Substrate	K_m	V_{max}	K_d
	mM	$nmol \cdot mg^{-1}$ protein $\cdot$ min^{-1}	$nmol \cdot mg^{-1}$ protein $\cdot$ $min^{-1} \cdot mM^{-1}$
Arginine	0.040 ± 0.005	1.0 ± 0.1	3.2 ± 0.1
Homoarginine	0.040 ± 0.007	1.5 ± 0.2	3.7 ± 0.2
Lysine	0.030 ± 0.004	0.67 ± 0.1	7.4 ± 0.2
Ornithine	0.09 ± 0.02	0.7 ± 0.1	4.2 ± 0.2
GPA	0.9 ± 0.3	1.6 ± 0.4	0.5 ± 0.1

maintenance of a potential difference in excess of −80 mV. Furthermore, exchange of homoarginine for endogenous amino acids does not generate the gradient because the same distribution is approached whether or not the cells have been depleted of endogenous amino acids.

A Study of Concentration Dependence on the Influx of Cationic Amino Acids—Fig. 3*A* shows the relation between external concentration and influx of cationic amino acids into human skin fibroblasts. The typical kinetic curve, for arginine in this case, is resolvable into a hyperbolic component and a first order component that cannot be saturated up to 20 mM. Subtraction of the calculated nonsaturable influx, represented by K_d, from the total measurable accumulation of arginine, yields the linear Lineweaver-Burk plot illustrated in Fig. 3*B*. Although linear double reciprocal plots do not prove transport homogeneity any more conclusively than curvilinearity proves heterogeneity (24), subsequent experiments indicate that the saturable transport component may be attributed to a single mediation. As usual, our decision for homogeneity of mediation must be provisional.

The apparent kinetic constants collected in Table II describe the influx of several positively charged amino acids. The estimated initial uptake velocity was fitted to Equation 1 which is the Michaelis-Menten equation modified to include a first order term, $K_d \cdot [S_1]$ (25). The kinetic constants for arginine transport in the presence and absence of Na^+ were indistinguishable. GPA, a metabolism-resistant, cyclic arginine analog (14), proved a relatively poor substrate for uptake by System y^+ with a value of V_{max}/K_m only 1/20 as large as the natural compounds tested.

Groth and Rosenberg (21) suggested that after correction for physical diffusion (a term in which they unfortunately encompass all nonsaturable migration) suspensions of human fibroblasts show two saturable systems serving for dibasic amino acids, a high affinity component probably identical with System y^+, and a second saturable system with K_m values near 20 mM. We failed to see evidence for a second saturable mediation in our experiments. Correspondence among analogs between their *trans*-port-inhibitory and *trans*-stimulatory actions indicate that System y^+ is the predominant membrane component catalyzing basic amino acid flows.

The values of K_m for arginine uptake plotted in Fig. 4 indicate that System y^+ is independent of pH in the interval of 5.0 to 8.0. In contrast, the values of K_m for ornithine influx increase steadily as the pH of the medium rises to 9. The insensitivity of V_{max} for both amino acids within this pH range (data not shown) suggests that ionization of the carrier substrate complex is not responsible for this effect (26). Therefore, the steady and significant decrease of the ratio, V_{max}/K_m, for ornithine but not for arginine between pH 7 and 9 implies that the predictable removal of a proton from ornithine ($pK_2' = 8.7$ (27)) converts it to an unsuitable substrate.

The nonsaturable component of influx described by the first order constant, K_d, does not necessarily represent nonspecific physical diffusion of amino acids across the plasma membrane even though the component fails to saturate within a reasonable concentration interval (0 to 20 mM) (28). It may include considerable mediated uptake by a system whose K_m lies well above the highest substrate concentration tested, or whose value of V_{max}/K_m may be less than or equal to K_d, the component therefore being masked. Its presence may arise from an inadequate correction for radioactivity trapped within the cell mass but still in the extracellular compartment. A part of the nonsaturable component can also result from trace impurities in the radioactive preparation that are taken up without saturation.

Analog Inhibition of Cationic Amino Acid Transport—Lineweaver-Burk plots describing the inhibition of arginine influx by 0.25 mM L-homoarginine, L-arginine, L-2-amino-4-guanidinobutyrate, and L-2-amino-3-guanidinopropionate are shown in Fig. 5. Table III summarizes the kinetic parameters obtained by fitting arginine influx over a range of concentrations to Equation 2, first deducting the nonsaturable uptake. These results show that as the number of methylene groups between the α-carbon atom and the guanidino group are increased, the values of the inhibition constants decreased. This result can arise from a combination of kinetically indistinguishable factors. First, constraints on the side chain at the

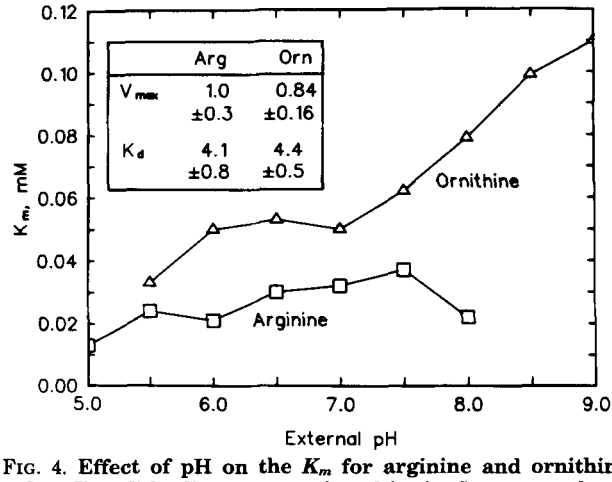

FIG. 4. **Effect of pH on the K_m for arginine and ornithine uptake.** The Michaelis constants plotted in the figure were determined for the transport of [^{3}H]arginine (□) between pH 5.0 and 8.0 or for [^{3}H]ornithine (△) between pH 5.5 and 9.0. The initial velocity of uptake was estimated from 30-s incubations over a concentration interval of 0.010 to 0.5 nM for arginine and between 0.01 and 1.0 mM for ornithine. These kinetic parameters were obtained by fitting the data to Equation 1. The values of V_{max} (nanomoles·mg^{-1} of protein·min^{-1}) and K_d (nanomoles·mg^{-1} of protein·min^{-1}·mM^{-1}) for both substrates tested were constant within these pH intervals. The average values of the parameters ± S.D. are shown on the figure.

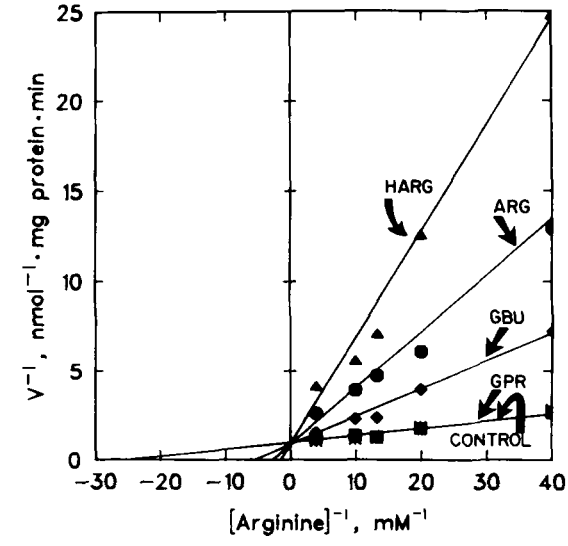

FIG. 5. **Inhibition of arginine influx by its homologs.** The initial uptake of [^{3}H]arginine was measured during 30-s incubations in the presence of 0.25 mM homoarginine (▲), arginine (●), 2-amino-4-guanidinobutyric acid (◆), 2-amino-3-guanidinopropionic acid (■), or no inhibitor (*). The data were corrected for the nonsaturable uptake with a K_d of 1.6 nmol·mg^{-1} of protein·min^{-1}·mM^{-1}. The calculated inhibition constants are listed in Table III.

TABLE III

Competitive inhibition of arginine uptake

Kinetic constants were determined for [^{3}H]arginine uptake (0.01 to 0.25 mM) in the presence of various cationic amino acids (0.25 and 0.5 mM). Initial velocities were determined from 30-s uptakes in sodium-containing EBS. K_i ± S.E. was determined by fitting the data to Equation 2 after deducting the nonsaturable component K_d = 1.6 nmol·mg^{-1} of protein·min^{-1}·mM^{-1}.

Inhibitor	K_i
	mM
L-Homoarginine	0.019 ± 0.002
L-Arginine	0.041 ± 0.003
L-2-Amino-4-guanidinobutyric acid	0.12 ± 0.01
L-2-Amino-3-guanidinopropionic acid	1.6 ± 0.7
L-Lysine	0.040 ± 0.004
GPA	0.54 ± 0.09
D-Arginine	0.70 ± 0.06
N^{α}-Methyl-L-arginine	>20
N^{α}-Methyl-L-lysine	>20

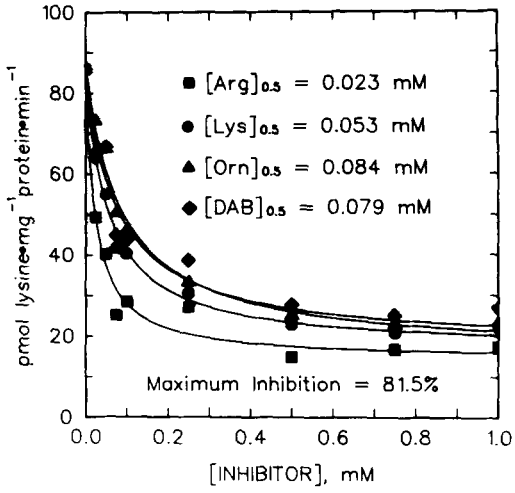

FIG. 6. **Inhibition of [^{14}C]lysine influx.** The uptake of 2.4 μM [^{14}C]lysine was measured during 30-s incubations in the presence of unlabeled arginine (■), lysine (●), ornithine (▲), or 2,4-diaminobutyrate (◆). The resultant hyperbolic curves were fitted to Equation 3 to determine the concentration of half-maximal inhibition $[I]_{0.5}$, and the maximum level of inhibition obtainable by the four compounds, 81 ± 2%. Inhibitor concentrations were varied from 0 to 20 mM.

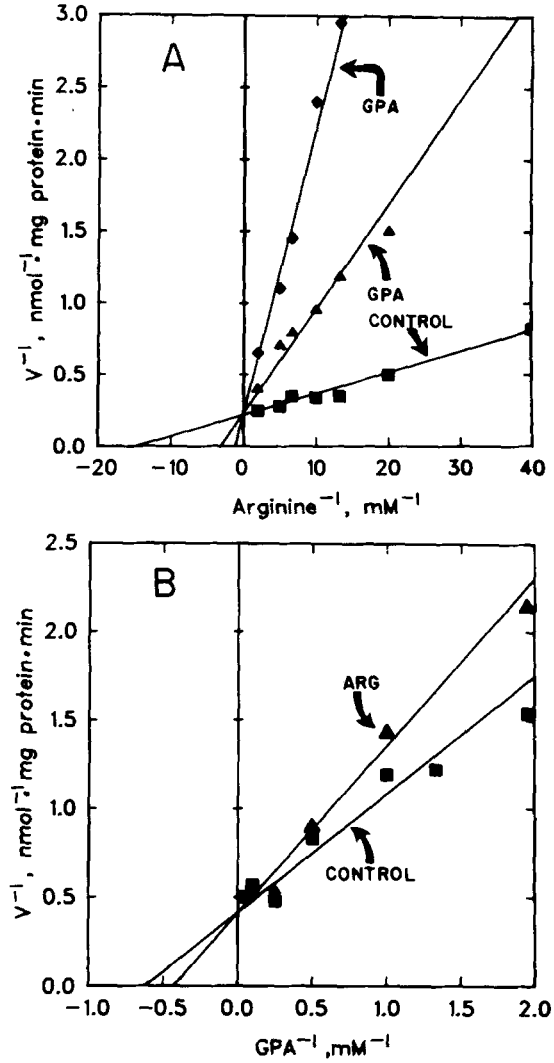

FIG. 7. **Mutual inhibition of arginine and GPA uptake.** Lineweaver-Burk plots are constructed for the influx of [^{3}H]arginine (30 s) or [^{14}C]GPA (2 min). The uptake of arginine (*A*) was measured in the presence of no inhibitor (■), 5 mM GPA (▲), or 20 mM GPA (◆). The data shown here have been corrected for a nonsaturable uptake with K_d = 1.5 nmol·mg^{-1} of protein·min^{-1}·mM^{-1}. The K_i determined for GPA is 1.1 ± 0.1 mM. Uptake of GPA (*B*) was measured in the presence (▲) and the absence (■) of 0.1 mM arginine. A competitive inhibition constant of 0.07 mM was obtained after correction for the nonsaturable influx with a K_d of 0.42 nmol·mg^{-1} of protein·min^{-1}·mM^{-1}.

receptor site as the length is decreased could change the alignment of the guanidino group which may be crucial for optimal recognition. Secondly, loss of methylene groups may decrease hydrophobic binding energy. Finally, alterations of the acid-base properties of the α-amino group due to the proximity of the positively charged guanidino group will almost certainly alter recognition.

The inhibitory action of lysine shown in Table III indicates a reactivity with System y^+ commensurate with that of arginine or homoarginine. Fig. 6 shows that the uptake of [^{14}C]lysine at 2.4 μM is inhibited to similar degrees of completeness by excessive quantities of unlabeled arginine, ornithine, 2,4-diaminobutyric acid, or lysine. The results of Table III and Fig. 6 point to a sharing of a transport system, y^+, by these cationic amino acids. Although the arginine analog built on the piperidine ring, GPA, proves a considerably weaker inhibitor than the linear analogs (Fig. 7*A*, K_i = 1.1 mM), nevertheless its behavior is consistent with a shared transport by System y^+ as indicated by arginine inhibition of GPA uptake (Fig. 7*B*, K_i = 0.07 mM). The N^{α}-methylation of arginine or lysine led to K_i values on arginine uptake so high as to be consistent with their exclusion from the transport system, as in the Ehrlich cell (29). These observations are consistent with the previously proposed distribution of subsites participating in the recognition of substrates by a similar or identical system (3).

Inhibition of Arginine Transport by Neutral Amino Acids—The neutral amino acid inhibition of cationic amino acid influx previously seen in Ehrlich cells and rabbit reticulocytes depends strictly on the presence of an alkali metal ion, $Na^+ > Li^+ \gg K^+$ (30). The reactivity is enhanced by a terminal hydroxyl group on the neutral amino acid, and maximized when the carbon length is 5 atoms (30–32). Fig. 8*A* shows similarly a sodium-dependent inhibition of arginine influx by glutamine, serine, and homoserine. The effect of varying concentrations of these amino acids on arginine influx is shown in the presence of Na^+ (Fig. 8*A*) and in a Na^+-free medium (Fig. 8*B*). The results indicate that, as in other cases, homoserine with its 4-carbon chain reacts much more strongly than serine with its 3-carbon chain, or than glutamine with no hydroxyl

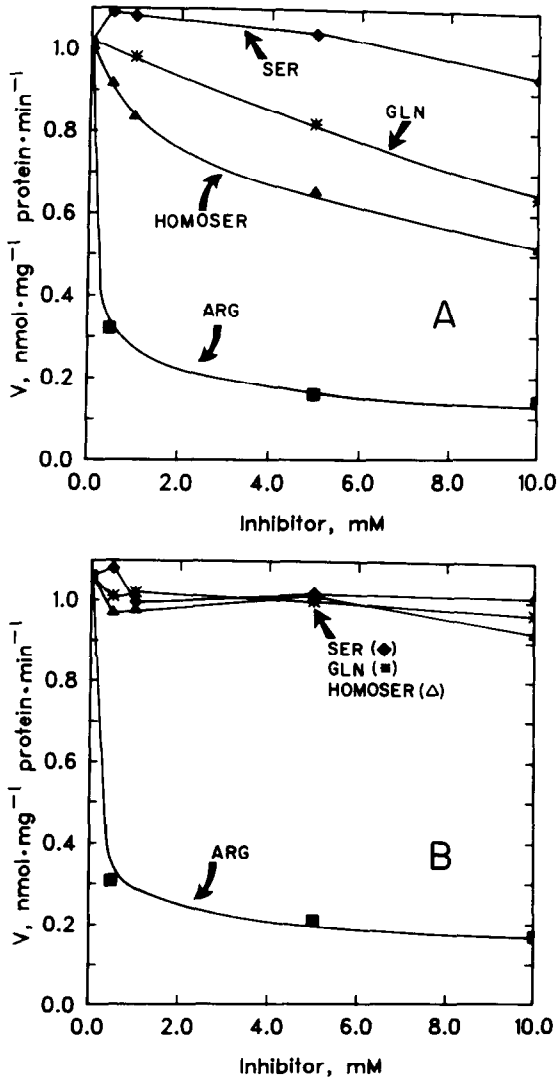

FIG. 8. **Neutral amino acid inhibition of arginine uptake.** The influx of 0.1 mM [^{3}H]arginine was determined in the presence of varying concentrations of L-serine (◆), L-glutamine (*), L-homoserine (▲), or L-arginine (■). Prior to the uptake test, the cells were washed and incubated for 15 min in EBS with all Na^+ replaced with choline. At the time of the experiment, the cells were changed to a sodium-containing medium (*A*) or a sodium-free, choline medium (*B*) containing the appropriate amino acid inhibitor at the indicated concentrations.

group. Removal of the Na^+ eliminates the effect, whereas the uptake of [^{3}H]arginine and its inhibition by unlabeled arginine is independent of added Na^+. Note the later inhibition by external Na^+ when no neutral amino acid had been added (Fig. 2*B*). As in the prior work (30), the optimal separation of the two cationic groups studied in Fig. 5 with the arginine homologs is recognized better with serine and its homologs, making use of their Na^+-dependent inhibition of cationic amino acid transport. By this device, one avoids the overwhelming effect of the changes of pK_2' seen in the homologous series of arginine analogs or α,ω-diamino acids. The optimal separation observed for the α-amino and ω-hydroxyl group can be used to characterize transport systems (33).

Trans-Stimulation of Arginine Flux—The observation that prior incubation of human fibroblasts in amino acid-free medium (Fig. 1) decreases the influx of System y^+ substrates suggested that this mediation responds to *trans*-stimulation by endogenous amino acid pools. To test this hypothesis and determine the specificity of the response, the influx of arginine was measured after loading human fibroblasts with various amino acids. The results of this experiment, summarized in Table IV, show that straight chain cationic amino acids interact strongly on the internal side of the plasma membrane to accelerate influx of arginine, but GPA largely fails to produce this effect. The results in Table V show that external levels of cationic amino acids, including GPA, and of neutral amino acids in the presence of Na^+ stimulate the exodus of accumulated [^{3}H]arginine from fibroblasts. An asymmetry in the *trans*-stimulatory effect of GPA was reported previously for the Ehrlich cell (34). In agreement, external GPA stimulated arginine efflux from the fibroblast (Table V), whereas internal GPA scarcely stimulated arginine influx (Table IV). External GPA may be acting largely as a neutral amino acid, since this small effect was observed in the presence of external Na^+

TABLE IV

Trans-stimulation of arginine uptake

After amino acid depletion, human fibroblasts were incubated for 30 min with 10 mM solutions of the amino acids listed below. Following a brief wash (<5 s) with EBS at 37 °C, 1 min uptake ± S.D. of 0.1 mM [^{3}H]arginine was determined in triplicate. Cells tested in Na^+-free medium were treated for an additional 10 min with a 10 mM amino acid solution in choline EBS before the measurement of uptake.

Amino acid loaded	Uptake Rate		Relative stimulation	
	Sodium	Choline	Sodium	Choline
	nmol·mg^{-1} protein·min^{-1}			
	0.60 ± 0.02	0.46 ± 0.04	1.0	1.0
Arginine	4.4 ± 0.4	3.0 ± 0.3	7.3	7.3
Lysine	4.2 ± 0.4	2.7 ± 0.3	7.0	6.7
Ornithine	3.55 ± 0.06	2.3 ± 0.3	5.0	5.6
Homoarginine	3.4 ± 0.3	2.4 ± 0.4	5.7	5.0
Histidine	1.6 ± 0.1	1.1 ± 0.1	2.7	2.6
Homoserine	1.4 ± 0.2	0.70 ± 0.08	2.3	1.8
Serine	0.8 ± 0.1	0.56 ± 0.07	1.3	1.3
Alanine	0.81 ± 0.07	0.60 ± 0.06	1.4	1.4
Glutamine	0.84 ± 0.06	0.51 ± 0.05	1.4	1.1
GPA	0.73 ± 0.04	0.66 ± 0.04	1.2	1.5
Phenylalanine	0.70 ± 0.1	0.43 ± 0.07	1.2	1.0
Leucine	0.73 ± 0.05	0.40 ± 0.04	1.2	1.1
MeAIB	0.61 ± 0.03	0.46 ± 0.03	1.0	1.0
BCH	0.53 ± 0.05	0.01 ± 0.04	1.0	1.0

TABLE V

Trans-stimulation of arginine exodus

Fibroblasts were incubated for 40 min with 1 mM [^{3}H]arginine, briefly washed (<5 min) with EBS and incubated for 2 min with 2 ml of EBS containing the indicated amino acids at a concentration of 10 mM. A parallel experiment was carried out in which all the Na^+ in the final incubation medium was replaced with choline. The arginine remaining in the cells ± S.D. was determined in triplicate. Before exodus, the cells contained 15.7 ± 0.4 nmol of arginine/mg of protein. The stimulation of exodus during the 2-min interval can be obtained as the difference between the values in the absence (None) and in the presence of 10 mM amino acid.

External amino acid	Arg retained (Na^+)	Arg retained (choline)
	nmol·mg^{-1} protein	*nmol·mg^{-1} protein*
None	10.6 ± 0.6	10.6 ± 0.6
Arginine	5.2 ± 0.1	5.0 ± 0.3
Homoarginine	6.1 ± 0.3	6.0 ± 0.2
Lysine	5.5 ± 0.2	5.7 ± 0.3
Ornithine	4.4 ± 0.3	4.0 ± 0.1
GPA	7.7 ± 0.2	9.5 ± 0.1
Homoserine	4.5 ± 0.2	10.2 ± 0.4
Serine	7.9 ± 0.4	10.5 ± 0.5
Glutamine	5.0 ± 0.2	9.3 ± 1.0
Phenylalanine	9.6 ± 0.4	10.3 ± 0.7
Alanine	7.1 ± 0.4	9.1 ± 0.3
BCH	10.2 ± 0.4	12.2 ± 0.4
MeAIB	10.8 ± 0.5	12.3 ± 0.2

(Table V). In contrast to their external action in Na^+-containing media (Table V), neutral amino acids show little corresponding reactivity with System y^+ when placed at the Na^+-poor inner face of the plasma membrane (Table IV). Histidine, about 10% cationic at physiological pH, and homoserine are the only strongly effective neutral amino acids of those tested for *trans*-stimulation of inward flux. BCH and MeAIB, substrates for Systems L and A, respectively, in fibroblasts (1) and Ehrlich cells (28), have no detectable influence on arginine flux. Removal of Na^+ from the medium had no effect on the degree of *trans*-stimulation observed for cationic amino acids in either direction. The contrast between the weak ability of neutral amino acids to stimulate arginine uptake and the strong stimulation produced by positively charged amino acids adds support to the conclusion that System y^+ primarily mediates cationic amino acid fluxes in human fibroblasts.

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