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Pathways of L-Glutamic Acid Transport in Cultured Human Fibroblasts*

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The transport of L-glutamic acid has been studied in skin-derived diploid human fibroblasts. Competition analysis in the presence and absence of Na^+ and mathematical discrimination by nonlinear regression indicated that L-glutamic acid enters the cell by at least three transport systems: 1) a high affinity Na^+-dependent system which has been found to be identical to the previously described system for anionic amino acids (Gazzola, G. C., Dall'Asta, V., Bussolati, O., Makowske, M., and Christensen, H. N. (1981) *J. Biol. Chem.* 256, 6054–6059) and which is provisionally designated as System X_{AG}^-; this route was shared by L-aspartic acid; 2) a low affinity Na^+-dependent system resembling the ASC System for neutral amino acids (Franchi-Gazzola, R., Gazzola, G. C., Dall'Asta, V., and Guidotti, G. G. (1982) *J. Biol. Chem.* 257, 9582–9587); its reactivity toward L-glutamic acid was strongly inhibited by L-serine, but not by 2-(methylamino)isobutyric acid; and 3) a Na^+-independent system similar to System x_C^- described in fetal human lung fibroblasts (Bannai, S., and Kitamura, E. (1980) *J. Biol. Chem.* 255, 2372–2376). The x_C^- system served for L-glutamic acid and L-cystine, the latter amino acid behaving as a potent inhibitor of L-glutamic acid uptake. Amino acid starvation did not change the uptake of L-glutamic acid by the two Na^+-dependent systems, but enhanced the activity of System x_C^- by increasing its V_{max}. L-Glutamic acid transport was also affected by the density of the culture. An increased cell density lowered the uptake of the amino acid by Systems ASC and x_C^- and promoted the uptake by System X_{AG}^-. All these variations were dependent upon changes in V_{max}.

A number of transport systems for amino acids has been identified or described in human fibroblasts. Among them at least three systems, namely System A, ASC, and L, serve for neutral amino acids (1), one for cationic amino acids (System y^+) (2), and two for anionic amino acids, one of them Na^+-dependent (3), the other Na^+-independent (4, 5) (System x_C^-) (6). L-Glutamic acid occupies a unique position in intermediary metabolism. It is known to be involved in energy production, movement of reducing equivalents between mitochondria and cytosol, urea synthesis, glutathione synthesis, and neurotransmission beside being a constituent of proteins. Therefore, it is not surprising that cells contain considerable quantities of free L-glutamate (7). Cultured human fibroblasts have become one of the most common materials for the study of human metabolism and genetics. This model has been selected for a detailed study of the transport pathways designed to ensure adequate intracellular levels of L-glutamic acid.

The results reported here provide evidence that the *inward* transport of L-glutamic acid in cultured human fibroblasts occurs by at least four distinct routes, mediated by a Na^+-dependent anionic system, System x_C^-, System ASC, and perhaps, System L. Their relative contribution has been evaluated and some indication of their physiologic role is offered.

EXPERIMENTAL PROCEDURES

Cell Culture—Human fibroblasts were derived from normal adult skin biopsies as described previously (1). Cells were routinely grown in 10-cm diameter dishes (Costar) in Medium 199 containing 10% fetal calf serum. The conditions of culturing were: pH 7.4; atmosphere, 5% CO_2 in air; temperature, 37 °C. All measurements of amino acid transport were made on fibroblast subcultures resulting from 4×10^4 cells seeded on to 2 cm^2 wells of disposable 24 well trays (Costar) and incubated for 2–7 days in 1 ml of growth medium. The culture medium was always renewed 24 h before the experiment. Human fibroblasts were used between the 10th and the 20th passage; in this interval, they retained a normal complement of chromosomes, as assessed by a Trypsin-Leishman banding technique (8).

Uptake Assay—Unless otherwise specified, amino acid uptake was measured under conditions approaching initial entry rates (1 min), as follows: after a 90-min incubation in Earle's balanced salt solution (containing 10% dialyzed fetal calf serum), as required to deplete intracellular amino acid pools (*cf.* Ref. 1), human fibroblast monolayers were washed and incubated for 1 min at 37 °C in the presence of the labeled amino acid and any other designated compound. During this period, the incubation medium was essentially unchanged, except that serum was omitted and sufficient Tris(hydroxymethyl)-aminomethane was present to neutralize the acidity of the amino acid substrate or inhibitor. In some experiments, we used a Na^+-free medium in which choline replaced Na^+ in the sodium salts of the Earle's mixture. Assays were terminated by rapidly rinsing the cell monolayers with ice-cold 0.9% NaCl. Acid-soluble pools were extracted with 10% trichloroacetic acid and counted in a liquid scintillation spectrometer. The cells were dissolved in 1 N NaOH and assayed for protein directly in the wells using a modified Lowry procedure (9), as described previously (10). The protein content of each well was used as an indirect estimate of cell density (expressed as μg of protein/cm^2). One mg of cell protein corresponded roughly to 3×10^6 cells.

Details of the transport assay and of the equipment used have been published (10).

Calculations—The intracellular fluid volume was estimated from the difference between total water ([^{14}C]urea space) and extracellular fluid volume ([^{3}H]inulin space) as described previously (1). Amino acid uptake, expressed as nanomoles or micromoles (as indicated) of amino acid · ml^{-1} of intracellular water · min^{-1} was calculated from the primary data by a computer (Hewlett-Packard 9845 S, equipped with

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CRT graphic option and X-Y peripheral plotter HP-9872C). The analysis of initial velocity kinetic curves was performed using a BASIC program applying Marquardt's algorithm, a general procedure for least squares estimation of nonlinear parameters (11). The equations used were

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \cdot \frac{1 - e^{-K_D}}{K_D} + [S] \cdot (1 - e^{-K_D}) \quad (1)$$

for a saturable system plus diffusion (12),

$$v = \frac{V_{max_1} \cdot [S]}{K_{m_1} + [S]} + \frac{V_{max_2} \cdot [S]}{K_{m_2} + [S]} \quad (2)$$

for two contributing saturable systems,

$$v = v_0 - \frac{I_{max} \cdot [I]}{I_{0.5} + [I]} \quad (3)$$

for a competitively inhibited system (where v_0 is the uptake in the absence of inhibitor, I_{max} is maximal inhibition and $I_{0.5}$ is the inhibitor concentration required for half-maximal inhibition),

$$v = \frac{V_{max_1} \cdot [S]}{K_{m_1} + [S]} + \frac{V_{max_2} \cdot [S]}{K_{m_2} \cdot (1 + [I]/K_i) + [S]} \quad (4)$$

for two contributing systems one of which was competitively inhibited, and

$$v = \frac{V_{max_1} \cdot [S]}{K_{m_1} \cdot (1 + [I]/K_{i_1}) + [S]} + \frac{V_{max_2} \cdot [S]}{K_{m_2} \cdot (1 + [I]/K_{i_2}) + [S]} \quad (5)$$

for two contributing, competitively inhibited systems. In Equations 4 and 5, K_{m_1} and K_{m_2} values were those of the relevant noninhibited transport system, as determined by Equation 2.

Materials—All sera, growth media, antibiotics, and trypsin solution were from Gibco. L-[1-^{14}C]Glutamic acid (49.6 mCi/mmol), and [*methoxy*-^{3}H]inulin (102.5 mCi/g) were purchased from New England Nuclear; [^{14}C]urea (59 mCi/mmol) was obtained from Amersham. 2-(Methylamino)isobutyric acid was from Aldrich. BCH[1] (isomeric form *b*(±)) and GPA were generous gifts from Professor Halvor N. Christensen, University of Michigan, Ann Arbor. Sigma was the source of all unlabeled amino acids and other chemicals.

RESULTS

Na^+-dependence of L-Glutamic Acid Uptake by Human Fibroblasts—Fig. 1 shows that L-glutamic acid enters the human fibroblast primarily by a Na^+-dependent route; however, a Na^+-independent component contributes substantially to the total uptake of this amino acid. Eadie-Hofstee plots for Na^+-dependent and for Na^+-independent uptake are shown in Fig. 2. In either case, the uptake rates deviated from the straight line expected for a single saturable transport system. The velocity data for the Na^+-dependent component were best fitted by an equation (Equation 2 see "Experimental Procedures") compatible with the operation of two saturable systems formally distinguishable by independent K_m and V_{max} (see Table I). The Na^+-independent component could be accounted for by the operation of a single saturable transport system associated with an apparently nonsaturable process (best fitting by Equation 1, see "Experimental Procedures") formally indistinguishable from diffusion ($K_D = 0.029$ min^{-1}).

The kinetic parameters reported in Table I allowed us to estimate the relative contribution of the three discriminated (two Na^+-dependent, one Na^+-independent) transport systems to the total saturable uptake of L-glutamic acid by human fibroblasts over a range of amino acid concentrations extended to include the "physiological" values present in blood plasma of newborn and adult humans (13). The relevant theoretical curves are presented in Fig. 3. One can see that

[1] The abbreviations used are: BCH, 2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid, isomeric form *b* (±); GPA, 4-amino-1-guanylpiperidine-4-carboxylic acid; MeAIB, 2-(methylamino)isobutyric acid.

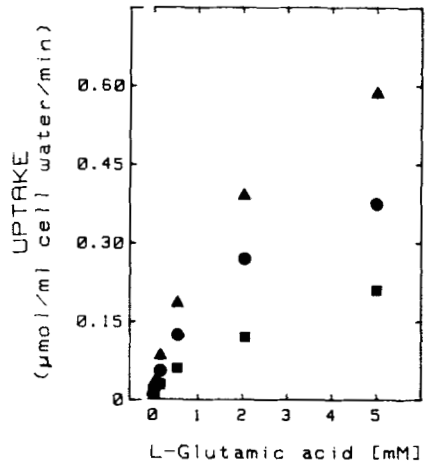


FIG. 1. **Na^+-dependence of L-glutamic acid uptake by cultured human skin fibroblasts.** Subconfluent cultures of fibroblasts were depleted of intracellular amino acids (90-min incubation at 37 °C in Earle's balanced salt solution containing 10% dialyzed fetal calf serum). Cell monolayers were washed and the transport assay was made during 1 min at 37 °C over the indicated range of values of L-[1-^{14}C]glutamic acid concentration, either in a Na^+-containing medium in the form of Earle's balanced salt solution (total uptake, ▲), or in a Na^+-free medium in which choline replaced the cation (Na^+-independent uptake, ■). The *points* are means of triplicate determinations in each of 4 experiments. Na^+-dependent uptake (●) was calculated by difference.

the contribution of the Na^+-independent mediation (*curve C*) increased slightly in the concentration range 0.01 to 0.1 mM and then remained relatively constant up to 0.6 mM; within this interval, the activity of the system accounted for 20–30% of the total saturable L-glutamic acid uptake. As expected from the relevant kinetic parameters, the contribution of the Na^+-dependent mediations as a function of increasing substrate concentrations varied markedly and oppositely. The high affinity system (*curve A*) accounted for the largest fraction of saturable L-glutamic acid uptake at very low external amino acid concentrations; its contribution decreased sharply up to 0.15 mM and more slowly thereafter reaching about 10% of the total transport at 0.5 mM substrate concentration. The relative contribution of the low affinity (high capacity) system (*curve B*) increased rapidly at low substrate concentrations (up to 0.15 mM) and then more steadily reaching about 60% of the total transport activity at 0.5 mM concentration of external L-glutamic acid.

Identification of the Na^+-independent Transport System—Inhibition analysis of the Na^+-independent transport system was performed in a Na^+-free medium (see "Experimental Procedures") by the use of amino acids known to be preferential substrates of specific transport systems in human fibroblasts. They included the analogs BCH and GPA as inhibitors of System L and of System y^+, respectively (1, 2), and the naturally occurring L-cystine, as inhibitor of System x_c^- (4). The latter system has been recently characterized in fetal human lung fibroblasts (4); it shows reactivity toward L-glutamate and L-cystine in its anionic form ($NH_2(NH_3^+)R(COO^-)_2$). As shown in Fig. 4, L-cystine strongly inhibited the Na^+-independent uptake of L-glutamic acid in our biologic preparation. The limited solubility of L-cystine prevented us from extending the inhibition analysis beyond 0.5 mM concentration of the inhibitor amino acid. Nevertheless, the available inhibition data were best fitted by an equation for competitive inhibition (Equation 3 under "Experimental Procedures"); the extrapolation of the resulting hyperbola to infinite inhibitor concentration led to an almost

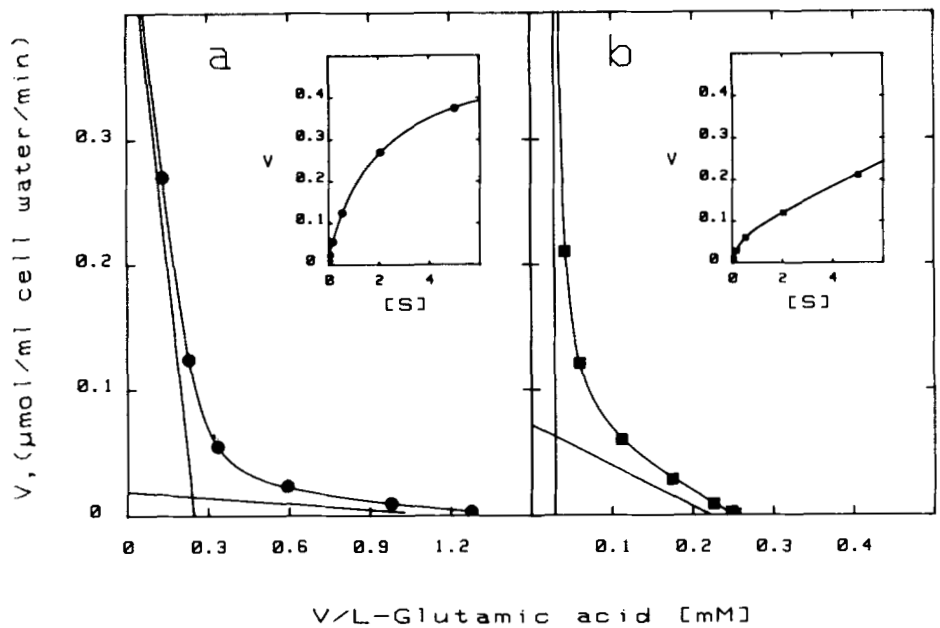


FIG. 2. **Discrimination of Na^+-dependent (*a*) and of Na^+-independent (*b*) transport systems for L-glutamic acid.** Human fibroblast subconfluent cultures were depleted of intracellular amino acids (see Fig. 1). Transport was measured for 1 min over a range of 0.004–5 mM labeled L-glutamic acid, either in a Na^+-containing or in a Na^+-free medium. Initial rates of entry of L-glutamic acid as a function of its external concentrations, shown in the *insets*, are plotted according to an Eadie-Hofstee graphical representation. In *a*, the Na^+-dependent fraction of L-glutamic acid uptake was calculated by subtracting Na^+-independent from total uptake (as measured in the presence of Na^+). The experimental data were best fitted by computer on the basis of the sum of two independent rectangular hyperbolae (Equation 2 under "Experimental Procedures"), whose parameters defined the two components presented as *straight lines*. In *b*, the Na^+-independent fraction of L-glutamic acid uptake was resolved into two components by computer on the basis of the sum of a single rectangular hyperbola and of a linear (nonsaturable) component (Equation 1 under "Experimental Procedures").

TABLE I

Kinetic parameters for the saturable uptake of L-glutamic acid by human fibroblasts

Conditions of cell culture, transport assay, and calculations, as described in Fig. 2. Parameters are shown as means ± S.D.

System	K_m	V_{max}
	mM	*µmol/min/ml cell water*
Na^+-dependent (high affinity)	0.016 ± 0.004	0.019 ± 0.002
Na^+-dependent (low affinity)	2.04 ± 0.09	0.50 ± 0.01
Na^+-independent	0.32 ± 0.02	0.07 ± 0.01

complete inhibition (93%) of the uptake of L-glutamic acid. GPA failed to inhibit the Na^+-independent inward transport of L-glutamic acid (Fig. 4). Under the same Na^+-free conditions, BCH inhibited the uptake of L-glutamic acid in a competitive manner, but the inhibition was largely incomplete, accounting only for less than 10% of total Na^+-independent uptake.

Identification of the Na^+-dependent Transport Systems—The occurrence of two systems for the Na^+-dependent uptake of L-glutamic acid by human fibroblasts (Fig. 2) and the dissimilarity of the kinetic parameters defining them (Table I) required that the inhibition analysis be carried out at proper substrate concentrations, close to the concentrations for half-maximal transport in each system (*cf.* Ref. 14). Fig. 5, where the per cent uptake of L-glutamic acid by each Na^+-dependent system has been calculated as a function of the external substrate concentration on the basis of the relevant kinetic parameters, elucidates this point. The pattern of the two symmetrical curves, calculated for the high affinity system (*curve A*, concave upwards) and for the low affinity system (*curve B*, concave downward), predicts that a given inhibitor (specific for one of the two systems) may or may not reveal

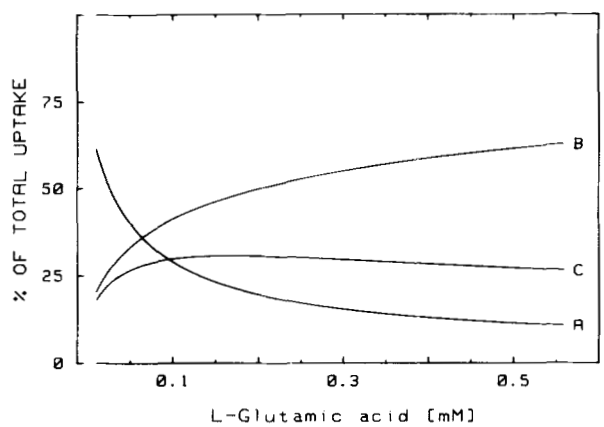


FIG. 3. **Uptake of L-glutamic acid by human fibroblasts as a function of increasing substrate concentrations. Expected contribution of individual saturable systems.** The curves have been calculated on the basis of the kinetic parameters presented in Table I. They express the per cent uptake of L-glutamic acid attributable to a high affinity Na^+-dependent system (*curve A*), to a low affinity Na^+-dependent system (*curve B*), and to a Na^+-independent system (*curve C*).

through its inhibitory effect the occurrence and significance of the pertinent system, depending on the relative contribution of the system to the total uptake of L-glutamic acid at the concentration selected for the assay.

The inhibition analysis of the Na^+-dependent transport systems took advantage of the analog MeAIB, known to be the best characterizing substrate of System A (1), and of such natural amino acids as L-serine and L-aspartic acid, known to be preferential substrates of System ASC (15) and of a recently described system for anionic amino acids in human

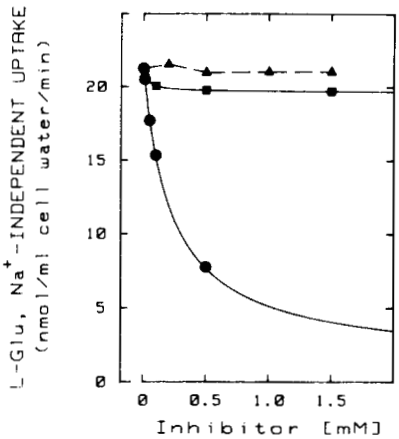


FIG. 4. **Inhibition profiles of L-glutamic acid Na^+-independent uptake by L-cystine, BCH, and GPA.** Conditions of cell culture as described in Fig. 1. Transport assay at 0.1 mM L-glutamic acid concentration was performed in a Na^+-free medium (see Fig. 1) in the absence and in the presence of increasing concentrations of L-cystine (0.01 to 0.5 mM, ●), BCH (0.01 to 1.5 mM, ■), GPA (0.2 to 1.5 mM, ▲). *Continuous lines* are computer-derived best fits of the experimental data for rectangular hyperbolae (Equation 3 under "Experimental Procedures"); the *dotted line* connects the experimental points (a fit to a rectangular hyperbola was not justified here). The data points are from a typical experiment; the experiment was repeated twice with similar results.

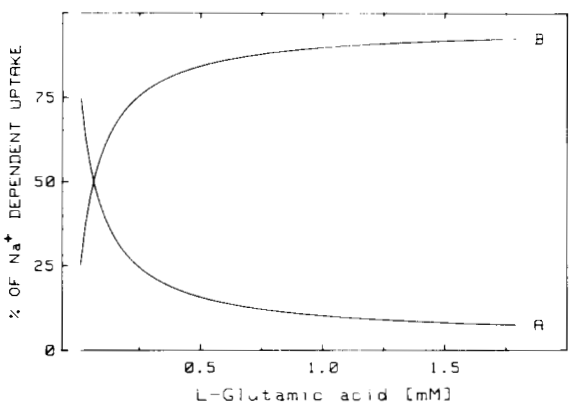
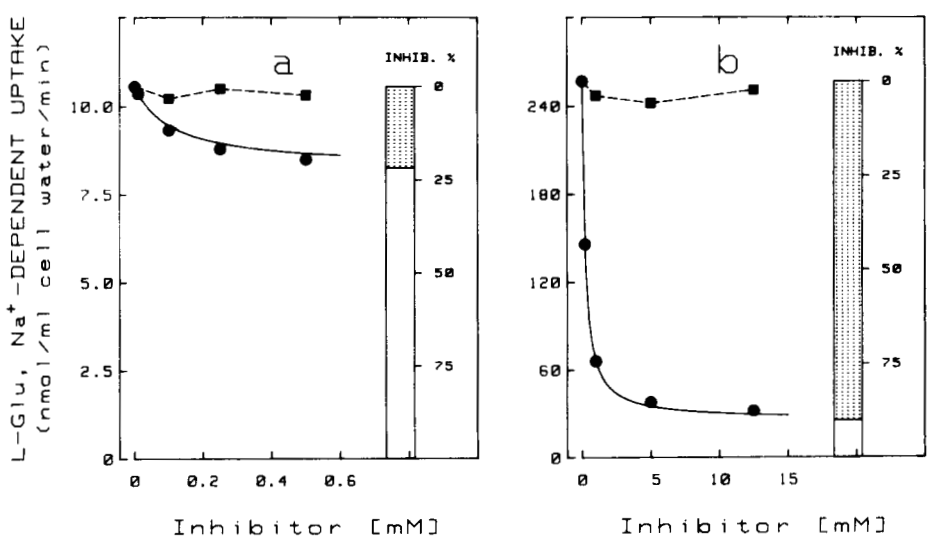


FIG. 5. **Expected per cent contribution of saturable systems to the total Na^+-dependent uptake of L-glutamic acid by human fibroblasts, as a function of substrate concentration.** The curves have been calculated on the basis of the kinetic parameters presented in Table I for Na^+-dependent systems. *Curve A*, high affinity system; *curve B*, low affinity system.

fibroblasts (3), respectively. Data reported in Fig. 6 show that MeAIB is unable to depress the transport of L-glutamic acid at both of the substrate concentrations adopted for the inhibition analysis. Conversely, L-serine inhibited the uptake of L-glutamic acid. The degree of maximal inhibition, as calculated by fitting the data to Equation 3 (see "Experimental Procedures"), was much higher at 2 mM than at 0.01 mM substrate concentration (not shown), indicating that L-serine is a putative competitive inhibitor of the low affinity system for the Na^+-dependent transport of L-glutamic acid. The experimental data obtained at either substrate concentration were then jointly fitted to Equation 4 (see "Experimental Procedures") for the estimation of the kinetic parameters of two systems (one of which competitively inhibited), contributing simultaneously to the uptake of the amino acid. The quality of this fit is illustrated in Fig. 6. The K_i value resulting from this inhibition analysis (0.1 mM) was remarkably similar to the K_m values for the inward transport of L-serine through the ASC System in human fibroblasts (15).

Fig. 7 shows that L-aspartic acid inhibits the Na^+-dependent entry of L-glutamic acid in human fibroblasts. The experimental data indicated that the degree of inhibition was rather high at either substrate concentration but the fitting of the data by an equation for a single competitive inhibition (Equation 3 under "Experimental Procedures") was relatively inaccurate (not shown). The values of K_i calculated from the $I_{0.5}$ values obtained by these fitting procedures at low and at high substrate concentration were quite dissimilar; moreover, the predicted maximal inhibition pointed to a complete suppression of the Na^+-dependent uptake of L-glutamic acid. Differences in derived K_i values and complete inhibition of substrate transport by the inhibitor are results incompatible with our earlier assumption that L-aspartic acid inhibits only one (the "anionic" System, Ref. 3) of the two kinetically heterogeneous Na^+-dependent systems for L-glutamic acid uptake (*cf.* Fig. 2 and Table I). These difficulties and the relative inaccuracy of the fitting of the data by a single rectangular hyperbola suggested that L-aspartic acid entered the human fibroblast by two Na^+-dependent systems shared with L-glutamic acid, thus inhibiting both routes of entry of the latter amino acid. The experimental data reported in Fig. 7, therefore, were jointly fitted by an equation accounting for two independent competitive inhibitions (Equation 5 under "Experimental Procedures"). Following this treatment, the accuracy of the fitting was greatly enhanced and the inhibition analysis allowed discrimination of the two independent inhibitory effects of L-aspartic acid on the Na^+-dependent trans-

FIG. 6. **Inhibition profiles of L-glutamic acid Na^+-dependent uptake by MeAIB and L-serine.** Conditions of cell culture and transport assay, as described in Fig. 1. The concentration of labeled L-glutamic acid was 0.01 mM in *a* and 2 mM in *b*. Inhibitors (MeAIB, ■ and L-serine, ●) were present at the indicated concentrations. *Continuous lines* (inhibition by L-serine) are the computer-derived best fit of the combined experimental data to Equation 4; *dotted lines* (presence of MeAIB) connect the experimental points. At right, *bars* represent the Na^+-dependent uptake of L-glutamic acid; the *shaded* portion refers to maximal inhibition by L-serine. In *a* and *b*, the data points are from a typical experiment; the experiment was repeated twice with similar results.



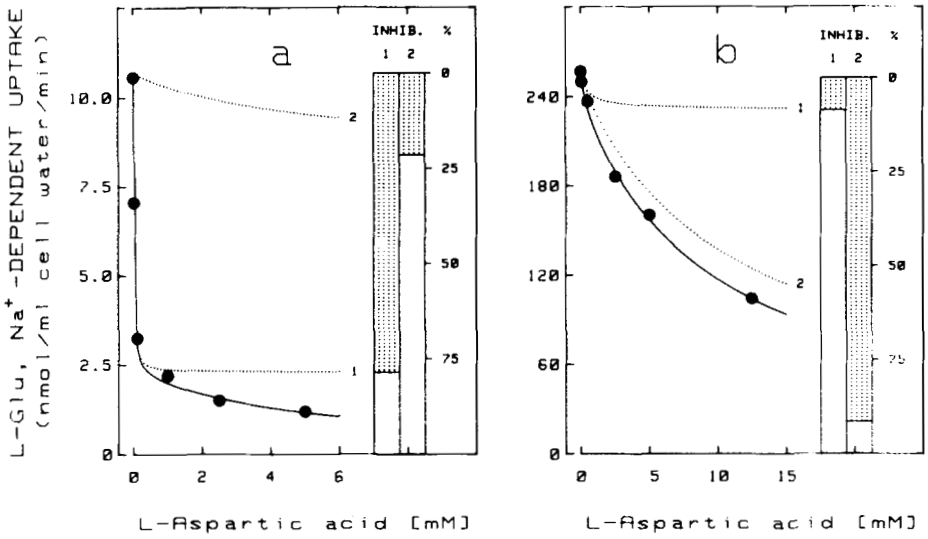


FIG. 7. **Inhibition profiles of L-glutamic acid Na^+-dependent uptake by L-aspartic acid.** Conditions of cell culture and transport assay, as described in Fig. 6. L-Aspartic acid was present at the indicated concentrations. *Continuous lines* are the computer-derived best fit of the combined experimental data to Equation 5 on the basis of the sum of two independent rectangular hyperbolae shown in each panel as *dotted lines*. At right, in both panels, *bars* represent total Na^+-dependent uptake of L-glutamic acid. *Shaded* portions define the extrapolated maximal inhibition (asymptotic value) for each rectangular hyperbola, discriminated by the fitting procedure. The data points are from a representative experiment; the experiment was repeated twice with similar results.

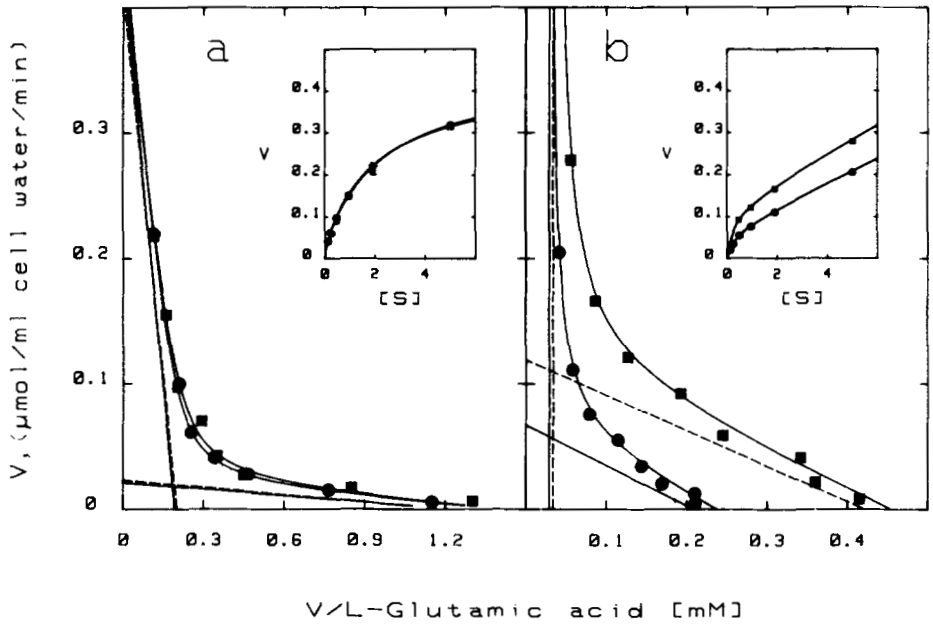


FIG. 8. **Uptake of L-glutamic acid by human fibroblasts: effect of amino acid starvation.** Subconfluent cultures of human fibroblasts were incubated for 12 h in Earle's balanced salt solution containing 10% dialyzed fetal calf serum. Transport activity was measured after 1.5 h (●, unstarved cells) and 12 h (■, starved cells). Uptake assay, calculations, and graphic representations, as described in Fig. 2. The data were best fitted by computer on the basis of the sum of two independent rectangular hyperbolae (Equation 2 under "Experimental Procedures") for Na^+-dependent uptake (*a*) or of the sum of a single rectangular hyperbola and a linear nonsaturable component (Equation 1 under "Experimental Procedures") for Na^+-independent uptake (*b*). *Continuous straight lines*, transport in unstarved cultures; *dotted lines*, starved cultures.

TABLE II

Kinetic parameters for the uptake of L-glutamic acid by "unstarved" and "starved" human fibroblasts

Conditions of cell culture, transport assay, and calculations, as described in Fig. 8. Parameters are shown as means ± S.D.

System	Unstarved			Starved		
	K_m	V_{max}	K_D	K_m	V_{max}	K_D
	mM	*µmol/min/ml cell water*	min^{-1}	*mM*	*µmol/min/ml cell water*	min^{-1}
Na^+-dependent (high affinity)	0.017 ± 0.003	0.021 ± 0.002		0.019 ± 0.01	0.023 ± 0.008	
Na^+-dependent (low affinity)	2.25 ± 0.15	0.43 ± 0.015		2.05 ± 0.42	0.41 ± 0.05	
Na^+-independent	0.32 ± 0.006	0.067 ± 0.008		0.28 ± 0.05	0.12 ± 0.01	
Non-saturable			0.029 ± 0.003			0.034 ± 0.003

port of L-glutamic acid. One inhibition (component 1 in Fig. 7) primarily affected the high affinity system, whereas the other one (component 2 in Fig. 7) was largely directed against the low affinity system. The resulting K_i values (K_{i_1} for the high affinity system = 0.01 mM; K_{i_2} for the low affinity system = 5.1 mM) agreed rather well with the values of K_m for L-aspartic acid uptake, as calculated from a set of data (not shown) obtained in the course of the present study over a range of substrate concentrations more extended toward high values than in previous experiments (3). The newly determined values are: K_{m_1} = 0.01 mM and K_{m_2} = 5.6 mM.

The results presented in Figs. 6 and 7 agree with the prediction of the theoretical analysis provided in Fig. 5 and justify the kinetic approach at two different substrate concentrations.

Effect of Amino Acid Starvation on the Transport of L-Glutamic Acid—Prolonged incubation of several animal tissues and cells in an amino acid-free medium has been reported to enhance the rate of uptake of some amino acids and to decrease the uptake of others (16). In the human fibroblast,

the latter change has been ascribed to a release from *trans*-stimulation (1), while the former appears to result from a combined process of release from *trans*-inhibition and of starvation-induced derepression of transport activity (adaptive regulation (1, 17)). Using procedures adopted in previous studies (1, 17), we have investigated the effect of amino acid starvation on the inward transport of L-glutamic acid in human fibroblasts. The transport rate has been assessed after 90 min of preincubation in Earle's balanced salt solution (period required to deplete the cells of their intracellular amino acid pool) and at the end of a 12-h incubation in the same medium. Measurements were performed at different substrate concentrations varying from 0.004 to 5 mM in the presence and in the absence of Na^+. The results are presented in Fig. 8. In *a*, the Eadie-Hofstee plots do not reveal any change in the rate of transport of L-glutamic acid by the two Na^+-dependent systems. In contrast, starvation does affect the Na^+-independent uptake of L-glutamic acid (*b*, *inset*). The Eadie-Hofstee plot indicates that the starvation-induced enhancement of the transport rate is dependent upon a marked increase in V_{max} of the saturable system. The plot does not reveal any change in K_m for the same system or in K_D for the nonsaturable component of transport. The relevant kinetic parameters are summarized in Table II. They allow us to estimate the relative contribution of the Na^+-dependent and Na^+-independent transport systems to the total saturable uptake of L-glutamic acid in unstarved and starved cells. Fig. 9 shows that the per cent contribution of the Na^+-independent system (*curves C*) increases upon starvation from approximately 30% to more than 45%. Under the latter conditions, this system accounts for the largest fraction of L-glutamic acid uptake in a wide range of substrate concentrations. As a consequence, the relative contribution of the Na^+-dependent systems (*curves A* and *B*) decreases with starvation.

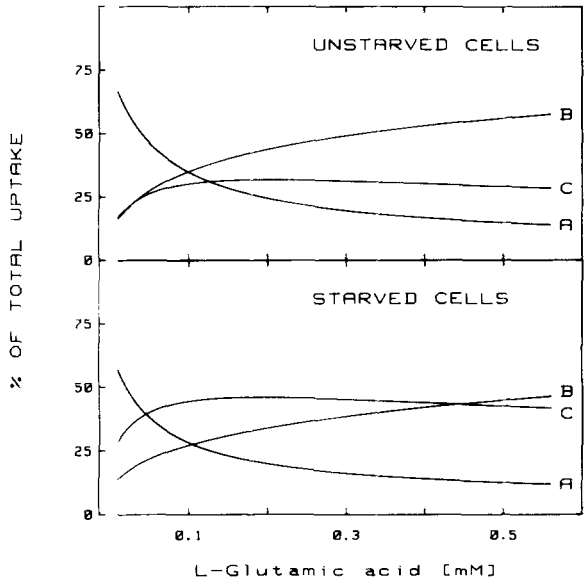


FIG. 9. **Effect of amino acid starvation on the expected contribution of saturable systems to the uptake of L-glutamic acid by human fibroblasts.** The *curves*, calculated on the basis of the kinetic parameters reported in Table II, represent the per cent uptake of L-glutamic acid attributable to a high affinity Na^+-dependent system (*curves A*), to a low affinity Na^+-dependent system (*curves B*) and to a Na^+-independent system (*curves C*). *Upper panel*, unstarved cells; *lower panel*, starved cells.

Effect of Cell Density on the Transport of L-Glutamic Acid— The activity of several transport systems for amino acids is known to vary with culture density in a number of biologic preparations (16, 18, 19). Fig. 10 shows the changes in the Na^+-dependent (*a*, *inset*) and in the Na^+-independent (*b*,

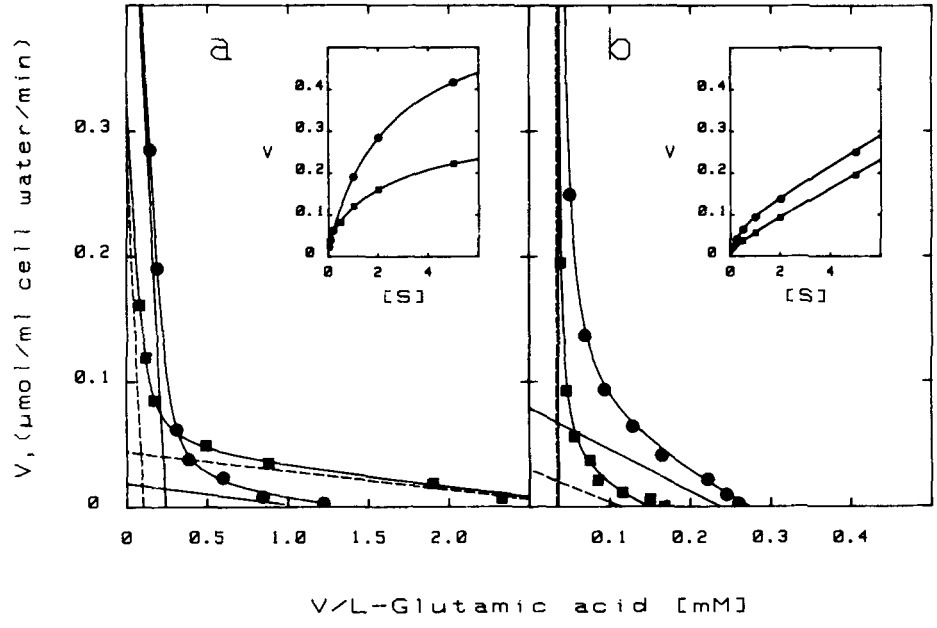


FIG. 10. **Uptake of L-glutamic acid by human fibroblasts: effect of culture density.** Human fibroblast subcultures (from comparable initial plating densities, see "Experimental Procedures") were allowed to grow for 2 days ("low density", ●) and for 7 days ("high density", ■). Transport assay, calculations and graphic representations, as described in Fig. 2. The data were best fitted by computer on the basis of two independent rectangular hyperbolae (Equation 2 under "Experimental Procedures") for Na^+-dependent uptake (*a*) or of the sum of a single rectangular hyperbola and of a linear nonsaturable component (Equation 1 under "Experimental Procedures") for Na^+-independent uptake (*b*). *Continuous straight lines*, transport in low density cultures; *dotted lines*, transport in high density cultures. In both panels, the data points are from a representative experiment in which cell densities (expressed by protein content) were 10 $\mu g/cm^2$ for low density cultures and 45 $\mu g/cm^2$ for high density cultures. Each experiment was repeated twice with comparable results.

TABLE III

Kinetic parameters for the uptake of L-glutamic acid by human fibroblasts at different culture densities

Conditions of cell culture, transport assay, and calculations, as described in Fig. 10. Parameters are shown as means ± S.D.

System	Low density			High density		
	K_m	V_{max}	K_D	K_m	V_{max}	K_D
	mM	*μmol/min/ml cell water*	min^{-1}	*mM*	*μmol/min/ml cell water*	min^{-1}
Na^+-dependent (high affinity)	0.018 ± 0.005	0.019 ± 0.002		0.015 ± 0.003	0.044 ± 0.003	
Na^+-dependent (low affinity)	2.46 ± 0.20	0.59 ± 0.02		2.64 ± 0.45	0.27 ± 0.02	
Na^+-independent	0.33 ± 0.10	0.08 ± 0.014		0.26 ± 0.09	0.03 ± 0.006	
Non-saturable			0.036 ± 0.004			0.034 ± 0.004

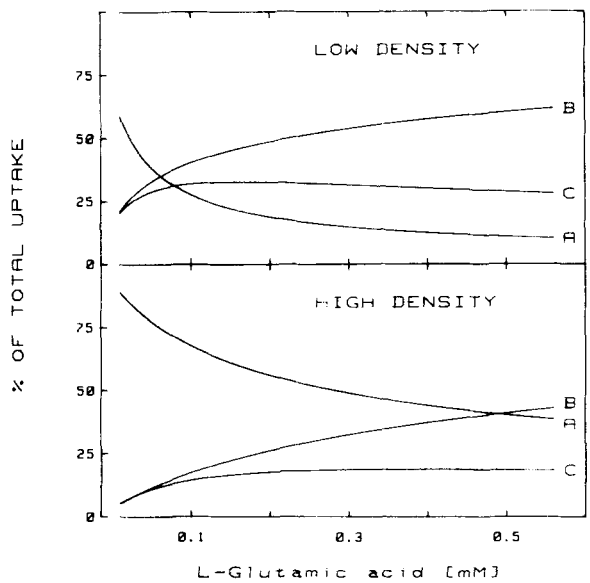


FIG. 11. **Effect of culture density on the expected contribution of saturable systems to the uptake of L-glutamic acid by human fibroblasts.** The curves, calculated on the basis of the kinetic parameters reported in Table III, represent the per cent uptake of L-glutamic acid attributable to a high affinity Na^+-dependent system (*curves A*), to a low affinity Na^+-dependent system (*curves B*) and to a Na^+-independent system (*curves C*). *Upper panel*, low density cultures; *lower panel*, high density cultures.

inset) uptake of L-glutamic acid by human fibroblasts grown at two different culture densities. In *a*, the Eadie-Hofstee plots indicate that both Na^+-dependent saturable systems for L-glutamic acid uptake exhibit a change in V_{max} values with cell density without substantial modification in K_m. However, the V_{max} of the low affinity system decreases at high cell density, whereas the opposite is observed for the high affinity system (Table III). In *b*, the Eadie-Hofstee plots for the Na^+-independent saturable system show a marked decrease in the V_{max} value at high cell density, without appreciable changes in K_m. The value of K_D of the nonsaturable component does not change significantly at the two culture densities selected for this study (Table III).

Fig. 11 describes the relative contribution of the Na^+-dependent and Na^+-independent transport systems to the total saturable uptake of L-glutamic acid, as predicted on the basis of the kinetic parameters reported in Table III for low and high culture densities. One can see that the per cent contribution of the Na^+-dependent high affinity system (*curves A*) is lower at low than at high cell density. At high cell density, this system becomes predominant, particularly when the substrate concentration is low. The per cent contribution of the other systems (*curves B* and *C*) is lower at high than at low culture density.

DISCUSSION

The results recounted in this paper indicate that L-glutamic acid enters cultured fibroblasts derived from human skin by at least three transport systems (two Na^+-dependent and one Na^+-independent). The relevant kinetic parameters allowed the differentiation of the two Na^+-dependent mediations into a high affinity and a low affinity system. The former has been identified by competition analysis with the previously described system for anionic amino acids (3). This system, which requires an extra negative charge on the ω group of the amino acid substrate, is characterized by a reactivity toward the naturally occurring L-aspartate, L-cysteate, L-glutamate, L-aminoadipate and by an anomalous stereoselectivity allowing access of D-aspartate and D-cysteate, but not of D-glutamate, into the fibroblast (3). The system has been provisionally designated as System ω^- (20, 21). We deviate here from this designation, preferring X^-_{AG}, in order to make the symbolism conform with that used to identify another anionic amino acid-transporting system (x_C^- for cystine and L-glutamate, *cf.* Ref. 6). The capital X underlines Na^+-dependence of the system, as contrasted with Na^+-independence of System x_C^-; subscript AG defines major naturally occurring substrates, L-aspartic and L-glutamic acid. As far as the transport of L-glutamate is concerned, System X^-_{AG} exhibits low K_m and low V_{max} values (Table I). Its transport maximum does not change upon amino acid starvation (Table II) and characteristically rises with an increase in culture density (Table III). This observation is peculiar since the activity of other Na^+-dependent mediations, such as System A and System ASC (for neutral amino acids), is known to decrease with increasing cell density in normal and virus-transformed cell cultures (18, 19). The low affinity Na^+-dependent mediation of L-glutamic acid uptake could be identified with System ASC. This mediation was fully inhibitable by L-serine with a K_i (0.1 mM) very close to the previously determined K_m for L-serine uptake via System ASC (0.16 mM, Ref. 15) and was insensitive to MeAIB (Fig. 6). Its activity decreased with an increase in culture density (Fig. 10), in agreement with the expected behavior of System ASC (18, 19).

The system which mediates the transport of the largest Na^+-independent fraction of L-glutamic acid in fibroblasts derived from adult human skin (Fig. 2) resembles the x_C^- System described in fibroblasts from human fetal lung (4) and in rat hepatoma cell line HTC (6). However, the activity of this system does not account in full for the Na^+-independent uptake of L-glutamic acid in our cells, where the contribution of an apparently nonsaturable component cannot be overlooked (Fig. 2). On the other hand, as shown in Fig. 4, BCH exerts a moderate inhibitory effect on the Na^+-independent uptake of L-glutamic acid under conditions in which this analog behaves as a transport-specific substrate of System L in human fibroblasts (1). If this inhibition identifies the activity of System L, the entry of L-glutamic acid by this

route could be explained by the observation that dicarboxylic amino acids react, in the neutral (protonated) form, with System L (3, 22). As predicted by the titration curve, at pH 7.4 the protonated form of L-glutamic acid represents only a small fraction (<0.1%) of the total solute. Hence, in the uptake experiments presented in Fig. 2, the concentration of this form is likely to remain in the region of first order kinetics even at the highest concentration of L-glutamic acid used in this study. As a consequence, the reported nonsaturable Na^+-independent component of L-glutamic acid uptake (Fig. 2) may well reflect at least in part a saturable uptake of the zwitterionic form of the amino acid via System L.

The relative contribution of the major transport systems (X_{AG}^-, ASC, and x_C^-) to the uptake of L-glutamic acid by cultured human fibroblasts has been found to vary as a function of the external concentration of the substrate as well as of the cell density and nutritional state. Let's consider a value of 0.06 mM as the average concentration of L-glutamic acid in the extracellular fluids of the adult man (13) and select this value as a reference point to draw information from the theoretical curves constructed on the basis of the kinetic parameters experimentally obtained at different culture densities. At low cell density (Fig. 3 and Fig. 11, *upper panel*), we can see that: (*a*) for external concentrations of L-glutamic acid well below the reference point, the activity of the Na^+-dependent System X_{AG}^- appears to predominate; (*b*) around the reference point, the contributions of all the systems considered become similar; (*c*) for external concentrations higher than the reference point, the major route of substrate entry shifts to System ASC. At high cell density (Fig. 11, *lower panel*), the activity of System X_{AG}^-, though decreasing with an increase of the external substrate concentration, largely predominates within a 10-fold range of concentrations around the reference point and approaches the contribution of System ASC at concentrations higher than 0.4 mM. Values of 0.5 mM L-glutamic acid have been reported as basal plasma concentrations in human premature newborns (13).

All these transport patterns, though useful and perhaps adequate to trace the movements of L-glutamic acid in cultured human fibroblasts, by no means provide a full description of the quantitative aspects of these movements *in vivo*. Indeed, no information is available whether cell density in mesenchymal tissues of man affects the uptake of L-glutamic acid by fibroblasts; in these tissues, the number of fibroblasts/unit volume varies over a wide range of values and the picture is further complicated by the presence of other cell types and extracellular structures. Within these limitations, we may attempt to draw some inferences about the properties and the physiologic role of the described transport systems.

System X_{AG}^-, endowed with a very low K_m value (0.016 mM), is likely to operate *in vivo* under saturating conditions for L-glutamic acid, the concentrations of the amino acid in human plasma being approximately 0.06 mM in adults and 0.5 mM in premature newborns (13). The fact that K_m of L-glutamic acid uptake by System X_{AG}^- does not change appreciably with cell density in fibroblast cultures (Table III) is compatible with this conclusion. On the other hand, competition for the transport of L-glutamic acid via System X_{AG}^- is substantially limited to L-aspartic acid, whose plasma concentration is much lower (2 nM in adults) (23) than that of L-glutamic acid, whereas its K_m for System X_{AG}^- is almost identical (0.01 mM) to that of L-glutamic acid (Table I). Calculations show that, under a 10 to 1 concentration ratio for L-glutamic acid/L-aspartic acid in the extracellular fluid, the K_m for L-glutamic acid increases from 0.016 mM to 0.019 mM, a value still well below the plasma concentration of L-glutamic acid.

The entry of L-glutamic acid through System ASC, a mediation commonly devoted to translocate neutral amino acids in human fibroblasts (15), was unexpected, although similar findings were reported in HTC cells (6). The high K_m value (2 mM) for this process renders it remarkably powerful at the highest substrate concentrations tested *in vitro* (see Figs. 3, 9, and 11). However, the much higher affinity of System ASC for small neutral amino acids (K_m values of about 0.05–0.2 mM, Ref. 15) suggests that this mediation is unlikely to function as an important route for L-glutamic acid uptake by fibroblasts *in vivo*, where the physiologic fluids contain many competing amino acids.

The Na^+-independent System x_C^- accounts for a definite, nontrivial fraction of L-glutamic acid uptake by human skin-derived fibroblasts (Fig. 3). Under conditions of cell starvation, it becomes the predominant mediation in a wide range of substrate concentrations including "physiologic" plasma levels. Much higher contributions of System x_C^- to the total uptake of L-glutamic acid have been reported by Bannai and Kitamura (4) for fetal lung fibroblasts grown in culture under slightly different conditions (50% confluency in minimal essential medium; uptake measured in phosphate buffer). A comparison of the kinetic parameters for System x_C^-, identified in fibroblasts derived from adult human skin (present study) and in lung fibroblasts from human fetus (4), shows that the value of K_m is essentially similar in either cell culture, whereas V_{max} is about 10-fold higher in fetal lung fibroblasts than in low density cultures of adult skin fibroblasts. Using a V_{max} value recalculated from Fig. 4 of Ref. 4, as the "capacity" parameter for System x_C^- in the set of equations used to estimate the relative contribution of the saturable systems for L-glutamic acid uptake, theoretical curves as those reported in Fig. 3 of the present paper would indicate that System x_C^- should predominate over the full range of substrate concentrations, its contribution being always higher than 80%. The large difference in V_{max} between fetal human lung fibroblasts and our preparation, together with the changes in V_{max} associated with cell density (Table III) and nutritional state (Table II) implies that System x_C^- is the target of a number of regulatory factors and conditions known to alter amino acid transport.

Addendum—After completion of this manuscript, our attention was drawn to a recent paper by Bannai and Kitamura (Bannai, S., and Kitamura, E. (1982) *Biochim. Biophys. Acta* **721**, 1–10) showing that System x_C^- is subject to adaptive regulation. Our results confirm this finding under somewhat different experimental conditions.

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