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The amino acid transport systems of the autotrophically grown green alga *Chlorella*

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The kinetic analysis of L-amino acid uptake by the green alga *Chlorella* revealed at least seven different uptake systems to be present in cells grown autotrophically with nitrate as nitrogen source. There is a 'general system' which transports most neutral and acidic amino acids, a system for short-chain neutral amino acids including proline, a system for basic amino acids including histidine, a special system for acidic amino acids, and specific systems for methionine, glutamine and threonine. The 'general system' is possibly the same as that which can be stimulated by incubation of cells in glucose plus ammonium (Sauer, N. (1984) Planta 161, 425–431). The incubation of *Chlorella* in glucose induces the increased synthesis of six amino acid uptake systems, namely the above-mentioned system for short-chain neutral amino acids, a threonine system, a methionine system, and a glutamine system. These results indicate that the uptake of L-amino acids by the green alga *Chlorella* is as complex as in other free-living organisms such as bacteria or yeast. The small number of amino acid uptake systems found in cells of higher plants, i.e. two or three, seems therefore to be a consequence of integration of the cells in a tissue supplying a relatively constant environment, and not a consequence of autotrophic growth on mineral carbon and mineral nitrogen.

Introduction

Organisms which depend on heterotrophic growth usually possess various transport systems for amino acids with narrow specificity For instance, *Escherichia coli* produces, depending on substrate availability, more than 15 different amino acid transport systems, many of which are very specific and work just for one or a few amino acids [1], in addition to a more general system, which accepts many different amino acids Therefore, each amino acid is taken up by *E coli* by at least two different uptake systems with different kinetic parameters and different regulation properties [1] Nearly the same situation exists in the yeast *Saccharomyces cerevisiae,* where about 10 systems could be identified kinetically and genetically (reviewed in Ref 2) Higher organisms have less diversity and specificity in amino acid transport, for instance, animal cells have four or five systems with rather broad specificity [3], and higher plants have three or perhaps even only two systems, which cover the whole range of protein amino acids [4–7] The question is whether the lack of diversity and the broad specificity of amino acid transport systems in higher plants is a consequence of the autotrophic growth conditions or a consequence of the integration of the cells in a complex tissue system with homeostatic control of the cells' environment Therefore *Chlorella,* a unicellular green alga, was used as a model for an autotrophic plant, which as a unicellular organism is subject to drastic environmental changes In addition the

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green algae are regarded as ancestors of the higher plants

It had been found previously that *Chlorella* can induce two rather specific amino acid transport systems during incubation with glucose, one specific for short-chain neutral amino acids and one for basic amino acids [8] The uptake of the other amino acids was not affected, which could indicate that most amino acids permeate either by passive diffusion or by an unspecific 'general system' which is not regulated by glucose An analysis of amino acid uptake systems of autotrophically grown *Chlorella* cells was undertaken to find out whether there is passive amino acid uptake or a 'general amino acid permease' or a great number of specific uptake systems

Material and Methods

Growth of cells

The strain of *Chlorella vulgaris* was the same as that used previously [8] The cells were grown in mineral salt medium, aerated with CO_2-enriched air and illuminated Usually 3 days after incubation the cells were harvested by low-speed centrifugation and suspended in 25 mM sodium phosphate, pH 6, at a cell density of 25 μl packed cells/ml

Uptake experiments

The cells were usually incubated in 25 mM sodium phosphate, pH 6 at 27°C The cell density varied from 0 1 to 35 μl packed cells/ml Radioactively labelled amino acid (Radiochemical Center, Amersham) was added at the start of the experiment at concentrations of 1–100 μM for K_m determination and of 50 μM to 1 mM for inhibition experiments The total test volume was 3 ml Samples of 0 5 ml were withdrawn at 30-s intervals, rapidly filtered on membrane filter, 0 8 μm pore size (Schleicher and Schull, Dassel), and washed with 10 ml ice-cold 25 mM sodium phosphate, pH 6 The filter with the cells was transferred to a scintillation vial and counted in dioxane-naphthalene-PPO cocktail

In inhibition and competition studies the competing unlabelled amino acid was added to the cells a few seconds before the labelled amino acid

Glucose pretreatment

For a few experiments cells were needed which had been pretreated with glucose to show induction of transport systems In these cases the cells were incubated for 3 h in 7 5 mM glucose and 25 mM sodium phosphate, pH 6 After 3 h all glucose had been taken up by the cells The cells were then used for uptake experiments as described above

Calculation of inhibition

Inhibition of uptake of labelled amino acid in the presence of another, unlabelled, amino acid was determined for all protein L-amino acids to obtain information about the amino acids which are transported by the same uptake system Since for all amino acids K_m and V_{max} values had been determined it was possible to calculate the degree of inhibition by another amino acid with the assumption that inhibition is competitive The calculated value was then compared with the actually measured value and similarity (within 10%) was taken as evidence that both amino acids share the same system The calculation was performed with the Michaelis-Menten equation where $v = V_m S/(K_m + S)$ for control conditions and $v' = V_m S/[K_m(1 + (J/K)) + S]$ in presence of the competing amino acid J

If the K_m value of the unlabelled amino acid was taken for K_i, the calculated $v' \times 100$ (i e percentage of activity of the control) was compared with the measured $v' \times 100$ Different phases of uptake were evaluated by extrapolation of the lines of Lineweaver-Burk plots [9]

Results

K_m and V_{max} values

The concentration-dependence of uptake of all protein amino acids by autotrophically grown *Chlorella* was followed For some amino acids such as isoleucine, valine, tyrosine, phenylalanine, arginine, lysine, proline and alanine a one-phasic kinetic shows up For other amino acids a dual uptake pattern is observed, e g for histidine (Fig 1), with a high-affinity phase (K_m 5–10 μM) and low-affinity phase (K_m 100–300 μM) All amino acids (perhaps with the exception of tryptophan) are taken up by at least one uptake system and not by diffusion, and furthermore it appears that there are several distinct uptake systems (Table I)

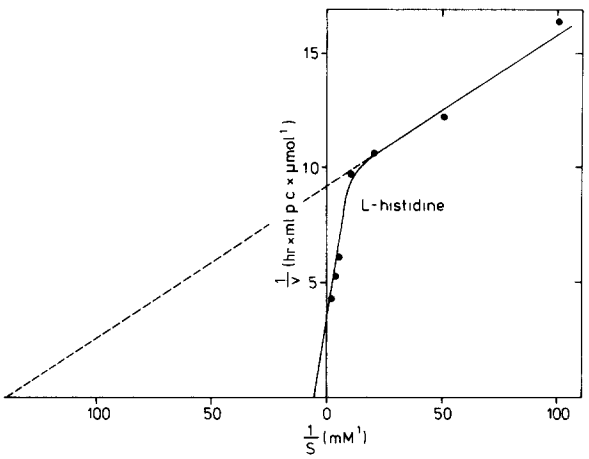

Fig 1 Lineweaver-Burk plot of L-histidine uptake by un-induced cells The cells (20 μl packed cells/ml) were incubated in 25 mM sodium phosphate, pH 6 and 0 01–0 5 mM L-histidine

TABLE I

K_m AND V_{max} VALUES FOR UPTAKE OF THE PROTEIN AMINO ACIDS

p c , packed cells

L-Amino acid	System 1		System 2	
	K_m (μM)	V_{max} (μmol/h per ml p c)	K_m (μM)	V_{max} (μmol/h per ml p c)
Alanine	40	0 55	–	–
Arginine	2	2 32	–	–
Aspartic acid	15	0 18	250	0 50
Asparagine	24	0 14	150	0 15
Cysteine	115	0 15	–	–
Glutamic acid	10	0 15	170	0 37
Glutamine	45	0 25	–	–
Glycine	10	0 17	95	0 37
Histidine	7	0 11	190	0 19
Isoleucine	13	0 17	–	–
Leucine	80	0 22	–	–
Lysine	6	0 55	–	–
Methionine	14	0 048	91	0 062
Phenylalanine	28	0 02	–	–
Proline	40	0 71	–	–
Serine	24	0 24	300	0 53
Threonine	7	0 064	77	0 14
Tryptophan	–	–	> 300	~1
Tryosine	15	0 22	–	–
Valine	13	0 18	–	–

Inhibition of amino acid uptake by alanine and proline

Alanine is one of the amino acids which seems to be taken up by only one system and therefore if amino acid uptake is tested in the presence of alanine the uptake of all those amino acids which share the same transport system as alanine should be inhibited in a predicted manner according to the individual K_m and V_{max} values (see Material and Methods)

If, for instance, system 1 of glutamine is identical with the alanine system the uptake rate over system 1 measured at 50 μM glutamine should decrease from 0 13 to 0 01 μmol/h per ml packed cells, whereas the rate over system 2 should stay constant at 0 05 μmol/h per ml packed cell (Table II) The overall uptake rate would therefore decrease from 0 18 to 0 06 μmol/h per ml packed cells, i e down to 34% If, however, system 2 of glutamine is identical with the alanine system then glutamine uptake over system 1 should stay constant and that over system 2 should decrease from 0 05 to 0 0027, and the overall rate from 0 18 to 0 133, i e to 75% (Table II) The experimental result gives 24% activity in the presence of 1 mM alanine, which is evidence that system 1 of glutamine is identical with the system for alanine uptake With that type of experiment all 'systems' which are identical with alanine uptake could be distinguished (Table II) In some cases it was difficult to decide because the predicted inhibition value was too similar for system 1 and system 2, e g for glutamic acid In these cases, in addition, the amount of inhibition by 1 mM alanine measured at 10 μM labelled amino acid was tested From all these studies it became apparent that arginine and lysine transport does not belong to the 'alanine system', whereas all other amino acids are transported by the 'alanine system' However, several amino acids also have another system which is not affected by alanine Curiously, there are exceptions, such as glycine and serine, where alanine inhibits both systems, because the inhibition is clearly stronger than was expected For example, for glycine an inhibition of 13% had been measured, a result which is only expected if both systems are competed for by alanine (Table II)

Since glucose pretreatment of cells induces an alanine-glycine-serine-proline system [8] it seemed

TABLE II

UPTAKE OF AMINO ACIDS IN ABSENCE AND PRESENCE OF L-ALANINE

V_1 is the calculated rate for labelled amino acid uptake at 50 μM and 10 μM via system 1, V_2 via system 2 (all in μmol/h per ml packed cells) The calculation was performed by use of the measured K_m and V_{max} values (Table I) The rates in the presence of 1 mM L-alanine were computed with the assumption that L-alanine (K_m 40 μM) competes for system 1 and system 2 p c, packed cells

L-Amino acid	Calculated uptake rate via system 1 or 2 (μmol/h per ml p c)			Calculated uptake rate of system 1 or 2 if alanine is competing with		Total ($V_1 + V_2$) if L-alanine competes				Experimental result activity (%)	Composition of L-alanine with system
	V_1	V_2	total ($V_1 + V_2$)	system 1 (V_1)	system 2 (V_2)	with system 1	%	with system 2	%		
50 μM											
Arginine	2 23	–	2 23	1 14	–	1 14	50	–	–	85	none
Aspartic acid	0 14	0 083	0 22	0 021	0 0038	0 104	46	0 144	65	63	2
Asparagine	0 091	0 061	0 15	0 010	0 048	0 071	47	0 096	62	58	1 or 2
Cysteine	0 045	–	0 045	0 0023	–	0 045	51	–	–	49	1
Glutamic acid	0 12	0 084	0 20	0 024	0 0047	0 108	52	0 125	61	57	1 or 2
Glutamine	0 13	–	0 18	0 010	–	0 06	34	–	–	24	1
Glycine	0 14	0 13	0 27	0 027	0 0073	0 157	58	0 147	55	13	1 and 2
Histidine	0 096	0 040	0 136	0 023	0 002	0 063	48	0 098	72	73	2
Isoleucine	0 14	–	0 14	0 022	–	0 022	17	–	–	31	1
Leucine	0 084	–	0 084	0 064	–	0 0064	8	–	–	12	1
Lysine	0 49	–	0 49	0 13	–	0 13	31	–	–	80	none
Methionine	0 037	0 022	0 059	0 006	0 0013	0 028	38	0 038	63	41	1
Phenylalanine	0 02	–	0 012	0 0022	–	0 0022	18	–	–	28	1
Proline	0 39	–	0 39	0 032	–	0 032	7	–	–	13	1
Serine	0 16	0 076	0 24	0 018	0 0033	0 094	39	0 163	70	2	1 and 2
Threonine	0 057	0 054	0 11	0 015	0 0033	0 069	63	0 060	55	50	1 or 2
Tryptophan	–	–	–	–	–	–	–	–	–	40	1
Tyrosine	0 17	–	0 17	0 024	–	0 024	17	–	–	20	1
Valine	0 14	–	0 14	0 047	–	0 047	16	–	–	11	1
10 μM											
Asparagine	0 041	0 009	0 05	0 0022	0 0004	0 011	22	0 041	82	19	1
Glutamic acid	0 075	0 021	0 096	0 0055	0 0008	0 027	28	0 076	79	25	1
Threonine	0 038	0 016	0 054	0 003	0 0007	0 019	35	0 039	72	41	1

TABLE III

EFFECT OF L-PROLINE (1 mM) ON UPTAKE OF ALANINE SERINE AND GLYCINE

Data were calculated as in Table II

Labelled amino acid	Calculated uptake rate			Calculated rate if L-proline is competing		Total ($V_1 + V_2$) if proline competes				Experimental result uptake rate (%)	Competition of L-proline with system
	via system 1 (V_1)	via system 2 (V_2)	total ($V_1 + V_2$)	V_1	V_2	for system 1	%	for system 2	%		
L-Alanine	not known		0 306	0 0252	–	0 0252	8 2	–	–	76	2 [a]
Glycine	0 142	0 128	0 270	0 0274	0 00734	0 1554	58	0 149	55	53	1 [b]
L-Serine	0 162	0 0757	0 238	0 0178	0 00338	0 0935	39	0 165	70	70	2

[a] Since the two uptake systems are not indicated by different K_m values, the assignation as system 2 is arbitrary

[b] Though the degree of inhibition is too similar to allow a decision on the system group the measured K_m values (Table I and Ref 8) indicate that glycine system 2 must be identical with the proline system

likely that a basic level of that system already exists in cells without glucose-pretreatment This alanine-transporting system could work in addition to the 'general system' which accepts alanine and most of the other amino acids but not proline, as determined in Table II In that case proline should inhibit alanine, serine and glycine uptake, but only that fraction which is transported via the inducible system From the results in Table III and previous measurements of K_m values [10] it appears that system 2 of glycine and serine and a quarter of the apparently single alanine system are inhibited by 1 mM proline Proline has no inhibitory effect on the uptake of any other amino acid, and proline uptake is also not affected by any other amino acids except alanine, glycine and serine (data not shown) This result implies that alanine uptake is mediated by two systems, which are apparently so close in K_m that they do not separate in the kinetics of concentration-dependence measurements, but they can clearly be separated by sensitivity to proline

In conclusion, there is a 'general system' and 'alanine-glycine-serine-proline system' in autotrophically grown *Chlorella* cells The general system has much resemblance with respect to specificity to the system which is stimulated by incubation of *Chlorella* cells with glucose plus ammonium [11] The system for alanine-glycine-serine-proline seems identical with the glucose-induced system for short-chain neutral amino acids [8] The question arises as to whether the transport systems which could not be grouped with the 'general system' are single specific systems or whether they together belong to another group

Transport of basic amino acids

Since the basic amino acids are taken up in many organisms by a special 'basic system' it was tempting to expect the same feature in *Chlorella* for uptake of arginine, lysine and possibly histidine Reciprocal inhibition studies indeed revealed that the uptakes of arginine, lysine and histidine belong to the same system The degree of inhibition is exactly the same as predicted by the respective K_m values (Table IV) Thus there is an arginine-lysine-histidine system, and L-histidine can be transported by the 'basic system' and by the general system The presence of L-alanine nearly eliminates the low-affinity part of histidine uptake (Fig 2A), whereas the presence of L-arginine mostly affects the high-affinity part (Fig 2B)

Transport of histidine by a 'neutral system' and a 'basic system' has also been found in Ehrlich ascites cells [12]

Transport of acidic amino acids

Often the acidic amino acids are transported by one special system and it was expected that those uptake phases which did not belong to the 'general system' (L-glutamic acid uptake with K_m 170 μM and L-aspartic acid uptake with K_m 15 μM) belonged to an acidic amino acid uptake system

TABLE IV

RECIPROCAL INHIBITION OF L-ARGININE, L-LYSINE AND L-HISTIDINE

The concentration of labelled amino acid was 50 μM, that of unlabelled amino acid also 50 μM The calculation for L-histidine uptake was done under the assumption that only system 1 of histidine uptake (K_m 7 μM) is affected by arginine or lysine The calculations were performed as described in Methods and Table II

Labelled amino acid	Unlabelled amino acid	Calculated activity (%)	Measured activity (%)
L-Arginine	L-lysine	76	72
	L-histidine	78	78
L-Lysine	L-arginine	30	39
	L-histidine	57	56
L-Histidine	L-arginine	24	20
	L-lysine	49	42

Since the acidic amino acids were also taken up by the general system it was important to suppress firstly the general system by the presence of 1 mM unlabelled L-alanine Then 1 mM unlabelled L-asparagine was added, since preliminary experiments had shown that asparagine strongly inhibits acidic amino acid transport The results (Table V) show that uptake of L-aspartic acid and L-glutamic acid is strongly inhibited by L-asparagine, whereas uptake of L-glutamine, L-threonine, etc , is not Thus there is a special uptake system for acidic amino acids which also transports asparagine, but not glutamine The affinity of this uptake system is strongest for aspartic acid, glutamic acid and

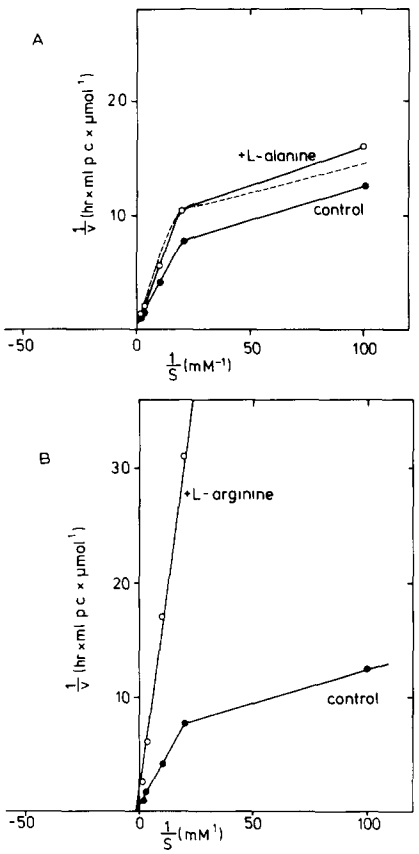

Fig 2 Lineweaver-Burk plots of L-histidine uptake by un-induced cells in the presence of L-alanine (A) or L-arginine (B) 20 μl cells (packed volume) were incubated in 25 mM sodium phosphate pH 6, with 0 01–0 5 mM L-histidine in the absence or presence of 1 mM L-alanine (A) or L-arginine (B) The broken line indicates the theoretically expected curve if L-alanine competes with the low-affinity system of L-histidine in this particular experiment

TABLE V

INHIBITION OF AMINO ACID UPTAKE BY L-ASPARAGINE

The labelled amino acids were added at 50 μM, the unlabelled L-alanine and L-asparagine at 1 mM each The calculation was performed as described in Methods and Table II The numbers in the first row (+L-alanine) indicate the inhibition by competition via the 'general system', the numbers in the second row indicate the expected activity if the other uptake system for aspartic acid glutamic acid, etc , is sensitive to competition with asparagine

Labelled amino acid	Calculated activity (%)		Measured activity (%) in the presence of 1 mM L-alanine +1 mM L-asparagine
	+1 mM L-alanine	+1 mM L-alanine +1 mM L-asparagine	
L-Aspartic acid	65	24	24
L-Glutamic acid	61	13	19
L-Methionine	38	2	39
L-Threonine	55	9	48

TABLE VI

RECIPROCAL INHIBITION OF L-THREONINE AND L-METHIONINE UPTAKE

The concentration of the labelled amino acid was 50 μM the concentration of the unlabelled amino acid 1 mM or 50 μM The calculation was performed as described in Methods and Table II

Labelled amino acid	Unlabelled amino acid	Calculated activity (%) if competition via		Measured activity (%)
		the general system	via both systems	
L-Threonine	L-methionine	55	12	24
L-Methionine	L-threonine	38	6	37

asparagine are taken up at a more than 10-fold higher K_m Since glutamic acid and asparagine are transported by the general transport system with much better affinity, this so-called acidic system seems mainly devised for aspartic acid transport

Transport of L-threonine and L-methionine

There are only a few uptake systems, such as the low-affinity systems for L-methionine and L-threonine, which remain unassigned to one of the so-far identified systems It was determined whether 1 mM of one unlabelled amino acid predictably inhibits the uptake of the other labelled amino acid From the results (Table VI) it appears that methionine (K_m 91 μM) and threonine (K_m 77 μM) probably do not share the same system, since the inhibition is not convincingly strong

TABLE VII

AMINO ACID TRANSPORT SYSTEMS OF *CHLORELLA*

	Systems (properties)	Amino acid specificity (with K_m)
System I	general system (probably the same as that stimulated by treatment with glucose plus ammonium [11])	alanine (40 μM), aspartic acid (250 μM) asparagine (24 μM) cysteine (114 μM) glutamic acid (10 μM), glutamine (45 μM) glycine (10 μM), histidine (190 μM), isoleucine (13 μM), leucine (80 μM), methionine (14 μM) phenylalanine (28 μM) serine (24 μM) threonine (87 μM), tryptophan (300 μM) tyrosine (15 μM) valine (13 μM)
System II	short-chain neutral system (strongly induced by glucose or nitrogen starvation [8 13])	alanine (40 μM), glycine (95 μM), serine (300 μM), proline (40 μM)
System III	basic system (induced by glucose or ammonium [8,13])	arginine (2 μM) histidine (7 μM), lysine (6 μM)
System IV	acidic system	aspartic acid (15 μM) glutamic acid (170 μM) asparagine (150 μM)
System V	methionine system (perhaps the same as that stimulated by glucose [8])	methionine (91 μM)
System VI	threonine system (perhaps the same as that stimulated by glucose [8])	threonine (77 μM)
System VII	glutamine system (stimulated by glucose [8])	glutamine (600 μM)

Transport of L-histidine, L-methionine, L-threonine and L-glutamine by glucose-induced cells

Since un-induced cells exhibit a complex series of transport systems which are somewhat difficult to separate by kinetic means, the particular regulation by induction (or derepression) can aid in identification of uptake systems It was found previously that induction with glucose [8] or nitrogen starvation [13] increases the activity of a 'proline system' and also leads to an increased uptake of histidine, threonine, methionine and glutamine, though the effect is not so drastic as for uptake of alanine or arginine It was speculated that histidine threonine methionine and glutamine were either poor substrates for the induced systems or their uptake was catalyzed by a system which accepts histidine, threonine, methionine and glutamine Therefore it was determined whether these amino acids were inhibitory in reciprocal-uptake experiments From the data it appears that L-threonine, L-methionine and L-glutamine are all taken up by specific, separate transport systems Comparison of the K_m values of the glucose-stimulated uptake systems for threonine and methionine with the respective systems in un-induced cells might indicate that these system are the same, whereas the system for glutamine seems special Uptake of histidine is strongly inhibited by arginine, in contrast to a previous report [14], and is therefore most probably transported via the basic system

Discussion

The uptake of amino acids by *Chlorella* is surprisingly complex and diverse There are at least seven distinct uptake systems (Table VII) The method of kinetic extraction of systems from double reciprocal plots can only separate those systems which are clearly different in K_m and similar in V_{max} [15], and the detection of transport systems is therefore limited and some systems might have been overlooked The rapid production of metabolic products could cause transinhibition, which could simulate inhibition due to competition Therefore the figure of seven transport systems is a lower estimate Six of the uptake systems are regulated and become dominant after incubation in glucose or glucose plus ammonium The intracellular signals for the regulation of amino acid uptake by *Chlorella* are not known, but it seems certain that there is induction of specific transport systems and not of the energy-providing machinery, e g the H^+-ATPase, since then a stimulation of all uptake systems would have been expected The presence of great number of specific transport systems and a general transport system shows great similarity to the situation in yeast and bacteria Thus also a photosynthetically, active autotrophically growing organism has many specific transport systems for organic compounds During evolution to higher plants the majority of systems have obviously disappeared and only the general system, the acidic system and the basic system have survived Obviously the presence of many specific systems is especially useful for free-living cells, which have to adapt to many and drastic changes of environment Cells of higher plants, which are embedded in a tissue, might have a much more constant nutritional situation so that a few uptake systems seem sufficient The V_{max} values of the different uptake systems in un-induced *Chlorella* appear low when compared with the rates of the inducible transport systems for hexoses, alanine or arginine, e g 0 1–0 5 μmol/h per ml packed cells, versus 50–200 μmol/h per ml packed cells However, even these low uptake activities are well in the range of those usually measured for cells of higher plants [5,16]

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