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Transport of Arginine and Aspartic Acid into Isolated Barley Mesophyll Vacuoles[1]

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ABSTRACT

The transport of arginine into isolated barley (*Hordeum vulgare* L.) mesophyll vacuoles was investigated. In the absence of ATP, arginine uptake was saturable with a K_m of 0.3 to 0.4 millimolar. Positively charged amino acids inhibited arginine uptake, lysine being most potent with a K_i of 1.2 millimolar. In the presence of free ATP, but not of its Mg-complex, uptake of arginine was drastically enhanced and a linear function of its concentration up to 16 millimolar. The nonhydrolyzable adenylyl imidodiphosphate, but no other nucleotide tested, could substitute for ATP. Therefore, it is suggested that this process does not require energy and does not involve the tonoplast ATPase. The ATP-dependent arginine uptake was strongly inhibited by *p*-chloromercuriphenylsulfonic acid. Furthermore, hydrophobic amino acids were inhibitory (I_{50} phenylalanine 1 millimolar). Similar characteristics were observed for the uptake of aspartic acid. However, rates of ATP-stimulated aspartic acid transport were 10-fold lower as compared to arginine transport. Uptake of aspartate in the absence of ATP was negligible.

In leaves, products of carbon and nitrogen assimilation, such as sucrose, malate, and amino acids, are rapidly transferred into the central vacuole during the light period (13). Amino acids were found to be in different cellular compartments, such as chloroplasts, the cytosol, and the vacuole (17). Exchange of amino acids between these compartments is a factor contributing to the regulation of nitrogen metabolism. Although the concentration of total amino acids in cytoplasmic compartments exceeds their vacuolar concentration, a considerable portion of the total cellular amino acid content may be localized in the vacuole, as a consequence of the large volume this organelle occupies in mesophyll cells. Some of the transport systems involved in the translocation of amino acids across the tonoplast have recently been investigated. Uptake of phenylalanine into the vacuole is inhibited by other aromatic amino acids and is driven by tonoplast-bound proton pumps (10, 11, 19, 22). The rate of the uptake of alanine and glutamine into barley mesophyll vacuoles was also found to be increased in the presence of ATP (5). However, in this case, ATP did not appear to act as an energy source but rather as an allosteric effector. Hydrophobic amino acids such as phenylalanine and leucine inhibited the ATP-dependent uptake of alanine and glutamine. Similar observations were made for the efflux of various amino acids from the vacuolar space into the surrounding medium (8). Exogenous ATP increases the release of amino acids from the vacuole, whereas hydrophobic amino acids inhibit this ATP-dependent effect.

Uptake of arginine and other amino acids with positively charged side chains by the vacuole has so far only been studied in fungi that frequently accumulate arginine at high concentrations as a nitrogen source (3, 21, 24). Paek and Weiss (18) identified a 40-kD protein involved in the transport of arginine across the tonoplast of *Neurospora crassa*. Analysis of the kinetic data reveals that the arginine carriers of yeast and *Neurospora* have quite different properties (3, 21, 24).

In the present study, we have investigated the uptake of arginine and aspartic acid into isolated barley mesophyll vacuoles.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Barley (*Hordeum vulgare* L. cv Gerbel) was grown in a growth cabinet with a 12 h photoperiod (45 μmol $\times$ m^{-2} s^{-1}) at day/night temperatures of 22/18°C. Relative humidity was 75%.

Isolation of Intact Vacuoles

Primary leaves of 8-d-old barley plants were harvested at the beginning of the light period. Mesophyll protoplasts were prepared according to the procedure of Kaiser *et al.* (13) as modified by Rentsch and Martinoia (20). In the latter reference, the modified methods for the isolation and purification of mesophyll vacuoles (15) are also described.

Uptake Experiments

Uptake of [^{14}C]arginine was measured as described by Martinoia *et al.* (14, 16), with a slight modification; for each condition and time point, the following components were added to five polyethylene microcentrifuge tubes (400 μL volume capacity): 70 μL of medium containing 400 mM sorbitol, 30 mM K-gluconate, 20 mM Hepes-KOH (pH 7.2), 2 mM KCl, 1 mM DTT, 29% Percoll, 0.1% BSA, 3.7 kBq [^{14}C]arginine, 3.7 kBq ^3H_2O, and solutes as indicated in the

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figures and tables. Uptake experiments were started by the addition of 30 μL vacuole suspension. The samples were rapidly overlayered with 200 μL silicone oil AR 200 (Fluka, Buchs, Switzerland) and 60 μL water. The incubation was terminated by centrifugation at 10,000 *g* for 15 s. ^3H_2O equilibrates rapidly between the medium and the vacuolar space and was used to quantify the number of vacuoles (10^7 vacuoles correspond to 160 μL). Where not stated otherwise, uptake was calculated from the difference in the radioactivity detected in the vacuoles at two time points (2 and 20 min).

Radiochemicals were purchased from Amersham, UK.

RESULTS

Kinetics of Uptake into Isolated Vacuoles

Uptake of [^{14}C]arginine into barley vacuoles was strongly increased in the presence of 5 mM ATP (Fig. 1). The uptake rates in the absence of ATP ranged from 0.32 to 0.87 nmol 10^{-7} vacuoles min^{-1}, whereas rates of 3 to 5 nmol 10^{-7} vacuoles min^{-1} were found in the presence of 5 mM ATP. Uptake in the absence of ATP was linear for at least 25 min. Interestingly, a lag period of a few minutes was observed for arginine uptake in the presence of ATP. Such a lag period had not been observed in the case of the ATP-stimulated uptake of alanine and glutamine (5). Once a steady-state rate was reached, uptake was linear for 15 to 18 min. In contrast to the uptake of alanine and glutamine, which was stimulated both by ATP and MgATP, arginine uptake was stimulated only by free ATP; MgATP, the substrate of the tonoplast ATPase, had no effect (data not shown).

Effect of Other Nucleotides on Arginine Uptake

Arginine uptake as a function of ATP concentration (Fig. 2A) exhibited a sigmoid saturation curve and thus differs from the neutral amino acid uptake system for which simple saturation kinetics were observed (5). The effect of ATP at low concentrations was negligible. A stimulation of uptake was only observed at ATP concentrations above 2 mM. Maximal stimulation is reached at ATP concentrations far in excess of those likely to be present in living cells. As mentioned above, Mg^{2+} was found to inhibit the ATP-stimulated arginine uptake. In consequence, rates of arginine uptake in the presence of 5 mM ATP decrease with increasing Mg^{2+} concentrations (Fig. 2B).

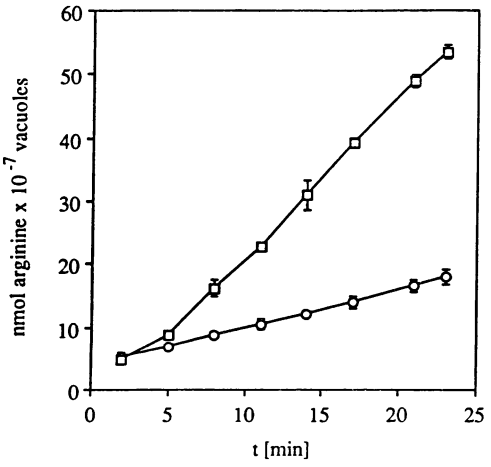

Figure 1. Time-dependent uptake of [^{14}C]arginine (0.4 mM) by isolated barley mesophyll vacuoles. Vacuoles were incubated in the absence (○) or presence (□) of 5 mM ATP. Means of five determinations. Bars indicate ± SD For experimental details, see "Materials and Methods."

The stimulation of arginine uptake by ATP is specific for this nucleotide (Table I) as was previously shown for the transport of neutral amino acids (5). Other nucleotides, such as ADP, UTP, and GTP, were not able to stimulate arginine uptake. Only the nonhydrolyzable ATP analog, AMPPNP,[2] showed a stimulatory effect. At equimolar concentrations, AMPPNP was less active than ATP; at 10 mM it was as active as ATP at 5 mM. Addition of 25 mM KNO_3, an inhibitor of the tonoplast ATPase (22), had no effect on the ATP-dependent arginine uptake (not shown), which also indicates that ATP does not act as an energy source for the translocation of arginine.

Within cells, ATP is mainly present as the MgATP complex and most of the known ATP-dependent processes are in fact MgATP-dependent. Some potassium channels in animal cells are known to be directly modulated by free ATP (4, 9). An inside slightly positive membrane potential can be observed in isolated vacuoles (2; G. Kaiser, E. Martinoia and Oberleitner, unpublished observation). In the presence of MgATP, this membrane potential shifts to more positive values (19, 22). It was tempting, therefore, to speculate that inhibition of the ATP-dependent arginine uptake by Mg^{2+} was due to the generation of a positive membrane potential and, therefore, was voltage-dependent. Indeed, a consistent stimulation of arginine uptake could be observed in the presence of valinomycin and nigericin (Table II). However, MgAMPPNP, which cannot be hydrolised and therefore utilized by the tonoplast ATPase to generate an inside positive membrane potential, shows no stimulatory effect on arginine uptake. When 10 mM ATP was present in the assay mixture together with 5 mM Mg^{2+}, thereby generating an inside positive membrane potential, arginine uptake rates corresponded to the rates in the presence of 5 mM ATP only (data not shown). This result indicates that, in isolated vacuoles, stimulation of arginine uptake is not a voltage-dependent process. The same conclusion can be drawn from an experiment in which the membrane-permeable anion isothiocyanate, which abolishes positive membrane potentials, was present in the assay. Isothiocyanate slightly inhibited the ATP-stimulated arginine uptake (Table II). In the presence of magnesium, isothiocyanate reduced the rate of arginine uptake below the control value. This result further indicates that the ATP-independent arginine transport system is either slightly inhibited by the chaotropic anion isothiocyanate or, alternatively, that the negative membrane potential, possibly generated in the presence of this permeable anion, inhibits the uptake of the positively charged arginine. The stimulation in the presence of the two ionophores, therefore, may be due to an unspecific increase in the permeability for arginine.

[2] Abbreviation: AMPPNP, adenylyl imidodiphosphate.

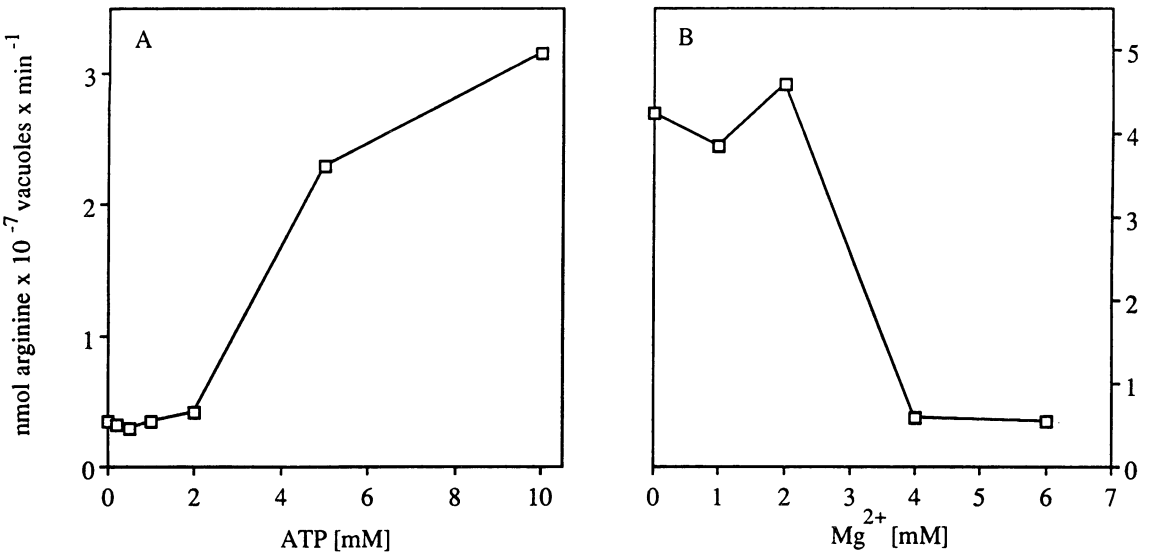

Figure 2. Uptake of [^{14}C]arginine (0.4 mM) as affected by increasing concentrations of ATP (A) or Mg^{2+} (B). In the latter experiment, the ATP concentration was 5 mM.

Concentration-Dependent Arginine Uptake

In the absence of ATP, arginine uptake into isolated barley vacuoles was saturable (Fig. 3), indicating the involvement of a carrier in the translocation of this amino acid across the tonoplast. It should be pointed out that uptake of the neutral amino acids, alanine and glutamine, was not saturable in the absence of ATP (5). The K_m for arginine, as determined in separate experiments, ranged from 0.3 to 0.4 mM. In the presence of ATP, no saturation of uptake was observed in the range of arginine concentrations employed (Fig. 4). The rate of uptake was rather an almost linear function of the external arginine concentration. Therefore, the relative stimulation by ATP of arginine transport at low concentrations of the amino acid was lower than at high concentrations (20-fold increase at 3.2 mM *versus* fivefold increase at 0.4 mM). The low rates of the ATP-independent arginine uptake could be due to protoplasts contaminating the vacuolar preparation. Comparison of the rates of uptake of arginine, either by intact protoplasts or by isolated vacuoles, however, gave similar values. Because our vacuolar preparation is contaminated at most with 3 to 4% protoplasts (15), this result shows that the saturable uptake system operating in the absence of ATP resides in the vacuoles.

Inhibition of Arginine Uptake by Other Amino Acids and their Analogs

The specificity of arginine uptake was tested by employing other amino acids and their analogs as putative inhibitors of arginine uptake. Because of the substantial differences in

Table I. *Effect of Nucleotides on [^{14}C]Arginine Uptake into Barley Mesophyll Vacuoles*

[^{14}C]Arginine (0.4 mM) was incubated in the presence of different nucleotides and pyrophosphate. Li_4 AMPPNP was converted to the potassium salt using Dowex 50 W. Vacuoles were incubated for 2 and 20 min, respectively, and uptake was calculated from the difference in the radioactivity detected in the vacuoles at the two time points. For details, see "Materials and Methods."

Effector	nmol Arginine / 10^7 vacuoles min^{-1}
Control (no addition)	0.32 ± 0.02[a]
5 mM ATP	4.36 ± 0.05
5 mM ADP	0.33 ± 0.04
5 mM UTP	0.29 ± 0.04
5 mM GTP	0.32 ± 0.07
5 mM PPi	0.38 ± 0.05
5 mM AMPPNP	1.03 ± 0.03
10 mM AMPPNP	4.50 ± 0.31

[a] Means ± SD of two experiments each with five replicates.

Table II. *Effect of Agents Affecting the Membrane Potential on the ATP-Stimulated [^{14}C]Arginine Uptake into Barley Mesophyll Vacuoles*

Uptake was performed as described in Table I.

Effector	% of Control[a]	*n*
Control	100[b]	
ATP (5 mM)	820 ± 170	12
ATP (5 mM) + Mg^{2+} (5 mM)	112 ± 17	8
ATP (5 mM) + Mg^{2+} (20 mM) + valinomycin (10^{-7} M) + nigericin (10^{-5} M)	217 ± 98	6
ATP (5 mM) + SCN^- (5 mM)	770 ± 50	2
ATP (5 mM) + Mg^{2+} (6 mM) + SCN^- (5 mM)	49 ± 13	2
AMPPNP (10 mM)	1021 ± 240	3
AMPPNP (10 mM) + MgGluconate (12 mM)	85 ± 5	3

[a] Means ± SD of *n* experiments each with five replicates. [b] Corresponds to 0.45 nmol 10^{-7} vacuoles min^{-1}.

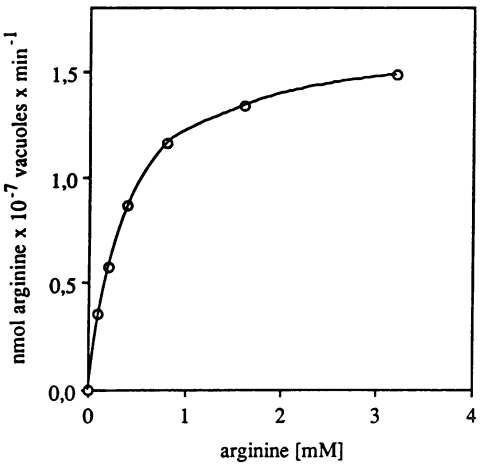

Figure 3. Concentration dependence of [^{14}C]arginine transport into isolated vacuoles in the absence of ATP. Vacuoles were incubated in the presence of 37 kBq mL^{-1} [^{14}C]arginine at the indicated concentrations. For experimental details, see "Materials and Methods."

Table III. *Inhibition of ATP-Independent and ATP-Dependent Arginine Uptake by L-Amino Acids*

The concentration of [^{14}C]arginine was 0.4 mM, concentrations of other amino acids 4 mM. Uptake rates were 0.69 nmol 10^{-7} vacuoles min^{-1} in the absence, and 3.60 nmol 10^{-7} min^{-1} in the presence, of ATP.

Effector	−ATP	+ATP
	% of control	
None (control)	100	100
Arginine	20 ± 1[a]	116 ± 5
Lysine	47 ± 2	77 ± 2
Histidine	103 ± 5	89 ± 9
Ornithine	66 ± 1	n.d.[b]
Alanine	103 ± 9	58 ± 9
Glutamine	99 ± 9	83 ± 5
Glutamic acid	93 ± 16	99 ± 18
Phenylalanine	63 ± 2	12 ± 2
Leucine	63 ± 3	11 ± 1
Methionine	70 ± 9	11 ± 1

[a] Means ± SD of three independent experiments each with five replicates. [b] n.d. = not determined.

arginine uptake in the absence and presence of ATP, both systems were investigated. As evident in Table III, the amino acids differentially inhibit the two systems. In the absence of ATP, uptake of label from arginine was greatly reduced in the presence of unlabeled arginine, as was to be expected from the data in Figure 3, but also in the presence of lysine. As shown in Figure 5, inhibition by lysine of arginine uptake into vacuoles, in the absence of ATP, is competitive, with a K_i of 1.2 mM. Ornithine also had an inhibitory effect, whereas histidine, the imidazole ring of which is predominantly uncharged at the pH of the incubation medium, had no effect on arginine uptake. The acidic amino acids, as well as alanine and glutamine, had no effect, whereas the hydrophobic amino acids were slightly inhibitory. In the absence of ATP, D-arginine, L-methylarginine, and tosylarginine methylester were slightly inhibitory (Table IV), suggesting that both the amino group as well as the guanidino moiety are essential for effective translocation of arginine.

In the presence of ATP, unlabeled arginine had no effect on the uptake of label (see also Fig. 4). Arginine uptake was only slightly inhibited by alanine and lysine, whereas the hydrophobic amino acids acted as very strong inhibitors. Similar results were previously obtained for the ATP-dependent uptake of alanine and glutamine (5) and for the release of amino acids from isolated barley vacuoles (8). Of various analogs of arginine tested (Table IV), only tosylarginine methylester inhibited the ATP-dependent uptake, whereas the other analogs had no effect.

To determine the specificity of the inhibition by phenylalanine, D-phenylalanine, phenylethylamine, and phenylpro-

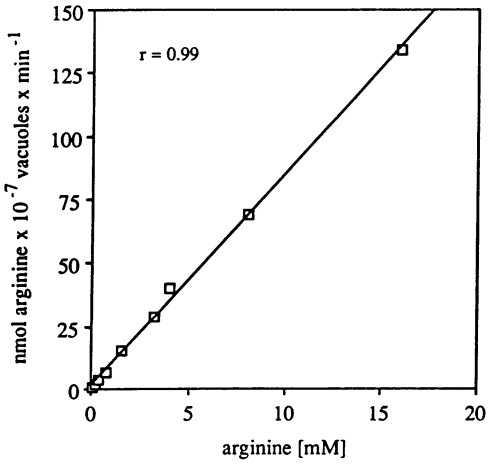

Figure 4. Effect of 5 mM ATP on [^{14}C]arginine uptake by isolated vacuoles at varying concentrations of arginine. Conditions were as in Figure 3. Note the different scales of both abscissa and ordinate with respect to Figure 3.

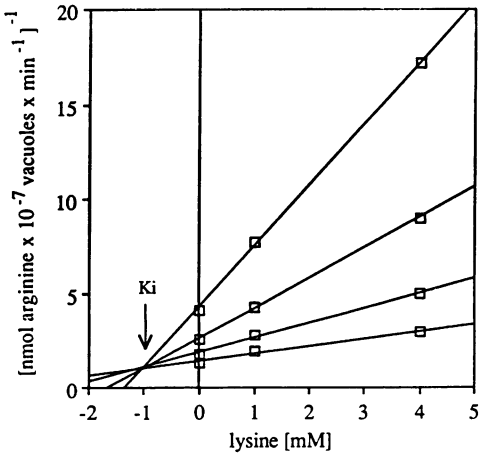

Figure 5. Dixon plot of the inhibition of arginine uptake by lysine. Arginine concentrations were 0.1, 0.2, 0.4, and 0.8 mM; lysine was added at concentrations of 0, 1, or 4 mM. The reciprocal uptake rate is plotted as a function of the inhibitor concentration.

Table IV. *Inhibition of [^{14}C]Arginine Uptake by Arginine and Phenylalanine Analogs*

The concentration of arginine was 0.4 mM, concentrations of the effectors 4 mM. Means of two to three independent experiments each with five replicates. The SD within one experiment were less than 10%. ± SD is given only where three experiments were conducted; in the other cases, the difference in the values between the two experiments was less than ± 5%. Results are given in percent of the respective control (100%: 0.61 nmol 10^{-7} vacuoles min^{-1} in absence and 3.42 nmol 10^{-7} vacuoles min^{-1} in presence of ATP).

Effector	−ATP	+ATP
	% of control	
None	100	100
L-Arginine	20	104
D-Arginine	53	108
L-Methylarginine	61	108
L-Tosylargininemethyester	66 ± 11	57
L-Canavanine	62 ± 12	55
L-Phenylalanine	63	12
D-Phenylalanine	n.d.[a]	103
Phenylethylamine	n.d.	114
Phenylpropanecarboxylic acid	n.d.	116

[a] n.d. = not determined.

panoic acid were tested as well and found to have no inhibitory effect on arginine uptake (Table IV). This indicates that the amino acid effective in inhibiting the ATP-stimulated arginine uptake must be present in the L-configuration and that, as shown previously (7), both the amino and the carboxylic groups are essential for the interaction with the carrier or channel.

Inhibition of the ATP-stimulated arginine uptake by phenylalanine as a function of phenylalanine concentration is shown in Figure 6. Inhibition is complete at about 4 mM, 0.8 to 1 mM phenylalanine causing 50% inhibition independently from the arginine concentration in the medium.

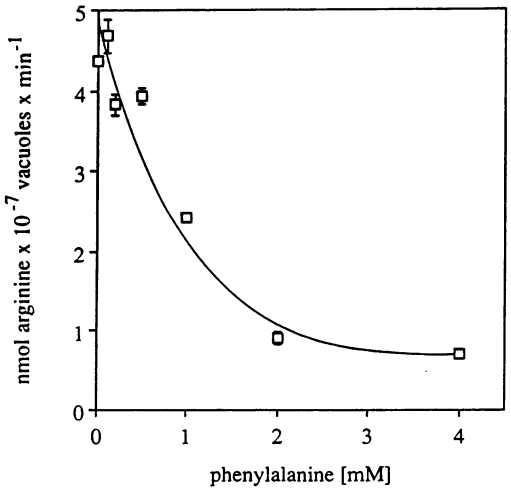

Figure 6. Inhibition of arginine uptake by phenylalanine. Vacuoles were incubated in the presence of [^{14}C]arginine (0.4 mM), 5 mM ATP, and increasing concentrations of phenylalanine.

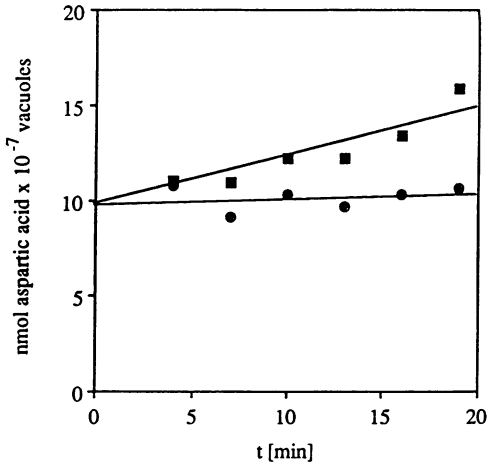

Figure 7. Time-dependent uptake of [^{14}C]aspartic acid (1.0 mM) into isolated barley mesophyll vacuoles in the absence (●) and presence (■) of 5 mM ATP. Aspartic acid contents at zero time reflect aspartic acid of the suspending medium that contaminates the vacuoles after their separation from the medium.

Comparison of ATP-Stimulated Arginine Transport with the Uptake of Other Amino Acids

We included the negatively charged amino acid aspartic acid in our investigations. Only very low rates of uptake of [^{14}C]aspartic acid were observed in the absence of ATP. In the presence of ATP, uptake was clearly demonstrable and was roughly linear over a period of 20 min (Fig. 7). The rate of uptake was a linear function of the aspartate concentration up to 10 mM (Fig. 8). As shown above for arginine, the ATP-dependent uptake is inhibited by Mg^{2+} as well as by the hydrophobic amino acid leucine (Table V).

DISCUSSION

In the absence of Mg^{2+}, ATP does not serve as a substrate for the tonoplast ATPase (12). Therefore, a stimulation of

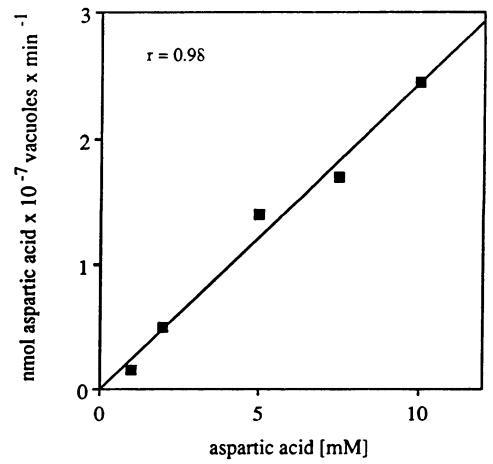

Figure 8. Concentration dependence of [^{14}C]aspartic acid uptake by isolated vacuoles in the presence of 5 mM ATP. Vacuoles were incubated in the presence of 60 kBq mL^{-1} [^{14}C]aspartic acid at the indicated concentrations.

Table V. *Uptake of Aspartic Acid (1 mM) into Barley Mesophyll Vacuoles*

Effect of ATP, Mg^{2+}, and leucine.

Condition	nmol 10^{-7} Vacuoles min^{-1}
Control	0.06 ± 0.06[a]
+ATP (5 mM)	0.26 ± 0.05
+ATP/Mg^{2+} (5 mM/20 mM)	0.11 ± 0.06
+ATP/leucine (5 mM/10 mM)	0.12 ± 0.08

[a] Means ± SD of four experiments each with at least five replicates.

amino acid transport by ATP in the absence of Mg^{2+} indicates that the amino acid transport is not dependent on the proton motive force. In fact, the rates of the ATP-dependent transport of neutral amino acids, such as alanine or glutamine, decreased after the addition of equimolar or excess of Mg^{2+}, whereas the electrogenic transport of anions, such as chloride or malate, is greatly enhanced under these conditions (6, 14, 16). In contrast to the transport of neutral amino acids, transport of charged amino acids must depend on the electrical potential across the membrane. The primary H^+ pumps of the tonoplast, the ATPase, and the pyrophosphatase, create an inside positive membrane potential at the tonoplast (19, 22). This membrane potential can drive anion influx and it inhibits cation uptake. However, in our short term experiments, the initially steep gradient between ^{14}C-labeled amino acids outside and inside is probably not reduced enough to observe an inhibition of arginine uptake. Alternatively, labeled arginine might be exchanged with vacuolar, unlabeled arginine or other amino acids.

The observation that arginine uptake is stimulated by ATP, but not by MgATP, may be explained by a number of different effects of the Mg^{2+} ion: (a) The transporter may be modulated only by free ATP. The MgATP complex would then be unable to stimulate translocation across the tonoplast. This situation appears unlikely because in the cell, ATP is present mainly as the MgATP complex; free ATP concentrations are reported to be only 5 to 10% of the total ATP concentration (23). However, it cannot be excluded that under specific conditions the concentration of free ATP may rise. (b) Apart from forming complexes with ATP, Mg^{2+} is known to form complexes with amino acids. Because the stability constants for the amino acid Mg^{2+} complexes are much lower than that of the Mg^{2+}-ATP complex, an inhibition of the uptake would occur only if Mg^{2+} is present in excess. (c) Alternatively, Mg^{2+} may affect the tonoplast membrane potential. An inside positive membrane potential should preferentially inhibit uptake of cationic amino acids. However, we have shown here (Table II) that the stimulation of arginine uptake is also reduced by Mg^{2+} when the nonhydrolyzable ATP analog AMPPNP is substituted for ATP, indicating that the ATP-dependent uptake is not affected by the membrane potential. (d) Mg^{2+} may affect other properties of isolated vacuoles, or isolated vacuoles behave somewhat differently *in vitro* as compared to the *in vivo* situation due to their totally different environment.

It should be mentioned that, in a preliminary communication, transport of neutral amino acids was reported to be stimulated by ATP as well as by MgATP (5). During the present investigation, in one laboratory (Würzburg), Mg^{2+} and ATP at equimolar concentrations stimulated transport, whereas in the other laboratory (Zürich), equimolar concentrations of Mg^{2+} and ATP had no stimulatory effect and higher Mg^{2+} concentrations were required to inhibit the ATP-dependent amino acid uptake (not shown). We have not yet found the cause for this discrepancy. However, in both cases, participation of the vacuolar H-ATPase was excluded. The fact that the different amino acids behave similarly in our two laboratories strongly suggests that only a single permease with the characteristics shown exists. It appears that the transport of the more hydrophilic amino acids is not saturable in the concentration range tested (20 mM). Hydrophobic amino acids are inhibitory, leading to an apparent saturation of the carrier. Channels are frequently saturated only at very high substrate concentrations; therefore, it is tempting to speculate that the amino acids cross the tonoplast via a channel, which may be regulated by ATP as well as by free hydrophobic amino acids. Arginine is the amino acid that is translocated most rapidly across the tonoplast. Aspartic acid, as well as methionine and leucine (6), are transferred into the vacuole at much lower rates. For aspartic acid, the ATP-stimulated uptake rate is only about one-tenth that of arginine (compare Figs. 1 and 7). Alanine and glutamine exhibit intermediate rates. A direct comparison of the uptake rates of all the different amino acids is not possible here because the experiments of Dietz *et al.* (5) were performed in the presence of 10 mM ATP. The occurrence of only small amounts of aspartic and glutamic acid in the vacuole (5) may be directly related to the property of the translocator.

CONCLUSIONS

From previously published data and from those presented in this contribution, we can conclude that at least three amino acid transport systems exist at the tonoplast. First, there is a system specific for aromatic amino acids (10, 11), which depends on the proton motive force generated by either the vacuolar ATPase or PPase (19, 22). Second, arginine, and probably lysine, may cross the tonoplast by a permease specific for positively charged amino acids (this report). Third, an ATP-dependent, but not energy-requiring translocator with rather broad specificity, catalyzes the exchange of various amino acids between the cytosol and the vacuole (5, this report). Because amino acids are normally more concentrated in the cytosol than in the vacuole (5, 15), an additional energy-requiring mechanism for the export of amino acids from the vacuole, as shown for *Chara* (1), has to be postulated for higher plants also.

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