# Amino Acid Uptake by Amino Acid Analog Resistant Tobacco Cell Lines

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Two tobacco cell lines resistant to *p*-fluorophenylalanine (PFP) and one resistant to 5-methyltryptophan (5-MT) are compared with wild type cells in their ability to absorb amino acids from the medium. One *p*-fluorophenylalanine-resistant cell line shows greatly reduced uptake of all amino acids so is resistant to growth inhibition by other amino acid analogs. The impaired absorption is noted with amino acids, amino acid analogs and shikimate, but not with cinnamate, salicylate, nicotine, glucose, 3-O-methylglucose and palmitate. The phenylalanine transport system of the PFP-resistant cell line and the wild type both have $K_m$ values of 90 $\mu$M, but have different $V_{max}$ values. Several analogs of phenylalanine and several neutral L-amino acids inhibit the phenylalanine transport system, while L-aspartic acid, L-arginine, D-phenylalanine or chlorogenic acid do not interfere with the L-phenylalanine uptake. The results indicate the presence of more than one transport system for amino acid uptake. The lessened uptake of all amino acids, the specificity of the uptake systems and the unchanged binding let us conclude that a pleiotropic mutation or that some inhibitor causes the reduced uptake of all amino acids by the PFP-resistant cell line.

Little is known about the mechanism by which organic molecules are transported into the cells of higher plants. Studies on sugar and amino acid transport have been carried out mainly with leaf and root slices. The kinetic data from these experiments have supported a carrier mediated, energy dependent transport dechanism for sugar [1, 2] and amino acids [3, 4]. A general amino acid transport system for all L-amino acids was proposed for root tips of *Cucumis*, *Caesalpinia* seedlings [4] and pea leaf fragments [3].

Plant tissue cultures provide a much more homogeneous material than sliced parts of whole plants. Therefore, plant cell suspension cultures should be a better system for studying the mechanism of uptake of organic materials. However, this system has been used in only a few uptake experiments for characterization of the transport systems. Using suspension cultures of sugarcane, Maretzki and Thom described two membrane transport systems for glucose [5, 6] and characterized specific transport systems for the basic amino acids arginine and lysine [7]. At least three different uptake systems for basic, neutral and acidic amino acids were found in soybean cultures on the basis of competitive inhibition experiments [8]. The conflicting results on the specificity of amino acid transport systems of higher plants may be due to different experimental systems and methods. Diphasic Lineweaver-Burk plots are often observed in uptake studies indicating the presence of more than one carrier system for an amino acid [9]. When more than one uptake system is found for a single compound, the uptake system with the low $K_m$ generally exhibits a high substrate specificity and those with a higher $K_m$ show a broader specificity [10, 11].

Besides being a relatively homogeneous plant material, cell cultures offer at least two other advantages: (1) the possibility of selecting and studying transport mutants and (2) the ease of conditioning the cells into a predetermined state. Thus, the previous incubation conditions have been shown to affect the rate of uptake and the transport system present [5–7, 12].

Amino acid analog resistant cell lines have been a useful tool for characterizing amino acid transport systems of microorganisms and mammalian cells [13, 14]. Amino acid analog resistant plant cell lines have been selected [15], and these may also be useful in uptake studies in higher plants. The resistance of a carrot line to growth inhibition by 5-methyltryptophan (5-MT) was apparently due to decreased uptake of the tryptophan analog [16].

*Abbreviations:* PFP, *p*-fluorophenylalanine; 5-MT, 5-methyltryptophan; AEC, aminoethyl-L-cysteine; Eth, ethionine.

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This line also showed decreased tryptophan uptake while the uptake of leucine remained unchanged, indicating an alteration of a tryptophan specific transport system. Carrot cells resistant to ethionine showed an increased resistance to some other amino acid analogs [17] indicating that the uptake of all amino acids may be reduced. During studies on p-fluorophenylalanine (PFP)-resistant cell lines of tobacco [18] a greatly decreased uptake of phenylalanine, tyrosine and shikimic acid and a cross resistance to 5-MT (unpublished) was noted.

This present study presents a more detailed characterization of the difference in uptake of several compounds by PFP-resistant and-sensitive tobacco cells. The possible reasons for these differences are discussed.

## Materials and Methods

*Plant Material:* Suspension cultures of tobacco pith (*Nicotiana tabacum* L. cv. Xanthi) were grown as described [19] in 70 ml liquid medium. Two of the cell lines have been described previously as PFP-sensitive (TX1) and resistant (TX4) tobacco cell lines [18, 20, 21]. TX44 is a recently selected PFP-resistant cell line and TX6 is a newly selected 5-methyltryptophan (5-MT)-resistant strain. All resistant cell lines were selected from the wild type TX1.

*Labelled compounds:* All $^{14}$C-labelled compounds were commercial products purchased from New England Nuclear and Isotopen Dienst West. The specific radioactivities varied from 20 mCi/mmol to 420 mCi/mmol.

*Uptake experiments*

(a) *Short time experiments:* Cells in logarithmic growth phase with a density of near 5 g fresh weight per flask were used in all uptake experiments. Typically, 6 flasks were combined and shaken without covering for 2 hours to overcome the so-called „shock effect" described by Doree *et al.* [22] and Thoiron *et al.* [23]. Ten ml of the suspension were then incubated with the appropriate labelled compounds. Two ml samples were removed within one minute as zero time [7] and after 10 min for TX1 cells and 30 min for TX4 cells. Uptake was linear for 15 min with TX1 cells and for more than one hour with TX4 cells. Cells were collected on filter paper disks and rinsed with 1 mM phenylalanine. These cells were rinsed into and homogenized in 10% trichloroacetic acid and heated for 15 min at 90 °C. The cool extract was filtered and aliquots were counted in a scintillation spectrometer. This gave a reasonable measure of phenylalanine uptake since the percent incorporation was shown to be the same in TX1 and TX4 cells [20] and since no other incorporation of phenylalanine into polymeric coumpounds was observed [18].

(b) *Long time experiments:* Uptake over periods of more than 30 min was monitored by measuring the radioactivity left in the medium. Cells were grown to a density of near 4 g per 70 ml for use in these experiments.

To determine the loss of label from cells a culture was divided into two equal parts and one part was incubated with $^{14}$C-labelled phenylalanine ($10^{-4}$ M) for two hours. The cells were collected on filter paper, rinsed three times with water and then placed into the filtered medium of the other half of the culture. Only traces of radioactivity (less than 1% of the total radioactivity taken up) were found in the medium within a 6 hour period.

## Results

One or more biochemical alterations may contribute to the resistance of an amino acid analog resistant cell strain including uptake variants which can be identified by their cross resistance to other amino acid analogs or by direct measurement of the absorption of labelled aminos acids.

*Checking for cross resistance:* When tested for resistance to several amino acid analogs (Fig. 1), the strain TX44, which was selected for PFP-resistance, was only resistant to PFP. Thus no general alteration of amino acid uptake had occurred in TX44 cells. The other PFP-resistant cell line TX4, however, was not only resistant to PFP, but was also quite resistant to 5-MT, aminoethylcysteine (AEC) and ethionine (Eth). The TX4 cells were even more resistant to 5-MT growth inhibition than the line TX6 which was recently selected for 5-MT resistance. The line TX6 did not show an uniform response to the tested amino acid analogs and was as sensitive to PFP and AEC growth inhibition as the wild type, but showed a high resistance to ethionine.

*Uptake of various amino acids:* These data suggested that the uptake of most if not all amino acids

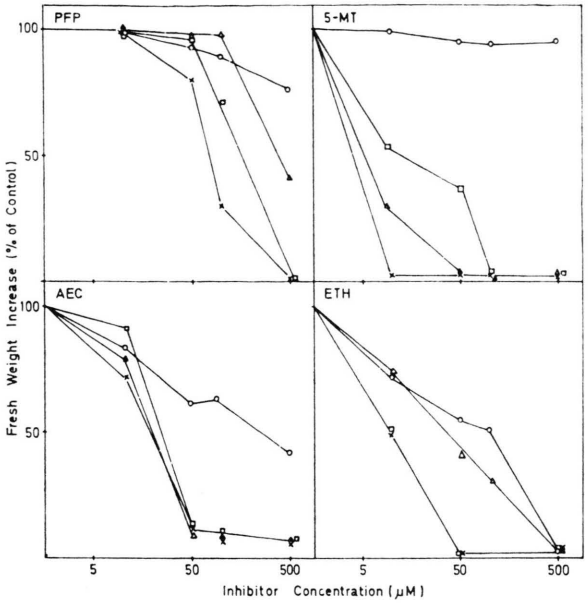


Fig. 1. The effect of *p*-fluorophenylalanine (PFP), 5-methyltryptophan (5-MT), aminoethyl-L-cysteine (AEC) and ethionine (Eth) on the growth of various tobacco cell lines with an inoculum of 0.8 g fresh weight. Cells were harvested after 10 days. Wild type cells TX1 (×—×), PFP-resistant line TX4 (○—○), PFP-resistant line TX44 (△—△), 5-MT-resistant line TX6 (□—□).

should be decreased in TX4 cells, while this would not be the case for the other lines. To study this, the uptake of various $^{14}C$-labelled amino acids and amino acid analogs was measured over a period of 4 hours. As expected, the uptake of all amino acids tested (Fig. 2) was decreased in TX4 cells while the other PFP-resistant cell line, TX44, showed the same uptake pattern as the wild type line. This generally lessened uptake of PFP, other amino acids and their analogs, may be responsible for the resistance of TX4 cells. While the reason for the resistance of TX44 cells has not been determined, decreased uptake can now be excluded. The TX6 cells generally showed a slightly impaired uptake of all amino acids so that resistance might be partly due to this. However, the main reason for the resistance of TX6 to 5-MT growth inhibition probably is a tenfold increase of free tryptophan found in this line (unpublished). The cell strains TX44 and TX6 were included in this study as examples of the different types of resistant cell lines that can be selected.

*Uptake of compounds other than amino acids:* During studies of shikimate metabolism in TX4 cells (unpublished), a greatly decreased rate of uptake was also noted for shikimate indicating that the TX4 cells may have an impaired uptake of all organic molecules. However, as shown in Fig. 3, the uptake of several organic compounds (cinnamic acid, nicotine, salicylic acid and glucose) was similar in TX4 and TX1 cells. Glucose and 3-O-methylglucose were not taken up from the regular medium, but were taken up rapidly when

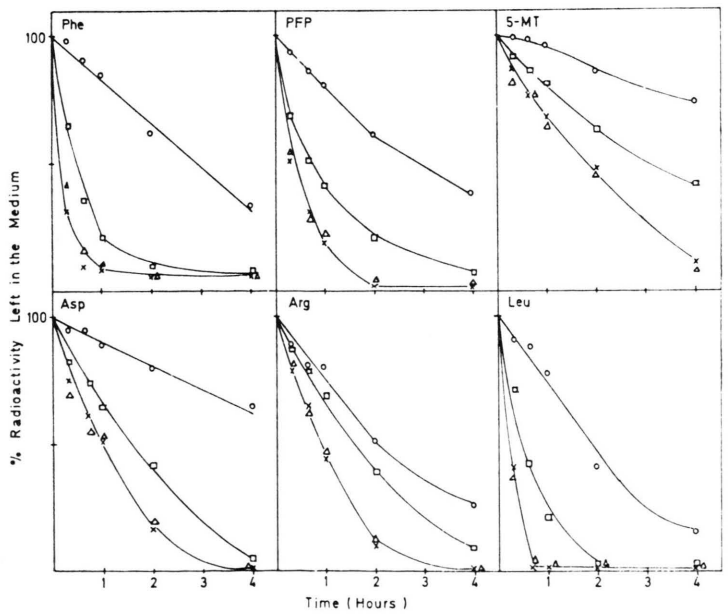


Fig. 2. Uptake of 1 μCi L-phenylalanine-[U-$^{14}C$] (Phe), DL-*p*-fluorophenyl[3-$^{14}C$]alanine (PFP), DL-[5-$^{14}C$]methyltryptophan (5-MT), L-[U-$^{14}C$]aspartic acid (Asp), L-[U-$^{14}C$]arginine (Arg) and L-[U-$^{14}C$]leucine (Leu) ($5\times10^{-5}$ M) monitored as radioactivity left in the medium by tobacco cell lines TX1 (×—×), TX4 (○—○), TX44 (△—△) and TX6 (□—□).

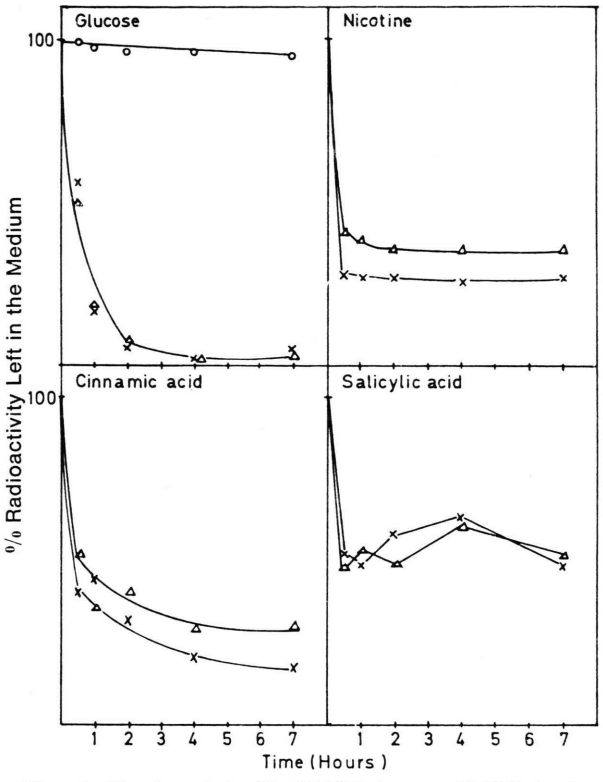


Fig. 3. Uptake of 1 $\mu$Ci [U-$^{14}$C]glucose, [2-$^{14}$C]nicotine, [7-$^{14}$C]salicylic acid and [3-$^{14}$C]cinnamic acid ($5\times10^{-5}$ M) monitored as radioactivity left in the medium by TX1 cells ($\times-\times$) and TX4 cells ($\triangle-\triangle$). Glucose uptake was completely inhibited in the normal medium (3% sucrose) by either line as shown for TX1 cells only ($\bigcirc-\bigcirc$), and was therefore measured at 0.3% sucrose in the medium.

the sucrose concentration was lowered to 0.3 from 3 percent. Palmitic acid (not shown) was also absorbed similarly by both cell lines. This data then rules out a general membran modification in the TX4 cells which results in an impaired uptake for all organic compounds.

*Kinetics of phenylalanine uptake:* The fact that all amino acids and amino acid analogs tested were taken up poorly by the PFP-resistant cell line (TX4) indicated that all amino acids might have the same or related transport systems. To learn more about the altered uptake, the rate of uptake of phenylalanine was measured in relation to the substrate concentration. Lineweaver-Burk plots (Figs 4 and 5) gave diphasic curves for both cell lines indicating that two phenylalanine transport systems are operative in both cell lines. The lower $K_m$ value for phenylalanine was about 90 $\mu$M in both TX1 and TX4 cells. However, the velocity was decreased by about a factor of ten in TX4 cells. This result clearly demonstrates that the uptake systems for amino acids are present in TX4 cells and that the affinity for the amino acids is not altered. During these experiments the $V_{max}$ values were found to vary with batches of one line in spite of well controlled conditions. The "shock effect" [22, 23] had to be taken into account since a 50 – 80% decreased uptake rate for phenylalanine was noted in the first two hours after the opening and mixing of flasks

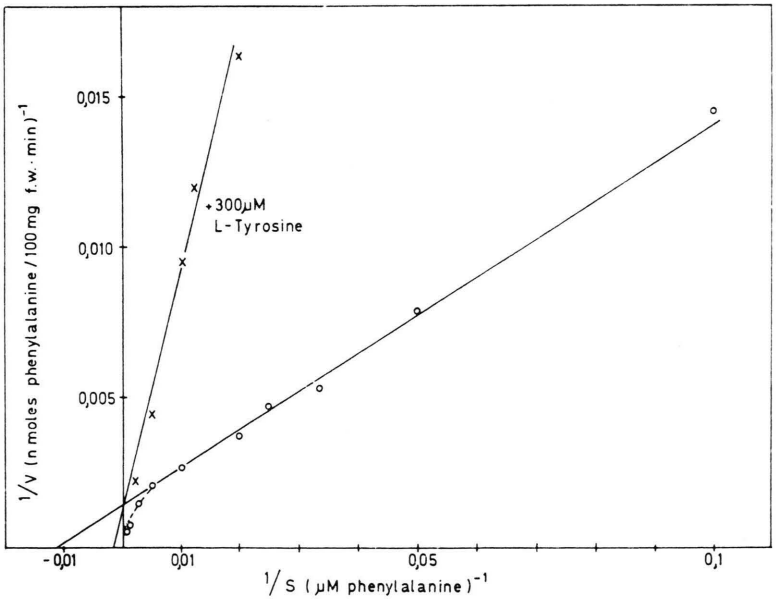


Fig. 4. Lineweaver-Burk plot of L-phenylalanine uptake by TX1 cells with and without 300 $\mu$M L-tyrosine. Phenylalanine uptake was measured using concentrations of 0.02 to 2 mM, in competition with tyrosine concentrations of 40 to 200 $\mu$M.

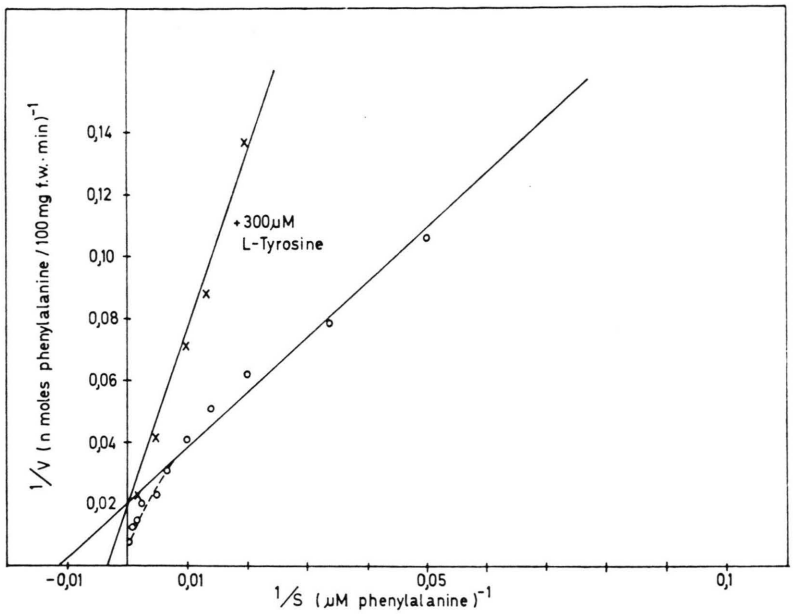


Fig. 5. Lineweaver-Burk plot of L-phenylalanine uptake by TX4 cells with and without 300 $\mu$M L-tyrosine. Phenylalanine uptake was measured using concentrations of 0.02 to 2 mM, in competition with tyrosine concentrations of 40 to 200 $\mu$M.

in kinetic experiments. After two hours the uptake velocity remained quite stable for each cell population, but different batches sometimes showed variations in velocities of up to 80% from the mean in 8 independent experiments. In these experiments little variation was found for the $K_m$ values, however.

Long time uptake experiments (Fig. 2) were carried out with substrate concentrations of $5 \times 10^{-5}$ M where the low $K_m$ phenylalanine transport system should be operative. If this system is a general amino acid transport system, other amino acids should competitively inhibit phenylalanine uptake. Therefore, the effect of amino acids, some amino acid analogs and some aromatic compounds on the uptake of L-phenylalanine was measured in TX1 and TX4 (Table I). The DL-analogs of phenylalanine inhibited the uptake while D-phenylalanine did not compete with the L-isomer. The neutral amino acids L-leucine and L-alanine reduced greatly the uptake of L-phenylalanine. L-tyrosine acted as a competitive inhibitor with both lines as shown in Fig. 4 and 5. Acidic and basic amino acids as well as several non amino acids did not reduce the uptake.

The uptake of phenylalanine was almost completely inhibited by sodium azide, potassium cyanide and 2,4-dinitrophenol while potassium fluoride and EDTA did not inhibit the rate of uptake (Table II).

Table I. Inhibition by amino acids, their analogs and non amino acids of phenylalanine uptake by TX1 and TX4 cells. The L-phenylalanine concentration was 0.1 mM in the short time experiments (see Materials and Methods) with 0.1 $\mu$Ci [U-$^{14}$C]phenylalanine.

| Added Inhibitor (0.25 mM) | Relative rate of uptake of 0.1 mM L-phenylalanine in cells of | |
|---|---|---|
| | TX1 | TX4 |
| None | 100 | 100 |
| L-Alanine | 8 | 12 |
| L-Arginine HCl | 98 | 109 |
| L-Aspartic Acid | 108 | 97 |
| L-Leucine | 16 | 36 |
| L-Tryptophan | 62 | 73 |
| L-Tyrosine | 21 | 24 |
| DL-*o*-Fluorophenylalanine | 50 | 56 |
| DL-*m*-Fluorophenylalanine | 49 | 47 |
| DL-*p*-Fluorophenylalanine | 53 | 51 |
| D-Phenylalanine | 106 | 100 |
| $\beta$-Thienylalanine | 70 | — |
| *p*-Methyl Cinnamic Acid | 96 | — |
| Cinnamic Acid | 116 | — |
| Shikimic Acid | 100 | — |
| Anthranilic Acid | 113 | — |
| Chlorogenic Acid | 107 | — |

## Discussion

The characterization of transport systems in higher plants has been impeded by the lack of homogeneous plant material and the lack of uptake mutants. The usefulness of tissue cultures for

Table II. Effect of metabolic inhibitors on phenylalanine uptake by TX1 cells. Cells were incubated for 1 or 5 min prior to addition of 0.1 mM phenylalanine and 0.1 $\mu$Ci [U-$^{14}$C]phenylalanine.

| Inhibitor (Conc.) | Relative rate of uptake of phenylalanine after a preincubation period of | |
|---|---|---|
| | 1 minute | 5 minutes |
| None | 100 | 100 |
| KF ($10^{-3}$) | 96 | 93 |
| $NaN_3$ ($10^{-3}$) | 13 | 8 |
| KCN ($10^{-3}$) | 8 | 6 |
| 2,4-dinitrophenol ($10^{-4}$) | 6 | 14 |
| EDTA ($10^{-3}$) | 102 | 94 |

studies on amino acid transport and glucose uptake has already been demonstrated [5 – 8, 12]. This is, however, the first time that uptake variants of plant tissue cultures have been used in uptake studies except for a brief account [16]. The frequency with which uptake variants may be found in selected resistant lines appears to be very low since in most cases other mechanisms have been shown to cause the resistance to an amino acid analog [15].

Only one of the resistant cell lines (TX4) used in this study can be considered to be a general uptake variant. This line was found to have an altered chorismate mutase which apparently causes an oversynthesis of phenylalanine and tyrosine [20]. The oversynthesized phenylalanine does not accumulate, but is converted into phenolic compounds by an elevated level of phenylalanine ammonia lyase [18, 20, 21]. It was first thought that the resistance to PFP was due to the lessened feedback control of the chorismate mutase by tyrosine and phenylalanine. However, the finding of a generally decreased uptake of amino acids and their analogs shows that the altered uptake may cause the resistance of the TX4 line. If this is true, it is difficult to see why the altered chorismate mutase, high phenylalanine ammonia lyase and high phenolic levels are found in these cells, unless these alterations, especially the high phenolic levels, cause the decreased uptake. On the other hand cinnamic acids, chlorogenic acid and other tested aromatic compounds did not effect the uptake of L-amino acids. However, it was found that the rate of L-phenylalanine uptake by TX1 cells can be reduced to 40% of the control by replacing half of the TX1 culture medium by TX4 conditioned medium (Berlin, unpublished).

Uptake studies with *Saccharomyces* show that amino acid uptake can be feedback-regulated by specific amino acids [24]. The amino acid analog resistant plant cell lines generally have an increased level of the corresponding free amino acid [25] or in the TX4 case, the phenylalanine pool shows an increased turnover rate [18]. These pool alterations might regulate uptake, but since TX4 absorbs all amino acids and analogs and shikimic acid poorly and since the the competition studies indicate that more than one L-amino acid transport system is present, some more general control would seem to be operative. The possibility that there was a generally decreased uptake of all organic molecules, as found in some Chinese hampster ovary cell mutants [26], was also considered. This possibility can be rejected since the uptake of sugars, fatty acids and several other organic compounds was not impaired in the TX4 line.

The competitive inhibition experiments indicate the existence of specific amino acid transport systems rather than a general uptake system. Without identifying all possible carriers (some of which will be characterized in the following paper [27], one can state that the binding affinities of these carriers have not been altered in TX4 cells. Something, however, affects the velocity of all amino acid uptake systems. Mutants from bacteria have been isolated which show pleiotropic or secondary effects in apparently independent amino acid transport systems [10, 28, 29]. Such effects indicate that these transport systems share a common component or could be inhibited by a common agent. A pleiotropic effect or a common inhibitor of amino acid uptake in TX4 may be responsible for the reduced uptake by this line.

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