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THE UPTAKE OF PHENYLALANINE AND TYROSINE BY SEEDLING ROOT TIPS

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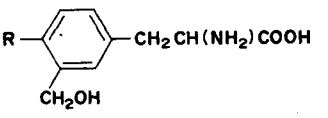
Key Word Index—*Cucumis melo*; Cucurbitoceae; *Caesalpinia tinctoria*; Leguminosae; amino acid uptake; inhibition by analogues.

Abstract—The uptake of phenylalanine and tyrosine into seedling root tips of *Cucumis melo* and *Caesalpinia tinctoria* depended on co-existing metabolic and non-metabolic processes. Competitive inhibition studies and kinetic experiments indicated that a single active uptake system operated for all α-amino acids. This active uptake system is compared to those occurring in other types of organism, and the possible role of cation-exchange in uptake is discussed. 3-Hydroxymethylphenylalanine and 4-hydroxy-3-hydroxymethylphenylalanine, natural products of *Caesalpinia tinctoria*, inhibited phenylalanine uptake more in *Cucumis* than in *Caesalpinia*.

INTRODUCTION

The mechanism of amino acid transport into bacterial, fungal and animal cells has been extensively studied and recently reviewed [1–4]. Much less is known about amino acid transport across plant cell walls. Some years ago an energy-dependent amino acid uptake system, selective for L-amino acids, was described for carrot slices [5–7]. More recently the transport of amino acids from xylem sap into the surrounding cells in apple stems was described as a two stage process, the first stage being reversible cation-exchange adsorption [8–9].

Our study set out to determine the nature of amino acid uptake into plant root tips and to compare the degree of selectivity of the system found with that of systems reported for other types of organism. We chose to examine the uptake of two aromatic amino acids, phenylalanine and tyrosine, but various structural analogues also were tested. In part, we wished to know whether structurally-modified phenylalanines or tyrosines could compete with the parent molecules for binding at the uptake site (the permease system), and whether the specificity governing the binding of particular compounds varied between species. It is well established that plants show considerable differential specificity when the binding of analogue molecules to aminoacyl-*t*RNA synthetases is considered. An analogue molecule may show little or no affinity for the synthetase activating the parent amino acid when the analogue is produced by the species from which the synthetase itself was isolated, but good substrate behaviour may be found for the analogue when tested with a synthetase preparation derived from a plant species not producing the analogue [10–12].



(1) R = H
(2) R = OH

Two modified phenylalanines, 3-hydroxymethylphenylalanine (**1**) and 4-hydroxy-3-hydroxymethylphenylalanine (**2**), have been identified as amino acid components of seeds and seedlings of *Caesalpinia tinctoria* [13]. The ability of these two amino acids, and a range of other substituted phenylalanines, to inhibit the uptake of phenylalanine and tyrosine by seedling root tips have been determined. Melon (*Cucumis melo*) seedlings were chosen as suitable for most experiments because they germinated readily and consistently to give uniform material. Seedlings of *Caesalpinia* were used only for comparative experiments involving the more important analogues.

RESULTS AND DISCUSSION

Radioactively-labelled amino acids accumulated rapidly in melon root tips exposed to low external amino acid concentrations, suggesting that an active transport process was operating. Accumulation was inhibited strongly by KCN, sodium arsenate and 8-hydroxyquinoline (Fig. 1), but these respiratory inhibitors did not completely abolish amino acid uptake, especially during the 15 min period following addition of the inhibitor. Some 30–40% of the normal amino acid uptake occurring in the initial 15 min period (Fig. 1) was not eliminated by these respiratory inhibitors, but they prevented any further accumulation after this time.

Non-inhibitable (non-energy dependent) uptake of amino acids into living cells has been accounted for in a number of ways. In bacteria amino acids can bind to the exterior of the cell wall, prior to transport, without the use of metabolic energy [14–16]; amino acids, bound in this way, can be released by washing with H_2O at 0°. However, when melon root tips were washed with ice-cold H_2O, amino acids taken up in the presence of respiratory inhibitors were not released. Oxender associated non-energy dependent binding in bacteria with specific amino acid binding proteins [4], which could be detached with their cognate amino acids, from the bacterial cell wall by osmotic shock [1, 17]. We obtained no evidence for the existence of similar specific amino acid uptake systems in melon root tips.

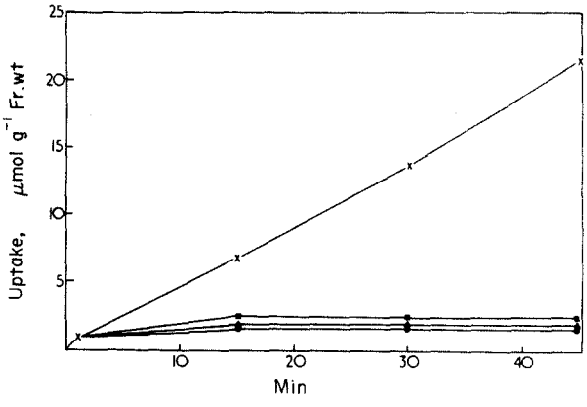


Fig. 1. Tyrosine uptake by 3-day melon seedlings: ×, 0·05 mM tyrosine; ■, 0·05 mM tyrosine with 10 mM 8-hydroxyquinoline; ▲, 0·05 mM tyrosine with 10 mM sodium arsenate; ○, 0·05 mM tyrosine with 10 mM KCN.

Alternatively non-inhibitable uptake may be due to a diffusion process, either between the root exterior and the intercellular spaces or between the cell exterior and the amino acid pools within the cell. A process of facilitated diffusion of selected amino acids has been reported in chloroplasts [18]; apparently, it depends on a limited number of sites able to bind the amino acid, and is subject to competitive inhibition [19]. Finally cation exchange has been demonstrated to be the first of a two-stage absorption process for amino acids from apple twig xylem sap [8]. The finding, reported below, that the amino acid uptake system present in melon root tips showed greater affinity for basic than for neutral, and for neutral than for acidic amino acids, suggests that cation exchange might be an important factor operating at the root cell surface.

The uptake of aromatic amino acid by melon root tips showd a temperature dependence typical of an enzyme-mediated process, uptake of phenylalanine being most rapid at about 35° and decreasing sharply at higher temperatures. A rather broad pH optimum in the region of 6·0–6·5 was established for the uptake process; other active transport systems have been reported to respond slowly to changes of pH [4]. The metabolic component of phenylalanine transport was shown to be a single process by measuring uptake in the presence of increasing concentrations of a competitive inhibitor (tryptophan). The curve relating depression of phenylalanine uptake with tryptophan concentration approximated to a rectangular hyperbola asymptotically approaching 70% inhibition (Fig. 2). If two active uptake systems were operating the curve would be the sum of two rec-

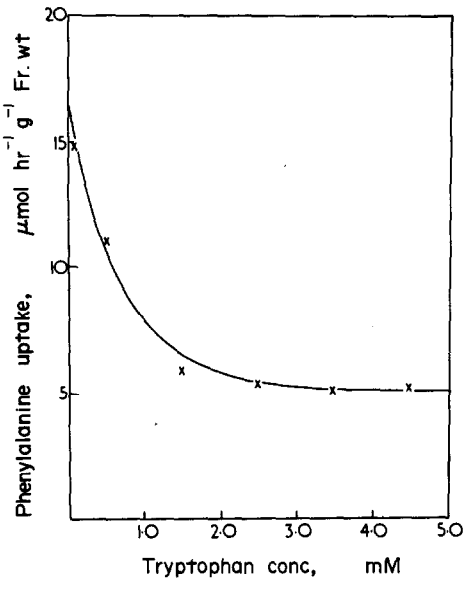


Fig. 2. Phenylalanine uptake by 3-day melon seedling root tips in the presence of increasing tryptophan concentrations. The initial phenylalanine concentration was 0·05 mM.

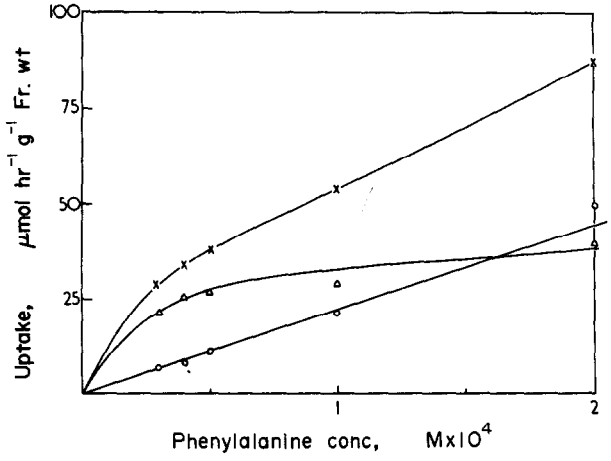


Fig. 3. Phenylalanine uptake by 3-day melon seedling root tips determined at varying phenylalanine concentrations. ×, gross uptake; ○, uptake in the presence of 2·5 mM tryptophan; △, (×–○), active uptake.

tangular hyperbolae [20]. If this result is accepted, then kinetic analysis of the active uptake system is possible. Phenylalanine uptake was measured at a range of concentrations and at the same low concentrations in the presence of a much higher concentration of tryptophan (2·5 mM), sufficient to always saturate the active uptake system. The difference between total uptake of labelled phenylalanine and uptake determined in the presence of a saturating concentration of tryptophan was taken as active uptake (Figs. 3 and 4). At substrate concentrations above 0·05 mM an increasingly substantial part of the total uptake is due to the non-metabolic process, the V_{max} for active transport calculated from Fig. 4 being 36 μmol hr^{-1} g^{-1} fr. wt. The corresponding K_m for uptake of phenylalanine was 2×10^{-5} M.

Many other amino acids were tested at 2·5 mM concentration as inhibitors of the uptake of labelled phenylalanine and tyrosine present at 0·05 mM. Initially, emphasis was placed upon aromatic amino acids, including compounds **1** and **2**, containing additional substituents on the phenyl ring or in the alanine side chain. A large number of compounds proved to be as inhibitory as tryptophan (see Table 1), and so the investigation was extended to include amino acids showing little structural resemblance to phenylalanine or tyrosine to ascertain the limits of specificity of the active transport system. Since active uptake represented about 70% of total uptake after an initial 15 min period, a compound was considered to completely inhibit active uptake if it reduced total uptake by 70%. Table 1 shows there was no significant difference between the inhibitory behaviour of a variety of ring-substituted phenylalanines: inhibitory action was retained when the β-phenyl moiety was replaced by other ring systems (e.g. pyrazolyl or thienyl). The transport system had

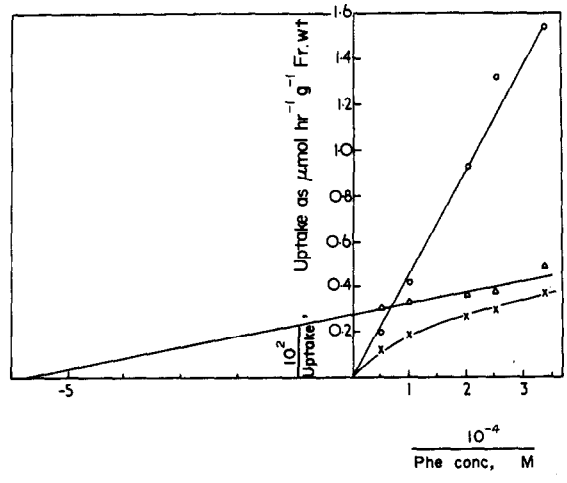


Fig. 4. Lineweaver–Burke plot for phenylalanine uptake by 3-day melon seedling root tips. ×, gross uptake; ○, uptake in the presence of 2·5 mM tryptophan; △, (×–○), active uptake.

Table 1. Inhibition of uptake of phenylalanine and tyrosine by melon seedling root tips

Inhibitor (2·5 mM L-form unless otherwise stated)	% Inhibition of Phe uptake	% Inhibition of Tyr uptake
Tryptophan	11·2	65·7
α-Methyltryptophan	22·1	28·6
Phenylalanine		62·0
Tyrosine	57·6	
α-Methyltyrosine	32·6	25·9
3,4-Dihydroxyphenylalanine (DOPA)	63·9	69·2
α-MethylDOPA	12·1	13·2
DL-4-Methylphenylalanine*	51·4	62·0
3-Methylphenylalanine	60·6	60·9
2-Fluorophenylalanine	43·9	41·9
3-Fluorophenylalanine	51·9	59·3
4-Fluorophenylalanine		57·3
5-Fluorotryptophan		58·3
3-Fluorotyrosine		77·5
β-Pyrazol-1-ylalanine		60·0
β-Thienylalanine		63·3
4-Methoxyphenylalanine		75·5
3-Methoxytyrosine	59·1	71·1
2-Hydroxy-4-methyl-phenylalanine	48·9	37·5
Tyramine	36·6	38·0
DL-2-Amino-4-methylhex-4-enoic acid*	66·0	57·8
DL-2-Amino-4-phenylbutyric acid*	59·9	60·2
3-Carboxyphenylalanine	48·8	50·9
3-Methyl-2,4-dihydroxypheny-lalanine	54·9	68·3
5-Methyl-2,4-dihydroxypheny-lalanine	47·9	63·3
α-Methyl-2,4-dihydroxypheny-lalanine	18·5	23·1
3-Hydroxymethylphenylalanine (**1**)	47·2	72·2
4-Hydroxy-3-hydroxymethyl-phenylalanine (**2**)	48·7	74·5
Alanine	74·3	
β-Alanine	25·2	
γ-Aminobutyric acid		
Glutamic acid	33·9	
N-Methylglutamic acid	30·7	
N-Acetylglutamic acid	16·9	
Glycine	62·0	
Sarcosine (*N*-methylglycine)	68·0	

* Used at 5 mM concentration.

low affinity for β-alanine and no affinity for γ-aminobutyric acid. *N*-Methylation of glutamic acid or glycine produced only small changes in inhibitory capacity, but *N*-acetylation of glutamic acid caused a more marked loss of inhibitory activity. Several α-methyl aromatic amino acids were tested but all were very poor inhibitors of phenylalanine and tyrosine uptake; the presence of a proton on the α-C atom of an amino acid is clearly important in conferring high affinity for the transport system.

The affinities of the protein amino acids for transport by the phenylalanine carrying system were tested by measuring inhibition of phenylalanine uptake: each amino acid was used alone and in the presence of 2·5 mM tryptophan to saturate the active uptake system. Surprisingly several amino acids caused substantially higher inhibition in combination with tryptophan (Table 2), indicating some competition for the non-metabolic component of uptake. This could be due to the saturation of selective transporter sites in a facilitated diffusion system [19], but since the more basic amino acids produced the greatest additive effects on inhibition, it seems likely that a cation-exchange adsorption process is involved. Glutamic acid and aspartic acid caused significantly lower inhibition than any of the neutral amino acids. The dicarboxylic amino acids are not transported by the general amino acid transport systems of other organisms [1, 3, 4], and this may be the case in plant tissue; if cation-exchange

Table 2. The inhibition of phenylalanine uptake by melon seedling root tips

Inhibitor (2·5 mM L-form)	Amino acid alone % inhibition (cf. tryptophan ≡ 100%)	Amino acid + tryptophan % inhibition (cf. tryptophan ≡ 100%)
Leucine	92	136
Valine	67	100
Methionine	109	128
Cystine	56	122
Tryptophan	100	100
Tyrosine	66*	100
Alanine	104	116
Glycine	87	100
Serine	107	111
Proline	73	119
Glutamine	93	100
Glutamic acid	58	93
Aspartic acid	57	97
Lysine	73	111
Arginine	110	131
Histidine	106	136
Ammonium tartrate	44	98
Ammonium chloride	42	104

* At limit of solubility.

The inhibition caused by 2·5 mM tryptophan was measured in each experiment and other results are expressed as a percentage of this inhibition.

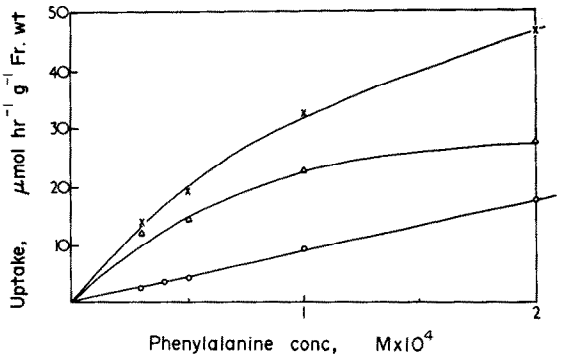


Fig. 5. Phenylalanine uptake by root tips of 5-day *C. tinctoria* seedlings at varying phenylalanine concentrations. ×, gross uptake; ○, uptake in the presence of 2·5 mM tryptophan; △, (×−○), active uptake.

absorption is the first of two stages of uptake, acidic amino acids would not be favoured [8].

These results indicate that the active uptake system for amino acids in melon root tips shows little selectivity. A more complex picture exists in many other organisms where amino acid transport has been studied. The most general amino acid transport systems reported in bacteria carry only members of chemically related amino acid groups [4, 21], and correspond to the specific systems of the fungi [1, 22]. The fungi and yeasts also have a very unspecific amino acid transport system, which operates in conditions of nitrogen starvation

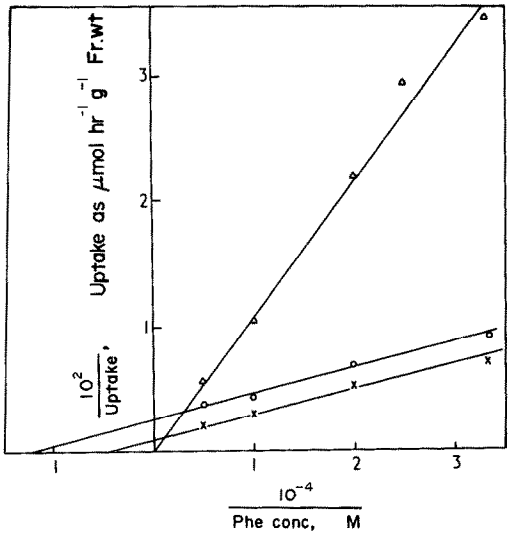


Fig. 6. Lineweaver–Burke plot for phenylalanine uptake by 5-day *C. tinctoria* seedling root tips. ×, gross uptake; ○, uptake in the presence of 2·5 mM tryptophan; △, (×−○), active uptake.

Table 3. Inhibition of phenylalanine and tyrosine uptake by root tips of 5-day *C. tinctoria* seedlings

Inhibitor (2·5 mM L-form)	% Inhibition of Phe uptake	% Inhibition of Tyr uptake
Phenylalanine		60·0
Tyrosine	57·3	
L-DOPA	48·2	56·4
α-MethylDOPA	8·5	5·5
2-Hydroxy-4-methylphenylalanine	46·0	19·1
3-Carboxyphenylalanine	49·1	54·5
3-Methoxytyrosine	46·9	32·6
Tyramine	31·5	5·2
2-Amino-4-methylhex-4-enoic acid (DL-form, 5·0 mM)	61·2	58·6
2-Amino-4-phenylbutyric acid (DL-form, 5·0 mM)		46·1
3-Hydroxymethylphenylalanine (**1**)	39·3	36·2
4-Hydroxy-3-hydroxymethylphenylalanine (**2**)	42·5	35·1

[23, 24]. Since ammonium salts inhibited amino acid uptake in melon root tips, a system similar to that in *N*-starved fungi may be envisaged.

Experimental work on active transport of amino acids in *C. tinctoria* was limited by the availability of seed, and the specificity of the system was not fully established. However, the kinetics governing phenylalanine uptake were broadly similar to those in melon, and there was a similar non-inhibitable component of uptake. For active uptake of phenylalanine K_m was $7{\cdot}8 \times 10^{-5}$ M and V_{max} 40 μmol hr^{-1} g^{-1} fr. wt. (Figs. 5, 6). The inhibition of phenylalanine and tyrosine uptake by various aromatic amino acids is shown in Table 3. The amino acids recently isolated from *C. tinctoria* (**1** and **2**) were less effective inhibitors than many other substituted phenylalanines; they also produced less inhibition of uptake in *Caesalpinia* than in melon root tips. A lowered affinity for natural analogues in the species in which they occur is consistent with findings for other enzymically-mediated systems [10–12].

EXPERIMENTAL

Materials

Plant materials. Melon seedlings (*Cucumis melo*, L.) were grown in sterile condition from seed. Seeds of *C. tinctoria* were kindly provided by Dr. E. Bornemisza (Lima, Peru). The melon seeds were dressed with the fungicide, Orthocide 75, which was

mostly removed when the seeds were soaked for 4 hr before they were allowed to germinate between sheets of moist filter paper. Dry seeds of *C. tinctoria* were soaked in conc. H_2SO_4 to soften the hard impervious testa and, after thorough washing, germinated in moist, sterile vermiculite.

Methods

Determination of amino acid uptake. All solutions were made up with sterile medium containing 0·1 g $CaCl_2$, 0·1 g KNO_3, 0·01 g Na_2HPO_4 in 500 ml H_2O, adjusted to pH 6·5. Seedlings were bound in bundles (melon, 10 per bundle; *C. tinctoria*, 3 per bundle), and the bundles carefully placed in pointed centrifuge tubes containing 1 ml of sterile medium and 0·1 ml streptomycin sulphate (1%, w/v), so that the distal 1 cm of radicle was immersed. The material was allowed to recover from the handling involved in transfer to tubes for at least 6 hr before experiments commenced. The streptomycin solution was then removed by suction and medium (1 ml) containing 0·05 μmol labelled amino acid ([^{3}H]-phenylalanine, tyrosine or alanine, 1 μCi), 2·5 μmol of each inhibitor and 0·1 mg streptomycin sulphate was introduced by hypodermic syringe. In the kinetic experiments, the concentration of the labelled amino acid was varied. After incubation for 15–30 min at room temp the seedling bundles were rapidly withdrawn from the tubes and the distal 1·5 cm of the radicles cut into a sintered glass funnel, where they were washed 6 times with H_2O at 0°. The washed root tips were transferred to scintillation vials contining 0·5 ml protosol tissue solubilizer (New England Nuclear). After 3 hr at room temp the material was transparent and membrane breakdown was considered complete; further treatment with the solubilizer did not increase measurable radioactivity. Radioactivity from the solubilized root tips was measured in a Packard Tri-Carb liquid scintillation counter, using fluid containing 75% (v/v) toluene, 25% (v/v) methanol and 0·6% (w/v) Butyl-PBD (Ciba). Although there was some variation in absolute uptake of amino acid between experimental runs, the percentage inhibition produced by particular analogues was a highly reproducible quantity. The results obtained with the above method were much more consistent than when similar measurements were made with excised root tips.

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