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Novel regulations of glutamate and aspartate uptake by HeLa cells

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Abstract

Pathways of L-glutamate and L-aspartate import by HeLa S_3 cells were investigated before and after the cells were depleted of internal amino acids by starvation. Two new regulations of transport were observed in starved cells. Aspartate entered nonstarved cells by two routes, one non-saturable and one, an apparent analog of saturable system X_{AG}^-, that was sodium-dependent and competitively inhibited by glutamate. Starvation for one hour in saline increased the efficiency of saturable aspartate import, increasing V_{max} and decreasing K_m, an effect not previously reported for system X_{AG}^-. Glutamate uptake by nonstarved cells appeared to occur through system X_{AG}^-; through an analog of system x_C^-, which was sodium-independent, cystine- and quisqualate-inhibitable; as well as through one or more nonsaturable pathways. Starvation in saline for one hour resulted in the appearance of a new low-affinity saturable glutamate uptake system. This new system was sodium-dependent but not inhibited by aspartate.

Keywords: Glutamate transport; Kinetics; Regulation; HeLa

1. Introduction

We have previously described how the use of iterative curve fitting and related numerical techniques can benefit analysis of whole cell transport experiments [1]. This was illustrated, in part, with new data on the uptake of glutamate by HeLa S_3 cells. In particular, a goodness-of-fit statistic helped reveal that starvation resulted in the appearance of an apparently new glutamate transport activity. The present report provides a more detailed picture of anionic amino-acid transport by HeLa.

The transport of the anionic amino acids, glutamate and aspartate, across mammalian cell plasma membranes has traditionally been studied from two perspectives, being seen either as a general cellular activity or as a function vital for the central nervous system where glutamate serves as an excitatory neurotransmitter. Studies of human fibroblasts have described a high-affinity, sodium-dependent uptake system which was named X_{AG}^-. The subscripts indicate that both aspartate and glutamate are substrates for this system [2]. A similar system was observed in cultured liver cells [3]. Studies of different human fibroblast cultures by other authors found only a sodium-independent system. It was named x_C^- to indicate that cystine competed with glutamate for uptake (while aspartate did not) [4]. The literature on the nervous system emphasizes ion-dependent activities which couple transmembrane flows of sodium, potassium, and/or chloride to glutamate uptake [5–11]. This uptake is required to terminate synaptic transmission initiated by glutamate release at the synapse. Recent cloning studies have identified genes coding for distinct sodium-dependent glutamate transporters in rat brain, GLAST [12], in glial cells, GLT-1 [13], and in neurons, EAAC1 [14]. None of these brain transporters appears to be widely expressed outside the nervous system, although EAAC1 was actually cloned from kidney epithelial cDNA and is expressed in specialized transporting epithelium as well as in neurons [14]. Quisqualate, a glutamate analog, has been reported to inhibit sodium-independent glutamate import by glia [9,15], and specifically to inhibit glutamate and cystine uptake through an x_C^--like transport system in cultured rat glioma cells [15]. Overall, the diversity of transport systems for these nonessential amino acids is intriguing.

We have been interested in further exploring anionic amino-acid uptake outside the nervous system. The human carcinoma line, HeLa S_3, has been extensively studied, though its uptake of glutamate had not been described

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before our initial report. Based on the literature and our experience with Chinese hamster ovary (CHO) cells [16], we were expecting to find transport activity analogous to system X^-_{AG} or x^-_C, or both, in HeLa. Both systems appear to operate in HeLa, and these experiments revealed additional complications. In particular, starvation was found to increase the efficiency of system X^-_{AG} and to induce the new glutamate-specific import system. Characterizing two or three saturable systems operating simultaneously proved to be an analytical challenge, and, as a consequence, many experiments must be described. The reader is here referred to Fig. 3, which provides a succinct graphic summary of the results.

2. Materials and methods

Reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted.

2.1. Cell culture

HC4, a randomly chosen subclone of HeLa S_3 [17], was grown in D/F H5C5 medium, consisting of 45% Dulbecco's modified Eagle medium with non-essential amino acids, 45% Ham F12 medium (Irvine Scientific, Santa Ana, CA), 5% horse serum (HyClone, Logan, Utah), 5% calf serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.45 g/l $NaHCO_3$. Cells were passed approximately twice per week, by incubation in a thin film of 0.01% trypsin for 10 min, with subsequent resuspension in fresh D/F H5C5. After 35 to 40 passages, cultures were replaced from frozen stocks.

2.2. L-Glutamate and L-aspartate uptake assays

Uptake of glutamate and aspartate was measured by the method of Gazzola et al. [18] with minor modifications [19]. Cells were plated on 24-well trays (Costar, Cambridge, MA), at a density of $1.6 \cdot 10^5$ per well, and grown for 24 h before the assay. Cells were rinsed twice with Hepes-buffered saline (Research Organics, Cleveland, OH) (HBS · MCD: 137 mM NaCl, 0.7 mM K_2HPO_4, 10 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 g/l D-glucose, pH adjusted to 7.4 with 1 N KOH, 37°C) or HBC · MCD (HBS · MCD with 137 mM choline chloride replacing NaCl) before addition of uptake solutions containing L-[^{3}H]glutamate (5 μCi/ml) or L-[^{3}H]aspartate (10 μCi/ml) (Amersham, Arlington Heights, IL). An uptake assay usually consisted of velocity measurements in triplicate at 16 different concentrations of substrate. Uptake was typically measured over a range of substrate concentrations of 1 μM to 5 mM. Amino acids were extracted from the cells with 10% trichloroacetic acid, ^{3}H dpm were determined with a Packard (Downers Grove, IL) 2000CA scintillation counter, and the total cell protein was measured by the bicinchoninic acid method [20]. Results are reported as weighted averages of two or more experiments, or as values from a 16-point experiment confirmed by one or more 8-point studies.

In the 'zero-time' protocol, uptake of glutamate or aspartate by cells in HBS · MCD or HBC · MCD was measured immediately after two rinses with the same saline used for the assay. Alternatively, the cells were starved for amino acids by rinsing twice with HBS · MCD, with preincubation in the second saline rinse for 1 h before replacement of the saline with radioactive uptake solutions. If uptake by starved cells was to be measured in HBC · MCD, then the cells were rinsed twice with HBC · MCD immediately before the uptake assay. For experiments involving 3 h starvation, cells were preincubated in a modified Earle's balanced salt solution (EBSS dH5C5: 116 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 6 mM D-glucose, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ containing 5% dialyzed horse serum and 5% dialyzed calf serum), and were rinsed twice in HBS · MCD immediately before the assay. Uptake was measured for 2 min, the longest interval over which velocity was constant with time under both zero-time and starvation treatments.

Inhibition by 2-(methylamino)isobutyric acid (MeAIB), a substrate of system A for neutral amino acids [21], was tested at 10 mM while L-cystine, a substrate of system x^-_C [4], was added as an inhibitor at 300 μM, limited to that concentration by the compound's low solubility. Quisqualate (QA), a substrate of a system x^-_C analog in glia [15], was added as an inhibitor at a concentration of 500 μM.

2.3. Computation of kinetic parameters

Fitting of velocity data to nonlinear equations was performed via Marquardt's algorithm [22], using KaleidaGraph (Synergy Software, Reading, PA) for the Apple Macintosh. Velocity data were fit to each of two models:

$$V = \frac{V_{max}[S]}{K_m + [S]} + k_D[S] \text{ and}$$

$$V = \frac{V_{max1}[S]}{K_{m1} + [S]} + \frac{V_{max2}[S]}{K_{m2} + [S]} + k_D[S]$$

for one and two saturable (Michaelis–Menten) uptake systems, respectively. V_{max} is expressed in units of (nmol substrate) (mg cell protein)$^{-1}$ min^{-1}, and K_m, in units of μM. The coefficient k_D is a term describing nonsaturable uptake, expressed as a slope with units of (nmol substrate) (mg cell protein)$^{-1}$ min^{-1} (μM glutamate)$^{-1}$. The linear term was converted to units of min^{-1} via multiplication by a constant of proportionality unique for each cell line. This constant, with units of (mg protein) (ml cell water)$^{-1}$, was calculated from the mass of protein per 10^6 cells and the water volume per cell (see Section 3). The better model

was chosen by comparison of the statistic q, the probability that the data could be derived from the model using the final fitted parameters. The q statistic was calculated from χ^2, a measure of the goodness-of-fit of the data reported by the Marquardt algorithm, and *d.f.*, the number of degrees of freedom of the data [1,23]. A model fit to a set of velocity data for which $q < 0.01$ was automatically rejected, and a model for which $q > 0.1$ was generally accepted. K_i for competitive inhibitors was estimated by the model

$$V = \frac{V_{max}[S]}{K_m(1 + I/K_i) + [S]} + k_D[S]$$

where V_{max} and K_m were determined from analogous experiments lacking inhibitor, and I is the inhibitor concentration in μM. Confidence limits for kinetic parameters were determined by Monte Carlo simulation [1]. The significance of differences between normally distributed parameters was assessed using the standard test of significance [1].

2.4. Measurement of cell volume, internal sodium, membrane potential, and amino-acid pools

Cell volume, determined by equilibration with [^{14}C]urea, was 2.55 ± 0.03 pl/cell and protein was 0.417 mg/(10^6 cells) (W.E. DeBusk, unpublished data). These values were used to convert nonsaturable uptake, k_D, from units of slope = (nmol substrate) (mg cell protein)$^{-1}$ min^{-1} (μM substrate)$^{-1}$ to units of min^{-1}. Sodium concentrations were measured by equilibrating cells in media containing [^{14}C]urea alone or [^{14}C]urea and $^{22}Na^+$ [19,24]. Cells were plated and grown in 24-well trays as described for transport assays. For measurements on non-starved cells, 265 μl medium was drawn from each of four wells, pooled, and brought to an activity of 4 μCi/ml with [^{14}C]urea (Amersham). For sodium determinations, $^{22}Na^+$ (New England Nuclear) was also added to ca. 8 μCi/ml. Radioactivity was determined for duplicate 20 μl samples. All the remaining medium in the wells was removed, and was replaced by 250 μl aliquots of the labeled media. For measurements of starved cells, four wells were rinsed twice with HBS · MCD and then 250 μl of HBS · MCD containing [^{14}C]urea or [^{14}C]urea and $^{22}Na^+$ at the same activities used for the growth medium was added. Cells were incubated for an hour before the radioactive medium was removed and the wells immediately rinsed four times with ice-cold PBS. Radioactivity was then extracted from cells with 10% trichloroacetic acid and quantitated by scintillation counting. For determining specific cell volumes from the [^{14}C]urea-incubated cells, protein remaining in each well was quantitated. Internal sodium concentrations were calculated directly from the ratios of $^{22}Na^+$ to [^{14}C]urea in the extracellular liquid (D/F H5C5: 148 mM Na^+, HBS · MCD: 137 mM Na^+) and that extracted from the cells. Thus, if the ratio fell tenfold in the extract from non-starved cells, the internal sodium level would be 14.8 mM.

Relative transmembrane potentials were estimated by measuring the 30 s influx of tetraphenylphosphonium (TPP^+) [25,26]. As these manipulations resulted in significant release of cells from their growth surface, $4 \cdot 10^5$ cells were plated on three 35 mm culture dishes, rather than in 24-well trays, and grown for 24 h in D/F H5C5. Uptake of 10 μM [^{3}H]TPP^+, 1 μCi/ml, was measured in HBS · MCD and in HBK · MCD (HBS · MCD with 137 mM KCl replacing NaCl) containing 1 mM ouabain. Cells were pre-rinsed twice with 2 ml of the same saline solution used for determining flux rates, and were post-rinsed 4-times with 4 ml ice-cold PBS. The cells were lysed in 0.1 M NaOH, 0.1 M SDS, and samples for counting ^{3}H dpm and for measuring cell protein were extracted directly from the lysates.

Intracellular pools of aspartate and (glutamate + glutamine) were determined by HPLC analysis of 2% sulfosalicylic acid extracts made from cells grown as described for transport assays [16].

Table 1
Aspartate uptake by HeLa cells

Inhibitor [a]	Saturable uptake [b]		Nonsaturable uptake [b]
	V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	Apparent K_m (μM)	k_D (min^{-1})
Nonstarved			
None [c]	0.184 ± 0.005	26.7 ± 1.1	0.0378 ± 0.0008
300 μM Glu	0.211 ± 0.020	*186* ± 19	0.0364 ± 0.0020
300 μM cystine	0.187 ± 0.001	*45* ± 5	0.0344 ± 0.0013
Starved			
None	0.274 ± 0.006	13.5 ± 0.6	0.0396 ± 0.0004
500 μM Glu	0.273 ± 0.006	*209* ± 12	0.0408 ± 0.0025
10 mM MeAIB	*0.343* ± 0.003	13.6 ± 0.4	0.0412 ± 0.0014

[a] 2-min uptake in HBS · MCD, in the presence of the listed inhibitor at the concentration indicated.
[b] Errors represent one standard deviation from the Marquardt fit, as verified by Monte Carlo simulation; $q > 0.1$ for each experiment. A value in italics for inhibited uptake differs significantly ($P < 0.05$) from that of the corresponding experiment in the absence of inhibitor.
[c] Weighted average of two identical experiments.

3. Results

3.1. Aspartate uptake by nonstarved HeLa cells

The velocity of aspartate accumulation by HeLa cells in the presence of Na^+, shown in Fig. 1, could be fit adequately to a model combining one Michaelis–Menten and one nonsaturable system. Kinetic parameters for aspartate influx by HeLa cells in HBS · MCD, measured with or without inhibitors, are listed in Table 1. Glutamate competitively inhibited aspartate uptake, raising the apparent K_m 7-fold; fitting the data for competitive inhibition gave an apparent K_i of 49 ± 6 μM. Cystine inhibited aspartate uptake weakly: $K_i = 420 \pm 100$ μM. Uptake of aspartate was also measured in HBC · MCD. In this sodium-free solution, saturable uptake almost disappeared, while nonsaturable uptake was increased by 75%: $V_{max} = 0.016 \pm 0.004$ nmol/(mg protein min), $K_m = 33 \pm 11$ μM, $k_D = 0.066 \pm 0.007$ min^{-1}. The V_{max} determined without sodium was only 9% of that with sodium present, and K_m was not significantly affected. These data show that aspartate uptake by nonstarved HeLa strongly resembled activity by system X^-_{AG}.

3.2. Aspartate uptake by starved HeLa cells

Influx of aspartate by starved HeLa cells could also be described in terms of a single saturable plus a nonsaturable process; velocity data from this condition are also shown in Fig. 1. However, aspartate uptake was more efficient after starvation: V_{max} was 60% greater and K_m was only half as large (Table 1). Import of aspartate by HeLa was again inhibited strongly by glutamate (Table 1); addition of 500 μM glutamate raised the K_m 15-fold ($K_i = 34 \pm 2$ μM). The presence of 10 mM MeAIB raised V_{max} by 25% for unknown reasons, but had no significant effect on K_m or k_D.

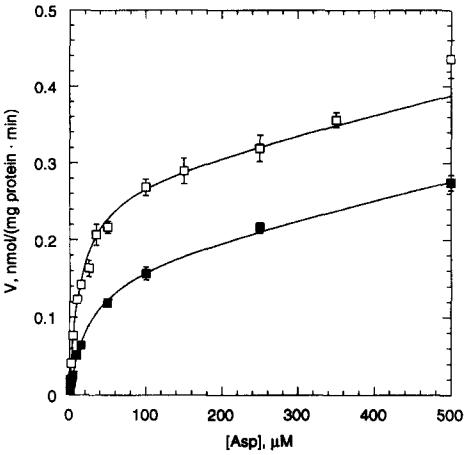

Fig. 1. Aspartate uptake by HeLa cells before and after starvation. Influx of [^{3}H]aspartate was measured before (■) and after (□) 1 h starvation in HBS · MCD. Each velocity point is the mean of three identical measurements; error bars, when larger than the symbols represent 1 S.D. Solid lines show the Marquardt fits of the data, whose uptake parameters are listed in Table 1. To emphasize saturable uptake, only the lower portion of the range of substrate concentrations is shown. Starvation has a clear effect on saturable influx, increasing V_{max} and decreasing K_m.

3.3. Glutamate uptake by nonstarved HeLa cells

Velocity data from all experiments on nonstarved cells could be fit adequately to the same model used for aspartate analysis. Kinetic parameters for uptake measured under several conditions are listed in Table 2. While removing Na^+ from the uptake solutions had a statistically significant effect on all parameters, saturable activity was only slightly affected: V_{max} was lowered by 12%, K_m was raised by 20% and k_D was lowered by 60%. According to the kinetic model adopted, aspartate (10 mM) appeared to be a weak competitive inhibitor of glutamate uptake, doubling K_m with or without extracellular sodium: $K_i \approx 10$ mM under either condition. This level of aspartate lowered

Table 2
Glutamate uptake by nonstarved HeLa cells

Condition [a]	Saturable uptake [b]		Nonsaturable uptake [b]
	V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	K_m (μM)	k_D (min^{-1})
HBS · MCD [c]	1.48 ± 0.05	101 ± 4	0.320 ± 0.006
HBS · MCD + 10 mM Asp	1.42 ± 0.06	*223* ± 10	*0.133* ± 0.006
HBS · MCD + 10 mM MeAIB	1.72 ± 0.24	112 ± 18	0.363 ± 0.029
HBS · MCD + 500 μM QA	*0.36* ± 0.05	82 ± 17	0.320 ± 0.013
HBC · MCD [d]	*1.30* ± 0.04	*121* ± 5	*0.134* ± 0.005
HBC · MCD + 10 mM Asp	*1.72* ± 0.05	*240* ± 8	*0.133* ± 0.004
HBC · MCD + 300 μM cystine	*0.92* ± 0.07	*490* ± 40	*0.128* ± 0.003
HBC · MCD + 500 μM QA	–	–	*0.180* ± 0.002

[a] 2-min uptake without starvation in the presence (HBS · MCD) or absence (HBC · MCD) of Na^+ ions, in the presence of inhibitors as listed.
[b] Errors represent one standard deviation from the Marquardt fit, verified by Monte Carlo simulation; $q > 0.1$ for each experiment. Values in italics type differ significantly ($P < 0.05$) from that of HC4 measured in HBS · MCD.
[c] Weighted average of three identical experiments.
[d] Weighted average of two identical experiments.

V_{max} of total glutamate uptake measured with Na^+ to a value similar to that measured in sodium-free medium without aspartate. Aspartate increased V_{max} measured in the absence of sodium — an unusual effect. Addition of 10 mM MeAIB did not change sodium-dependent glutamate uptake significantly. Cystine at 300 μM affected saturable, sodium-free uptake: V_{max} was lowered 30%, and K_m was quadrupled ($K_i = 98 \pm 10$ μM). Nonsaturable uptake was not affected by aspartate or cystine in the absence of Na^+.

The presence both of a large proportion of sodium-independent saturable glutamate uptake, which was largely aspartate insensitive, *and* of high-affinity, sodium-dependent aspartate uptake (Table 1) suggested that the model used for the analyses of Table 2, while statistically adequate, was probably too simple. It appeared that there were at least two saturable glutamate import systems in HeLa cells. It seemed reasonable to assume that, as in CHO-K1 [16], both systems X^-_{AG} and x^-_C were operating with similar K_m values for glutamate import into HeLa; although in HeLa cells, the sodium-independent system appeared to have the greater V_{max}. The sodium-dependent system was apparently kinetically isolated in experiments in which quisqualate was tested as a potential inhibitor of system x^-_C. Initial experiments suggested that quisqualate inhibited the sodium-independent system competitively, with $K_i < 25$ μM. The addition of 500 μM quisqualate lowered the V_{max} of glutamate import in HBS · MCD by 75% (Table 2). High-affinity uptake resistant to inhibition by quisqualate was presumed to correspond to system X^-_{AG}. This hypothesis was tested by measuring the uptake of glutamate in the presence of sodium, 50 μM aspartate, and 500 μM quisqualate. Aspartate potently inhibited the high-affinity quisqualate-resistant glutamate uptake (data not shown), verifying that this activity could be assigned to system X^-_{AG}. The Marquardt algorithm was unable to provide an accurate estimate of the K_i of aspartate inhibition of the quisqualate-resistant glutamate uptake. The quisqualate-resistant saturable uptake was also found to be sodium-dependent; the presence of 500 μM QA in HBC · MCD abolished all detectable saturable glutamate influx (Table 2). Though lower than the control with sodium present, the nonsaturable uptake with quisqualate in the absence of sodium was higher than that of the other sodium-free conditions. This increase most likely reflects activity of system x^-_C, whose apparent K_m in the presence of 500 μM QA was great enough to make system x^-_C appear as 'nonsaturable' uptake.

We assumed that in HeLa, as in CHO [16], the saturable, sodium-independent, cystine-inhibited uptake was mediated entirely by a system x^-_C activity, which also appeared to be potently inhibited by quisqualate in HeLa. It was not possible, however, simply to refit all experimental data described in Table 2 with the two system model and accurately determine both saturable systems' parameters, for reasons discussed below. Thus, the data presented in Table 2 remain a useful description of glutamate transport by nonstarved cells.

3.4. Glutamate uptake by starved HeLa cells

The results of two independent assays of glutamate uptake by HeLa cells after 1 h starvation in HBS · MCD are shown in Fig. 2. The most simple model to which the

Table 3
Glutamate uptake by starved HeLa cells

Condition [a]	High-affinity saturable uptake [b]		Low-affinity saturable uptake [b]		Nonsaturable uptake [b]
	V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	K_m (μM)	V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	K_m (μM)	k_D (min^{-1})
HBS · MCD, Expt. 1	0.71 (0.57–0.82)	29 (23.3–33.9)	1.67 (0.83–5.92)	937 (304–2080)	0.326 (0.229–0.369)
HBS · MCD, Expt. 2	0.60 ± 0.08	25 ± 3	2.67 (1.21–6.39)	1120 (410–2880)	0.313 (0.222–0.366)
HBS · MCD + 10 mM MeAIB, Expt. 1	*1.21* ± 0.09	30 ± 2	3.29 (1.64–10.1)	1040 (412–3420)	0.239 (0.098–0.312)
HBS · MCD + 10 mM MeAIB, Expt. 2	0.85 ± 0.09	22 (17.4–27.8)	4.20 (2.36–12.5)	1150 (461–3590)	0.206 (0.068–0.265)
HBS · MCD + 10 mM Asp	0.44 (0.33–0.58)	*107* (84.7–135)	1.61 (0.99–3.27)	2060 (1040–6110)	*0.202* ± 0.009
HBS · MCD + 500 μM QA	*0.40* ± 0.05	27 ± 3	ND [c]	ND [c]	ND [c]
HBC · MCD [d]	*0.54* ± 0.01	*43* ± 2	–	–	*0.107* ± 0.004
HBC · MCD + 10 mM Asp [d]	0.62 ± 0.07	*190* ± 20	–	–	*0.091* ± 0.005
HBC · MCD + 500 μM QA	0.40 ± 0.15	*180* ± 60	ND [c]	ND [c]	ND [c]
3 h starvation in HBS · MCD	0.84 ± 0.03	22 ± 1	1.25 (0.57–3.92)	1284 (495–4152)	0.376 (0.322–0.406)

[a] 2-min glutamate uptake after starvation in HBS · MCD. Cells were starved 1 h unless otherwise noted.

[b] Parameters are expressed as mean ± S.D., where normally distributed, or as mean (95% C.I.), where not normally distributed, as determined by Monte Carlo simulation. For normally distributed means, the 95% C.I. is equal to the mean ± 1.96 × S.D. A value in italics type differs significantly ($P < 0.05$) from that of both experiments of HC4 measured in HBS · MCD. For each experiment, $q > 0.05$.

[c] The Marquardt algorithm could not separate among low-affinity saturable uptake and quisqualate-inhibited saturable uptake, and thus the range of substrate concentrations was lowered to 1–1000 μM, and only the parameters for high-affinity uptake were determined.

[d] Only one Michaelis–Menten system was detectable; parameters appear under 'high-affinity uptake' due to numerical similarity with the high-affinity parameters of the control condition (1 h starvation in HBS · MCD).

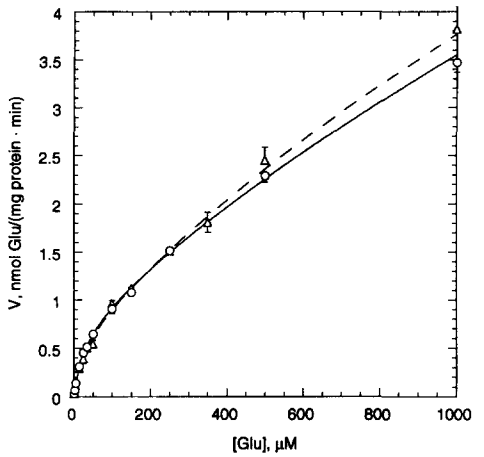

Fig. 2. Glutamate uptake by starved HeLa cells. Influx of glutamate was measured in HeLa cells after 1 h starvation in HBS · MCD. Velocity data and Marquardt curve fits are shown for experiment 1 (○, solid line) and experiment 2 (△, dashed line) from Table 4. The curve fits converge at high concentrations of glutamate, due to the larger estimated k_D of experiment 1. Corresponding velocity points are not significantly different between the two experiments, ($P > 0.01$ by Student's t-test with 4 *d.f.*). Only a portion of the range of substrate concentrations is shown, to emphasize the saturable portion of the curve fits.

velocity data could be fit required two saturable systems, one of which had a K_m of approx. 1 mM. While one-system fits of the two data sets gave $q < 10^{-6}$, two-system fits gave q values of 0.60 and 0.48 for Experiments 1 and 2, respectively. As shown in Table 3, the parameters of this low-affinity system differ between the two experiments, but the overlap of their 95% confidence intervals, as determined by the Monte Carlo method, showed that the differences were not significant. In fact, at each substrate concentration tested, the velocity measured in Experiment 1 did not differ significantly ($P > 0.01$) from the corresponding velocity from Experiment 2. Neither saturable system of starved HeLa cells closely resembled uptake modeled as a single system at zero time (Table 2). Two-thirds of the total V_{max} in starved cells was associated with the low-affinity system (V_{max2}). Though V_{max2} was nearly equal to the V_{max} of total uptake at zero time, K_{m2} was 8-fold larger than K_m at zero time, and this difference was significant even considering the very large uncertainty in K_{m2}. The V_{max} of high-affinity influx by starved HeLa cells was half that of nonstarved cells, while the K_m was only one-fourth that of nonstarved cells. Starvation did not affect k_D.

Additional analyses of these two saturable systems were made in starved HeLa cells and are also reported in Table 3. Previous experiments had suggested that MeAIB might be a competitive inhibitor of the low-affinity system [1]. Again, large variations in the calculated parameters were observed between the two experiments with MeAIB present. The only consistent effects of MeAIB, however, were an approximately 50% increase in V_{max} and a nonsignificant 40% reduction in k_D. MeAIB did not appear to inhibit the low-affinity system. The K_i for MeAIB inhibition of L-proline (a substrate of system A) import by HeLa was determined to be 200 ± 60 μM (data not shown). If system A was responsible for low-affinity saturable glutamate uptake, the addition of 10 mM MeAIB should have increased K_{m2} 50-fold, rendering it undetectable.

The presence of 10 mM aspartate reduced V_{max1} by two-thirds, raised K_{m1} nearly fourfold, and reduced k_D by 40%. These effects represent statistically significant inhibition of aspartate on the high-affinity and nonsaturable uptake systems. In contrast, aspartate had no detectable effect on the low-affinity system. Addition of 500 μM quisqualate halved the V_{max} of the high-affinity system without significantly affecting its K_m. System x_C^- took up glutamate with very low apparent affinity in the presence of QA, but this uptake did interfere with the determination of the low-affinity and nonsaturable uptake by the Marquardt algorithm, artifactually increasing V_{max2} and k_D. Therefore, the low-affinity and nonsaturable parameters are not reported in Table 3.

In the absence of sodium ions, glutamate uptake by starved HeLa cells could be fit by a single saturable system, and the new low-affinity system was not detected (Table 3). The saturable uptake had a V_{max} close to V_{max1} of starved HeLa in HBS · MCD, and a K_m 50% higher than K_{m1} of that condition. The lack of sodium reduced k_D by two-thirds, indicating the presence of sodium-dependent nonsaturable uptake. The presence of 10 mM aspartate competitively inhibited sodium-free uptake after starvation: V_{max} did not change significantly, while K_m was raised by a factor of 4.5 ($K_i = 2.9 \pm 0.4$ mM). In addition, aspartate lowered k_D by an additional 15% under sodium-free conditions.

System x_C^- is an amino-acid exchanger [4], and therefore, depletion of internal glutamate pools would be expected to lower both V_{max} and K_m of this system, assuming that the transporter alternates between two conformational states [27]. Consistent with this expectation, the parameters of the apparent single saturable sodium-independent uptake system of starved cells are both lower than those of cells at zero time, V_{max} being 60% lower and K_m, 65% lower. The ratio V_{max}/K_m of sodium-independent glutamate import was very nearly preserved over starvation — its numerical value was 0.107 for nonstarved cells and 0.127 for starved cells — which comprised additional evidence that the systems at zero time and after preincubation are identical. For these reasons, the bulk of the sodium-free saturable uptake was considered to belong to the same apparent system as the high-affinity saturable uptake by starved cells in HBS · MCD. It seemed certain that system X_{AG}^- functioned for glutamate uptake in starved cells as well, since high-affinity aspartate uptake was detected after preincubation.

To determine whether 1 h preincubation in saline exerted the maximum regulatory effect, glutamate influx by HeLa was also measured in HBS · MCD after 3 h starva-

tion in EBSS dH5C5. The additional preincubation time did not change any of the parameters of uptake, compared to 1 h starvation in HBS · MCD.

3.5. Steady state measurements

Amino-acid pools were measured in units of nmol (mg protein)$^{-1}$. Before starvation, cells contained 34.3 ± 0.5 nmol/mg of aspartate and 36.7 ± 2.1 nmol/mg of glutamate plus glutamine, which were not resolved by the chromatographic system used. After starvation, aspartate was not detected and glutamate plus glutamine levels fell to 6.4 ± 0.4 nmol/mg.

The flux of TPP^+ was used to compare membrane potentials at zero time and after 1 h starvation in HBS · MCD. The ratio of TPP^+ flux in HBS · MCD to that in HBK · MCD plus ouabain is largely dependent on the transmembrane electrical potential [25,26]. This ratio for nonstarved cells, 1.31 ± 0.11, was not significantly different from that for starved cells, 1.27 ± 0.06, indicating that 1 h preincubation in HBS · MCD did not change the membrane potential. Internal sodium concentration fell from 11.4 ± 0.7 mM in nonstarved cells to 8.4 ± 1.1 mM after 1 h starvation.

4. Discussion

These studies, though not exhaustive, have uncovered additional interesting and unexpected features of glutamate and aspartate uptake by HeLa cells. A summary of the results is presented in Fig. 3 to facilitate discussion, and the kinetic parameters of the three saturable uptake systems are listed in Table 4.

4.1. Aspartate uptake

The presence of high-affinity aspartate uptake ($K_m \approx 20$ μM), which is sodium-dependent and glutamate-inhibitable (Table 1), justifies assigning a system X_{AG}^- transport activity to non-starved HeLa plasma membranes in Fig. 3 [2]. As in CHO, the activity was not completely abolished in sodium-free medium [16]. Cystine appears to be a weak competitive inhibitor of aspartate uptake by system X_{AG}^- in HeLa, though the K_i (ca. 400 μM) is approximately the solubility of this compound at pH 7.4, meaning that cystine cannot be an effective inhibitor under physiological conditions.

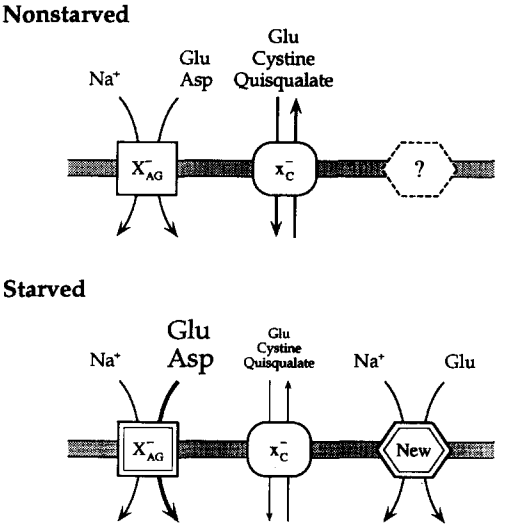

Fig. 3. Model of starvation-induced changes in saturable glutamate and aspartate uptake by HeLa cells. Three transporters on the plasma membrane of HeLa cells are shown. (A) At zero time ('Nonstarved'), systems X_{AG}^- and x_C^- are active, while the 'new' uptake system, if present, lies dormant. Quisqualate and cystine were found to be competitive inhibitors of glutamate uptake by system x_C^-, and are presumed here to be substrates of this system, although their transport via system x_C^- has not yet been tested directly in HeLa. (B) After starvation ('Starved'), system X_{AG}^- imports aspartate (and presumably, glutamate) with increased efficiency, while the reduction of trans-stimulation reduces glutamate transport through system x_C^-. The novel transporter is activated, and takes up glutamate in a sodium-dependent fashion. Double outlines on system X_{AG}^- and on the 'new' glutamate uptake system indicate regulation of these pathways, though not necessarily by the same mechanism.

Table 4
Summary of saturable acidic amino-acid uptake systems of HeLa cells[a]

Condition	Substrate	System X_{AG}^- [b]		System x_C^- [b]		New system [b,c]	
		V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	K_m (μM)	V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	K_m (μM)	V_{max} (nmol (mg prot.)$^{-1}$ min^{-1})	K_m (μM)
Nonstarved	Aspartate	0.169 ± 0.006	26.1 ± 1.5	– [d]	– [d]	– [e]	– [e]
	Glutamate	0.36 ± 0.05	82 ± 17	1.30 ± 0.04	121 ± 5	– [e]	– [e]
Starved	Aspartate	0.274 ± 0.006	13.5 ± 0.6	– [d]	– [d]	– [e]	– [e]
	Glutamate	0.40 ± 0.05	27 ± 3	0.54 ± 0.01	43 ± 2	2.0 ± 1.5	1000 ± 800

[a] Parameters are taken from experiments listed in Tables 1, 2 and 3, and represent uptake under conditions thought to isolate one uptake system, as described in the text.
[b] Parameters are expressed as mean ± s.d., where normally distributed, or as mean ± 68% C.I., where not normally distributed, as determined by Monte Carlo simulation.
[c] Parameters are expressed as the weighted averages ±68% C.I. from the two HBS · MCD experiments in Table 3.
[d] Initial experiments indicated a K_m greater than 5 mM for aspartate uptake by system x_C^-; more precise estimates have not been attempted.
[e] No activity detected.

The starvation-induced alteration of aspartate uptake (Fig. 1 and Table 4) is striking. For a simple Michaelis–Menten process, the ratio V_{max}/K_m (or k_{cat}/K_m), sometimes known as the catalytic efficiency or specificity constant, describes the rate at which free enzyme and substrate associate. This ratio increases threefold in starved HeLa cells without an effect on nonsaturable uptake. Although the actual transport mechanism is unknown, a significant alteration in the X_{AG}^- transporter or its environment should be required to produce this increase. It is clear from Fig. 1 that HeLa will transport low concentrations of aspartate much more effectively after saline incubation. Such a mechanism, if present in nervous system cells, could be significant for control of extracellular anionic amino-acid levels, assuming that it could be activated by signals other than general starvation [5].

We initially chose the short saline incubation times to seek so-called 'trans-effects': alterations of import velocity produced by changes in internal substrate levels. Trans-inhibition is the reduction in uptake caused by internal substrates [28]. In HeLa, levels of aspartate, a potential trans-inhibitor, dropped below detection after starvation. However, it would be expected, in the absence of other changes, that an increase in V_{max} produced by reduced trans-inhibition would be accompanied by an increase in K_m [27], yielding a constant V_{max}/K_m — not the effect seen here. Other up-regulations, known as 'adaptive regulations,' observed for amino-acid transport systems A [29] and x_C^- [30] typically require several hours to detect and are thought to result primarily from increased transporter synthesis. More transporters should simply result in a higher V_{max} without affecting K_m. We did not observe additional changes in aspartate transport parameters in cells starved for three hours, suggesting that adaptive changes are not involved here. Both the rapidity and the nature of the transport parameter alterations observed here argue for a mechanism different than one simply involving trans-effects or synthetic rates.

Glutamate transport is electrogenic in some tissues [10], and therefore starvation could affect glutamate transport through system X_{AG}^- by altering the membrane potential and/or the sodium electrochemical potential. Based on the lack of change in the relative rates of TPP^+ influx in starved versus non-starved cells, we did not find evidence for altered membrane potentials in starved HeLa. The internal sodium concentration in starved cells did fall 28%, likely due both to the lower extracellular sodium levels in the saline used to starve the cells and to the reduction in sodium entry by solute co-transport systems. This would, however, result in only a small increase in the sodium electrochemical potential, $\Delta\mu_{Na^+} = RT\ln([Na^+]_o/[Na^+]_i) + F\Delta\Psi$, in starved cells. If the contribution of the membrane potential is ignored, $\Delta\mu_{Na^+}$ would be 9.0% greater in starved cells. If the actual membrane potential was −60 mV, $\Delta\mu_{Na^+}$ would be only 4.8% greater in starved cells. It is clear that $\Delta\mu_{Na^+}$ can strongly influence steady state amino-acid distributions [24,31], but the relationship between $\Delta\mu_{Na^+}$ and transport rate constants is not known. Thus, while it is possible that a combination of indirect effects of starvation — a small increase in $\Delta\mu_{Na^+}$ and decreased *trans*-inhibition — could produce the threefold increase in V_{max}/K_m observed for aspartate uptake, we believe that other explanations, such as a direct modification of the transporter, are worth exploring.

4.2. Glutamate uptake

Although fitting the data did not require two saturable systems, the presence of sodium-independent, cystine-inhibitable glutamate influx supports the assignment of a system x_C^- activity, in addition to system X_{AG}^-, to non-starved HeLa membranes in Fig. 3 [4]. In fact, system x_C^- dominated saturable glutamate uptake by HeLa without starvation and obscured the activity of system X_{AG}^- on glutamate, which could still be measured, however, by inhibition experiments. Compared to glutamate uptake by system x_C^- of human fibroblasts [4], the K_m in HeLa was 2.5-fold lower and the K_i for cystine was twofold higher. Quisqualate proved to be an effective competitive inhibitor of system x_C^- in HeLa, as was observed for glial cells [9,15]. Thus, the K_m of system X_{AG}^- for glutamate operating in normal saline, 82 ± 17 μM, was determined using 500 μM quisqualate to inhibit system x_C^-, assuming no inhibition of system X_{AG}^- by quisqualate. The K_m values for glutamate uptake by system X_{AG}^- and system x_C^- differed by only 33%, and we have found that the Marquardt algorithm generally cannot separate two Michaelis–Menten processes with K_m values that differ less than 10-fold.

In contrast, an acceptable model for glutamate uptake by starved HeLa cells in HBS · MCD clearly required two saturable systems, although by inhibition studies we discovered at least three systems. High-affinity saturable uptake was still dominated by system x_C^-, now operating at lower velocity, presumably due to reduced trans-stimulation. The activity of system X_{AG}^- on glutamate after starvation was again seen indirectly. The inhibition of high-affinity aspartate influx by glutamate ($K_i = 34 \pm 2$ μM) indicated that glutamate transport by system X_{AG}^- persisted after starvation. This was confirmed by the presence of sodium-dependent, aspartate-inhibitable glutamate uptake measured in the presence of quisqualate to eliminate system x_C^-. The V_{max} for glutamate uptake by system X_{AG}^- did not change significantly after 1 h starvation, but the K_m was lowered by 67%. Thus, the catalytic efficiency of glutamate uptake was increased 3-fold with starvation, the same magnitude observed for aspartate uptake, although both V_{max} and K_m of aspartate uptake changed with preincubation. Starvation had no measurable effect on glutamate transport in CHO cells [16].

The sodium-dependent, aspartate-insensitive, low-affinity uptake system which rapidly responds to starvation

corresponds to no known glutamate transporter and we simply labeled it 'New' in Fig. 3. The K_m of this new system, ca. 1 mM, seems unusually high, especially if it is designed to operate under conditions of amino-acid starvation. It is possible that glutamate is not a principal substrate of this system. If such is the case, it might be expected that aspartate and glutamate, being chemically similar, would both be recognized. The new system, however, completely ignored aspartate. Future experiments will seek inhibition by other compounds. Besides better illuminating the identity and role of this new system, an effective inhibitor would allow a more precise determination of its kinetic parameters.

Initial experiments had suggested that MeAIB competitively inhibited low-affinity glutamate influx, raising the possibility that the low-affinity system was system A for neutral amino acids [1]. In that study, the low estimates of uninhibited K_{m2} likely resulted from the inherently large parameter uncertainties, which led us to propose that MeAIB might be an inhibitor. However, even the 6-fold difference in K_{m2} that we observed previously was not statistically significant when both low-affinity and high-affinity saturable uptake were fit simultaneously. In the present study, K_m in the presence of MeAIB was nearly identical to that without MeAIB, and we must conclude that system A does not appear to be involved with saturable glutamate transport in HeLa.

Approx. 60% of nonsaturable glutamate uptake before or after starvation was sodium-dependent. Several amino-acid transport systems could be involved in apparent nonsaturable uptake. System ASC (sodium-dependent) for small neutral amino acids has been shown to take up glutamate with low affinity in human fibroblasts before and after starvation [2] and systems A (sodium-dependent) and L (sodium-independent) are responsible for nonsaturable glutamate import into Ehrlich ascites cells [32]. The insensitivity of nonsaturable import to MeAIB in HeLa rules out system A's involvement. We have not yet tested the roles of systems ASC and L. There were several instances (Tables 1, 2, 4) where uptake measured in the presence of test inhibitors, MeAIB or aspartate, was associated with an increased V_{max} value. Although this was never a dramatic increase, nor always statistically significant, it has been routinely observed and is puzzling.

An efficient, reliable whole-cell transport assay [18,19] and careful attention to nonlinear regression analysis were required to obtain and interpret these results. In spite of our best efforts, however, we encountered difficulty analyzing what might appear to be a simple situation: two saturable systems operating in parallel. It is clear that the process of fitting velocity data — and not the data itself — can be responsible for variability and imprecision of calculated parameters. This can be appreciated if one calculates 'perfect' velocity data directly from an equation containing two saturable systems, using parameter values taken from Table 3 and assigning a 5% relative standard deviation to each velocity point, a typical error for our actual measurements. Fitting these data back to the equation that generated them yielded 95% confidence limits wider than $\pm 100\%$ for V_{max2} and K_{m2}, even though q was 1.0, indicating a perfect fit. A frustrating consequence of these large uncertainties is that some parameter estimates may have little utility: the fitting algorithm tells one that a second system exists, but cannot describe it with precision. In spite of these difficulties, a new system and two new regulations of acidic amino-acid import have been described in HeLa cells. The reasons for the intricate modulation of import of these nonessential substrates deserve further study.

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References

[1] Ash, J.F. and Igo, R.P., Jr. (1993) Biochim. Biophys. Acta 1149, 109–118.
[2] Dall'Asta, V., Gazzola, G.C., Franchi-Gazzola, R., Bussolati, O., Longo, N. and Guidotti, G.G. (1983) J. Biol. Chem. 258, 6371–6379.
[3] Makowske, M. and Christensen, H.N. (1982) J. Biol. Chem. 257, 5663–5670.
[4] Bannai, S. and Kitamura, E. (1980) J. Biol. Chem. 255, 2372–2376.
[5] Casado, M., Zafra, F., Aragón, C. and Giménez, C. (1991) J. Neurochem. 57, 1185–1190.
[6] Clark, J.A. and Amara, S.G. (1993) BioEssays 15, 323–332.
[7] Erecinska, M. and Silver, I.A. (1990) Prog. Neurobiol. 35, 245–296.
[8] Fletcher, E.J. and Johnston, G.A.R. (1991) J. Neurochem. 57, 911–914.
[9] Flott, B. and Seifert, W. (1991) Glia 4, 293–304.
[10] Lerner, J. (1987) Comp. Biochem. Physiol. 87B, 443–457.
[11] Nicholls, D. and Atwell, D. (1990) Trends Pharmacol. Sci. 11, 462–468.
[12] Storck, T., Schulte, S., Hofmann, K. and Stoffel, W. (1992) Proc. Natl. Acad. Sci. USA 89, 10955–10959.
[13] Pines, G., Danbolt, N.C., Bjørås, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E. and Kanner, B.I. (1992) Nature 360, 464–467.
[14] Kanai, Y. and Hediger, M.A. (1992) Nature 360, 467–471.
[15] Cho, Y. and Bannai, S. (1990) J. Neurochem. 57, 911–914.
[16] Ash, J.F. and Igo, R.P., Jr. (1993) Som. Cell. Mol. Gen. 19, 231–243.
[17] Puck, T.T., Marcus, P.I. and Ciecuira, S.J. (1956) J. Exp. Med. 103, 273–284.
[18] Gazzola, G.C., Dall'Asta, V., Franchi-Gazzola, R. and White, M.F. (1981) Anal. Biochem. 115, 368–374.
[19] DeBusk, W.E. and Ash, J.F. (1993) Som. Cell. Mol. Gen. 19, 331–345.
[20] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[21] Collarini, E.J. and Oxender, D.L. (1987) Annu. Rev. Nutr. 7, 75–90.

[22] Marquardt, D.W. (1963) J. Soc. Ind. Appl. Math. 11, 431–441.
[23] Press, W.H., Flannery, B.P., Teukolsky, S.A. and Vetterling, W.T. (1988) Numerical Recipes in C: The Art of Scientific Computing, p. 177, Cambridge University Press, New York.
[24] Pauw, P.G., Sheck, R.N. and Ash, J.F. (1989) Mol. Cell. Biol. 9, 116–123.
[25] Kimmich, G.A., Randles, J., Restrepo, D.R. and Montrose, M. (1985) Ann. N.Y. Acad. Sci. 456, 63–76.
[26] Pauw, P.G. and Ash, J.F. (1987) J. Cell. Physiol. 130, 199–206.
[27] Stein, W.D. (1986) Transport and Diffusion across Cell Membranes, pp. 231–361, Academic Press, San Diego, CA.
[28] Gazzola, G.C., Dall'Asta, V. and Guidotti, G.G. (1980) J. Biol. Chem. 255, 929–936.
[29] Shotwell, M.A., Kilberg, M.S. and Oxender, D.L. (1983) Biochim. Biophys. Acta 737, 267–284.
[30] Bannai, S. and Kitamura, E. (1982) Biochim. Biophys. Acta 721, 1–10.
[31] Eddy, A.A. (1985) Ann. N.Y. Acad. Sci. 456, 51–61.
[32] Garcia-Sancho, J., Sanchez, A. and Christensen, H.N. (1977) Biochim. Biophys. Acta 464, 295–312.