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THE ACIDIC AMINO ACID TRANSPORT SYSTEM OF THE BABY HAMSTER KIDNEY CELL LINE BHK21-C13

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Summary

The uptake of L-glutamate into BHK21-C13 cells in culture has been studied. This amino acid appears to be transported via a relatively high affinity, low capacity, Na^+-dependent transport system capable of the rapid accumulation of substrate amino acids.

Kinetic studies of the inhibition of L-glutamate uptake has provided information as to the substrate specificity and the molecular configuration required for transport via the glutamate transport system. This system exhibited marked substrate specificity and was only capable of transporting L-glutamate and aspartate and certain closely related acidic amino acid analogues.

Introduction

The transport of the naturally occurring acidic amino acids glutamate and aspartate has been studied in a number of mammalian and microbial organisms [1,2]. A number of differences in the relative activities and specificities of transport systems capable of transporting both these animo acids have been reported in mammalian [3—6] and microbial systems [7—11]. Separate aspartate and glutamate transport systems have also been observed in *Escherichia coli* [12,13].

Little work however, has been carried out involving the transport of glutamate and aspartate in cultured or isolated mammalian cells, with the exception of the studies with Ehrlich ascites tumour cells [14—17,32] and with isolated synaptosomes of rat central nervous tissues [4]. In this paper the transport of

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the naturally occurring acidic amino acids L-glutamate, L-aspartate and L-cysteate, and the properties of a proposed transport system specific for these amino acids are investigated in the baby hamster kidney cell line BHK21-C13.

Materials and Methods

Chemicals. Chemicals used were of reagent grade and were obtained from Sigma Chemical Co. Ltd., London, with the exception of L-homocysteate which was obtained from Calbiochem Ltd., London, and DL-4-fluoroglutamate which was obtained from Koch Light Ltd., Colnbrook, Bucks., England. L-[U-^{14}C]glutamate, L-[G-^{3}H]aspartate and L-[3,3-$^{3}H_2$]-cystine · HCl were obtained from Radiochemical Centre, Amersham, Bucks., England.

Cell culture techniques. BHK21-C13 cells were grown in Glasgow modified Eagles minimum essential medium [18], supplemented with 10% (v/v) foetal calf serum (Gibco-Biocult Ltd., Paisley, Scotland) and incubated at 37°C in a 5% CO_2 atmosphere. Cells were grown for 48 h in 60-mm petri-dishes (Gibco-Biocult Ltd., Paisley, Scotland) prior to amino acid transport studies.

Uptake assay procedures. Amino acid uptake was studied in log-phase cultures of BHK21-C13 cells by a modification of the method used for sugar transport studies, described by Hatanaka et al. [19]. The culture medium was removed by aspiration and the cells washed twice with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonate)-buffered salt solution of the following composition: NaCl (114.8 mM), KCl (5.4 mM), $MgSO_4$ (0.8 mM), $CaCl_2$ (1.8 mM), NaH_2PO_4 (0.9 mM), $Fe(NO_3)_3$ (0.003 mM), $NaHPO_4$ (0.9 mM), glucose (25 mM), HEPES (20 mM), pH 7.4, (subsequently referred to as buffered salt solution). This was then replaced with buffered salt solution (1 ml) containing radioactive amino acid (L-[U-^{14}C]glutamate, 225 Ci/mol; or L-[G-^{3}H]aspartate, 100—300 Ci/mol) plus carrier amino acid added to the required initial concentration. After incubation for the required time interval at 37°C the buffered salt solution was removed and the cells washed rapidly four times with warm (37°C) non-radioactive buffered salt solution. After the cells were air-dried 1 ml of Folin's solution C [20] was added and the cells maintained for 1 h at 4°C. Four 0.1-ml aliquots were then separately transferred to scintillation vials containing 5 ml of NEM 250 liquid scintillation fluid (Nuclear Enterprises Ltd., Edinburgh, Scotland) and the radioactivity of the samples measured. Four further 0.1-ml aliquots were transferred to Folin's solution C and the protein content of these samples determined, using the method described by Oyama and Eagle [20]. Initial rates of uptake (v) for various substrate amino acid concentrations (S) were determined from incubations of up to 10 min, during which time transport was approximately linear. This data was initially plotted graphically, using the S/v versus S plot [21] in order to determine whether the data appeared to deviate markedly from linearity. K_m and V values and their standard errors were determined from the method of least square regression described by Wilkinson [22].

Extraction and chromatography of endogenous radioactive amino acids. Following growth of monolayer cultures in petri dishes (60 mm) the growth medium was replaced with warm buffered salt solution containing radioactive amino acid, and incubated for the required time interval at 37°C. The cells

were then washed rapidly four times with warm buffered salt solution (37°C), 0.5 ml of ice-cold trichloroacetic acid (10%, w/v) added and the cells maintained for 1 h at 4°C. The cells were then removed by scraping and the trichloroacetic acid-insoluble fraction separated by centrifuging at 10 000 × *g* for 14 min. Amino acids present in the trichloroacetic acid-soluble fraction were then isolated by ether extraction. Radioactive L-glutamate was separated from the other amino acids by low-voltage ionophoresis for 3 h at 200 V (current 5 mA/3 cm wide strip), in buffer pH 1.9 (58 ml glacial acetic acid/26 ml 25% (w/w) formic acid, in a final volume of 2 l). The ionophoretogram was then cut into strips and the radioactivity of each determined. Intracellular levels of radioactive L-glutamate were calculated from an estimate of the total cell volume per dish. This value was calculated from the cell numbers and mean cell volumes (determined by the method of Magath and Berkson [23]) of duplicate cell cultures.

Preparation and purification of L-[^{3}H]cysteic acid. L-[3-^{3}H]Cysteic acid was synthesized from L-[3,3-$^3H_2$]cystine · HCl (625 Ci/mol) by a modification of the method of Moore [24] described by Hendrickson and Conn [25]. L-[3,3-$^3H_2$]Cystine · HCl was applied to 3MM Whatman chromatography paper, allowed to dry and then moistened with 0.02% ammonium molybdate. After drying the sample was moistened with performic acid, incubated for 1 h at 4°C and strip again dried. The L-[^{3}H]cysteic acid was separated from impurities by high voltage electrophoresis for 20 min, 300 V, in tank buffer of pyridine/acetic acid/water (250 : 10 : 250, v/v), pH 6.4.

Results

Accumulation of L-glutamate and general characteristics of its uptake

L-[^{14}C]Glutamate uptake by monolayer cultures of BHK21-C13 cells was approximately linear over the first 10 min of assay for all examined concentrations. In view of the long period of linearity of incorporation of this amino acid into these cells the nature of this intracellular radioactivity was investigated. After a 10 min incubation of the cells in buffered salt solution containing L-[^{14}C]-glutamate (initial concn. 10 μM) only 5% of the total sample radioactivity was found in the trichloroacetic acid-insoluble (protein) fraction. The amino acids preserved in the ether-extracted trichloroacetic acid-soluble fraction were separated by ionophoresis. A single major band, containing 94% of the applied total radioactivity, was observed. This band was seen to run coincidently with control samples of L-[^{14}C]glutamate. Comparison of the intracellular : extracellular concentrations of L-[^{14}C]glutamate indicated that this amino acid was concentrated approx. 30-fold over 10 min. No accumulation of radioactive glutamate was observed in cells that had been preincubated for 10 min (and throughout this transport assay) in the presence of NaN_3 (5 mM) and NaCN (2 mM).

A number of other inhibitors were also shown to reduce the transport of L-glutamate in BHK21-C13 cells; the most effective being ouabain (an inhibitor of cation transport) and the sulphydryl group inhibitors *N*-ethylmaleimide and *p*-chloromercuribenzoate (Table I). The inhibition of transport by the last two may indicate that sulphydryl groups are involved in L-glutamate uptake,

TABLE I

EFFECT OF INHIBITORS ON L-GLUTAMATE UPTAKE IN BHK21-C13 CELLS

Uptake of L-[^{14}C]glutamate ($2 \cdot 10^{-5}$ M) was examined in the presence of the above inhibitors, following preincubation of cells in their presence for 10 min. Rates of uptake were determined from 5-min incubations. Values are means of six samples ± S.E. t values were considered significant if P was less than 0.05 (t = 2.20).

Inhibitor	Rate of L-glutamate uptake (pmol/μg protein per min)	t	P
None	2.40 ± 0.22		
Cycloheximide ($1 \cdot 10^{-3}$ M)	1.70 ± 0.13	2.67	0.05 > 0.02
P-Chloromercuribenzoate ($1 \cdot 10^{-4}$ M)	1.35 ± 0.09	4.46	0.005
N-Ethylmaleimide ($1 \cdot 10^{-4}$ M)	1.10 ± 0.09	5.45	0.005
Ouabain ($1 \cdot 10^{-4}$ M)	0.72 ± 0.10	7.43	0.005
NaCN ($1 \cdot 10^{-3}$ M)	0.07 ± 0.01	10.53	0.005
NaN_3 ($1 \cdot 10^{-3}$ M)	0.19 ± 0.02	10.00	0.005

although this inhibition may not be directly at the carrier site.

The uptake of L-glutamate by these cells was also examined at various temperature and pH values. L-Glutamate uptake was minimal in cells maintained at temperatures less than 15°C and the transport rate rapidly increased to an apparent optimum at about 39°C (Fig. 1). The Q_{10} value for the increased rate at L-glutamate uptake between 25 and 37°C was 2.41. L-Glutamate uptake was also shown to vary with the pH value of the assay medium (Fig. 2) and showed an apparent pH optimum at approx. 7—7.3. At this value the α-amino group is fully protonated and carboxyl groups are in the non-protonated form (pK_a values: α-carboxyl, 2.19; γ-carboxyl, 4.25; α-amino, 9.67).

A vital role for Na^+ in the active transport of certain amino acids has been

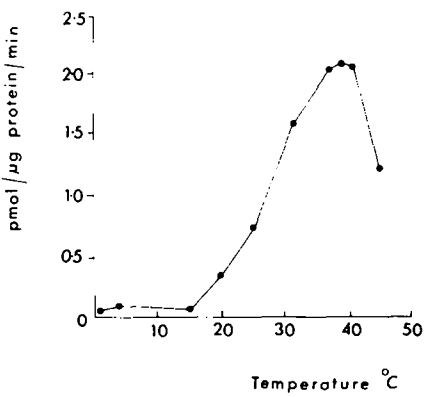


Fig. 1. Effect of temperature on the uptake of L-glutamate into BHK21-C13 cells. The uptake of L-[^{14}C]glutamate (20 μM) was examined at various temperatures following a 10 min preincubation of the cells prior to assay. Rates of uptake were based on 4-min incubations and each point represents the mean of six samples.

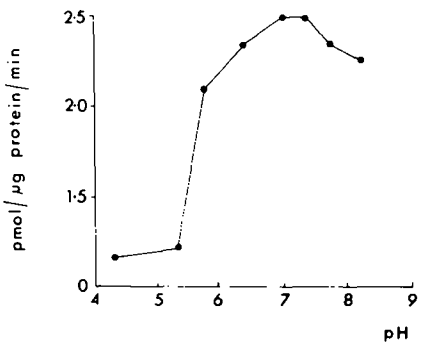
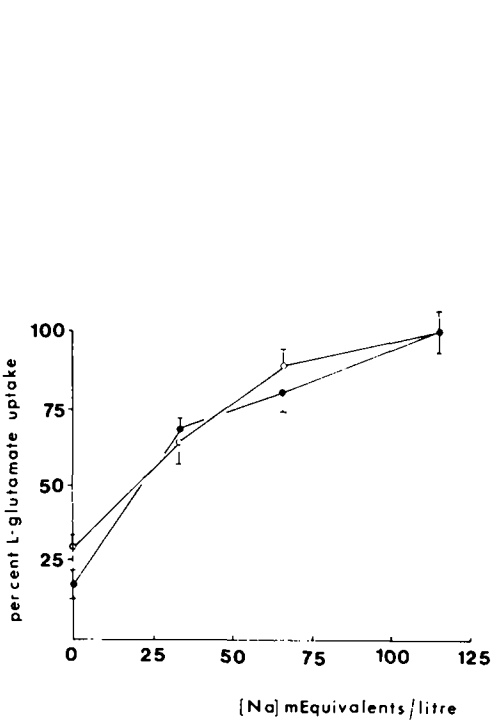


Fig. 2. Effect of pH on L-glutamate uptake into BHK21-C13 cells. The uptake of L-[^{14}C]glutamate (20 μM) was examined at various pH values between 4.3 and 8.3. Initial rates of uptake were based on 10-min incubations and each point is shown as the mean of six samples.

demonstrated in a number of mammalian tissues [3,26,27] and its possible importance in L-glutamate uptake was indicated from its marked inhibition by ouabain. L-Glutamate uptake was consequently studied in balanced salt solution containing various Na^+ concentrations (Fig. 3). The rate of L-glutamate uptake was reduced as the extracellular Na^+ concentration was reduced and was minimal when Na^+ were almost completely replaced by D-mannitol or choline chloride. Thus the transport of this amino acid exhibited a high degree of dependence on exogenous Na^+.

Kinetics of L-glutamate uptake

The variation of the initial rates of transport (based on uptake over 10 min) of L-[^{14}C]glutamate with the concentration of L-glutamate (20 μM—1 mM) in the assay medium was investigated. The S/v versus S plot of this data appeared to be linear (Fig. 4a) indicating that this amino acid was transported according to Michaelis-Menten kinetics, an observation consistent with the transport of



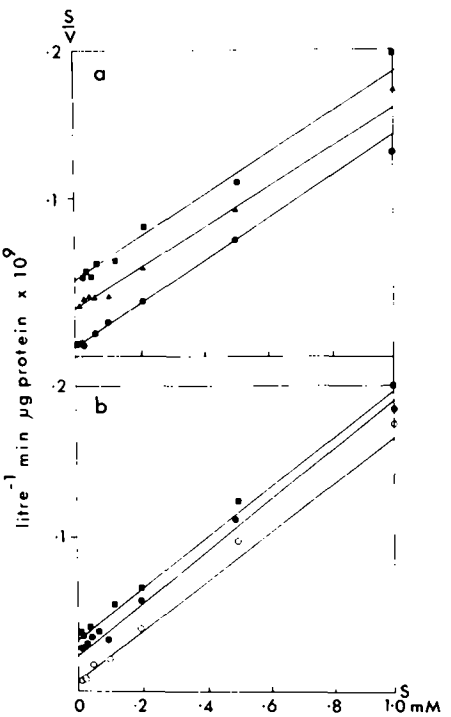


Fig. 3. Effect of Na^+ concentration on L-glutamate uptake into BHK21-C13 cells. The uptake of L-[^{14}C]-glutamate (20 μM) from uptake medium containing various Na^+ concentrations, was examined for 10-min incubations. The NaCl component of the uptake medium was replaced by equiosmolar quantities of D-mannitol or choline chloride. (●———●) represents uptake in the presence of D-mannitol and (○———○) in the presence of choline chloride. Each point is shown as the mean of six samples ± S.E.

Fig. 4. (a) S/v versus S plots of L-glutamate in the presence and absence of L-aspartate or L-cysteate. Uptake of L-[^{14}C]glutamate into BHK21-C13 cells was examined in the absence (●———●) and presence of 200 μM L-aspartate (▲———▲) or 500 μM L-cysteate (■———■). (b) S/v versus S plots of L-aspartate in the presence and absence of L-glutamate or L-cysteate. Uptake of L-[^{3}H]aspartate into BHK21-C13 cells was examined in the absence (○———○) and presence of 200 μM L-glutamate (●———●) or 500 μM L-cysteate (■———■). Rates of uptake were based on incubations of up to 10 min.

L-glutamate (over the examined concentration range) via a single transport agency. Statistically determined estimates of K_m and V produced values of 46 ± 7 μM and 7.5 ± 0.3 pmol/μg protein per min, respectively.

Specificity of the L-glutamate uptake system

The specificity of the L-glutamate transport system was investigated by examining the ability of a number of naturally occurring amino acids and certain glutamate and aspartate analogues to inhibit L-[^{14}C]glutamate uptake (Table II). Only L-glutamate, L-aspartate, L-cysteate and DL-4-fluoroglutamate produced any significant inhibition indicating that these amino acids may be transported by the same transport system. In order to investigate this possibility further a more detailed kinetic analysis of the transport and inhibition by these compounds was made.

The initial rates of L-aspartate and L-cysteate transport (concentration range 20 μM—1 mM) were determined from uptake over a 10 min period, during which time uptake was linear. S/v vs. S plots of the initial rates of transport (Fig. 4b shows L-aspartate uptake; L-cysteate uptake not shown) indicate that over the examined concentration range each of these amino acids are transported via a single, though not necessarily identical uptake system. Statistically

TABLE II

INHIBITION OF L-GLUTAMATE UPTAKE INTO BKH21-C13 CELLS BY DIFFERENT AMINO ACIDS

Uptake of L-[U-^{14}C]glutamate ($2 \cdot 10^{-5}$ M) was examined in the presence and absence of inhibiting amino acid or amino acid anologues (of concentration $5 \cdot 10^{-4}$ M, for L-amino acids, or $1 \cdot 10^{-3}$ M for DL-amino acid anologues). Incubations were made for 10 min. Values are the means of five samples and are expressed as percentage inhibition of L-[U-^{14}C]glutamate uptake alone.

Inhibiting substrate	Percentage inhibition of L-[U-^{14}C]-glutamate uptake	Inhibiting amino acids	Percentage inhibition of L-[U-^{14}C]-glutamate uptake
(a) α-Amino group modifications		L-Glutamate	89
α-Keto glutarate	−8	L-Aspartate	94
N-Acetyl-L-glutamate	12	L-Cysteate	79
N-Acetyl-DL-aspartate	6	L-Alanine	3
N-Formyl-L-aspartate	10	L-Serine	5
N-*p*-Nitrobenzoyl-L-glutamate	8	L-Valine	7
		L-Threonine	6
(b) α-Hydrogen group modifications		L-Phenylalanine	8
DL-2-Methylglutamate	6	L-Tyrosine	6
DL-2-Methylaspartate	7	L-Tryptophan	7
		L-Leucine	10
(c) α-Carboxylate group modifications		L-Isoleucine	8
α-Aminobutyrate	−3	L-Arginine	3
		L-Lysine	6
(d) γ-Carboxylate group modifications		L-Cysteine	6
L-Glutamatic acid-5-methyl-ester	9	L-Cystine	14
L-Homocysteate	3	L-Methionine	15
		L-Proline	7
(e) Side chain modifications		Glycine	6
DL-*erythro*-3-hydroxy aspartate	5	L-Glutamine	16
DL-*threo*-3-hydroxy aspartate	−6	L-Aspargine	10
DL-4-fluoroglutamate	71	L-Glutamine	16
DL-α-aminoadipate	14	D-Glutamate	13
		D-Aspartate	24

TABLE III

COMPARISON OF K_m AND K_i VALUES FOR ENTRY OF ACIDIC AMINO ACIDS INTO BHK21-C13 CELLS

K_m and K_i values for the uptake of these amino acids were determined from rates of uptake based on incubations of up to 10 min as described in Materials and Methods. K_p values, from which K_i values and their standard errors were calculated, are shown in parentheses.

Amino acid	K_m (μM)	K_i values when acting as an inhibitor of:	
		L-Glutamate	L-Aspartate
L-Glutamate	46 ± 7	–	58 ± 11 (151 ± 19)
L-Aspartate	34 ± 8	47 ± 10 (242 ± 18)	–
L-Cysteate	69 ± 5	77 ± 17 (346 ± 37)	91 ± 27 (221 ± 17)
DL-4-Fluoroglutamate	–	114 ± 24 (247 ± 18)	104 ± 31 (197 ± 17)

determined estimates of K_m and V produced values of 34 ± 8 μM and 6.1 ± 0.3 pmol/μg protein per min for L-aspartate and 69 ± 10 μM and 1.3 ± 0.5 pmol/μg protein per min L-cysteate uptake, respectively. The kinetics of DL-4-fluoroglutamate uptake were not examined as radioactivity labelled preparations of this amino acid analogue were not readily synthesized or commercially available.

The S/v vs. S plots of L-[^{14}C]glutamate uptake in the absence and presence of inhibiting amino acids, L-aspartate, L-cysteate and DL-4-fluoroglutamate were approximately parallel, indicating that these latter amino acids were competitive inhibitors of L-glutamate uptake (Fig. 4a demonstrates typical inhibition of L-glutamate uptake as exemplified by L-aspartate and L-cysteate). Similar results were obtained for L-aspartate uptake in the absence and presence of L-glutamate, L-cysteate and DL-4-fluoroglutamate (Fig. 4b shows inhibition of L-aspartate uptake by L-glutamate and L-cysteate). This data thus indicates that the inhibition of L-glutamate and L-aspartate uptake by each of these amino acids is competitive.

K_m values for the uptake of these amino acids and their K_i values when acting as inhibitors are summarised in Table III. K_i values for L-glutamate, L-aspartate and L-cysteate when acting as inhibitors, were not significantly different from their K_m values for uptake. Such observations are consistent with the requirements of the transport of these amino acids via a single transport system [28]. The similar K_p values obtained for DL-4-fluoroglutamate when inhibiting the uptake of glutamate and aspartate are also consistent with this amino acid analogue being transported via this "acidic amino acid transport system".

Discussion

The results presented in this paper indicate that for the examined concentration ranges the anionic forms of L-glutamate, L-aspartate and L-cysteate are transported via a single amino acid transport system specific for these amino acids. Uptake studies with L-glutamate have demonstrated that this transport system has a relatively high affinity but relatively low substrate capacity and is

energy, temperature, pH and Na^+ dependent.

Amino acid transport systems specific for L-glutamate and L-aspartate have also been demonstrated in a number of mammalian cells or tissues, including rabbit ileum and jejunum [3], kidney [31] and the synaptosomes of rat central nervous tissue [4,29]. Other mammalian acidic amino acid transport systems, however, have been shown to be less specific in their substrate specificity and have shown some overlap with the transport systems responsible for basic or neutral amino acids, e.g. the low affinity acidic amino acid transport system of brain [30,31]. L-Glutamate transport in Ehrlich ascites carcinoma cells, however, does not appear to be via a system specific for acidic amino acids but via the two major (A and L) system for neutral amino acids [32].

Although mammalian cells and tissues are capable of maintaining intracellular concentrations of glutamate and aspartate at levels greater than in the surrounding environment [33], levels of accumulation of these amino acids from their immediate environment is generally low, e.g. Ehrlich ascites carcinoma cells [17]. However, cultured BHK21-C13 cells were demonstrated in this paper to be similar to brain tissues slices [34] and most examined microorganisms [11,13], in that glutamate may be rapidly accumulated to levels in excess of those in the surrounding medium. Although other tissues such as intestine have been shown to transport these acidic amino acids at rates comparable to the readily accumulated neutral amino acids [3] the amino acids are not apparently accumulated. Such absence of glutamate or aspartate accumulation in these tissues may be due to the rapid transamination [31,34] or the conversion of glutamate to glutamine [35,36].

The affinity of the BHK21-C13 acidic amino acid transport system for substrate amino acids as indicated by the K_m values for L-glutamate (46 μM) and L-aspartate (34 μM), is very similar to those observed for the high affinity transport systems for acidic amino acids in isolated synaptosomes obtained from the spinal cord and cerebral cortex of rat [4]. These K_m values are approximately two orders of magnitude lower than the low affinity acidic amino acid transport system reported for brain slices [5] and two to three orders of magnitude (depending on the Na^+ concentration) lower than values observed for rabbit ileum and jejunum [3].

Inhibition studies using naturally occurring amino acids or chemical analogues of glutamate or aspartate provided additional information as to the required molecular configuration for transport via the acidic amino acid transport system of BHK21-C13 cells. This system exhibited a marked preference for the L-stereoisomers of glutamate and aspartate and removal or modification of the α-amino or α-carboxyl groups of substrate amino acids completely abolished the ability of these compounds to be transported via the carrier. Substitution of the α-hydrogen by an aliphatic group, increase in the glutamate side chain by a single methylene group (e.g. in α-amino adipate), the substitution of a β-hydrogen atom by a different group also completely abolished the affinity of the transport system for the compound. Substitution of a γ-hydrogen atom by a fluoro group, however, did not completely remove affinity for the transport system. A second anionic group was also demonstrated to be necessary in a transportable amino acid as esterification or amidation resulted in complete loss of affinity for the transport system, although substitution of

the β-carboxyl of L-aspartate by a sulphonate group (L-cysteate) appears to satisfy the general specificity requirement of the acidic amino acid transport system $R^- \cdot CH \cdot (NH_3^+)CO_2^-$.

Very few studies have investigated the structural requirements of the transport systems specific for acidic amino acid uptake. Limited studies with dog kidney [37] and more detailed studies with the high affinity acidic amino acid transport systems of rat synaptosomes [4,38,40] have demonstrated structural requirements similar to those of the BHK21-C13 system. More extensive studies have been carried out in micro-organisms and in nearly all cases the organism possesses at least one transport system for acidic amino acids. These systems generally exhibit a less marked stereospecificity than the BHK21-C13 system although different organisms vary in their tolerance to side chain, α-amino and α-carboxyl modifications [2].

We have previously demonstrated that at physiological pH L-glutamate appears to have little affinity for the alanine-(A) and leucine-preferring (L) transport systems which are responsible for the uptake of the neutral amino acids in monolayer cultures of the baby hamster kidney cell line BHK21-C13 [41]. The studies described in this paper have demonstrated the presence in these cells of an active transport system capable of the accumulation of the anionic forms of the naturally occurring acidic amino acids. This acidic amino acid transport system was shown to have a relatively high substrate affinity and to transport only those amino acids of very similar structural configuration.

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