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Amino Acid Transport in Protoplasts Isolated from Soybean Leaves[1]

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CHARLES D. VERNOOY AND WILLY LIN*
Central Research and Development Department, Experimental Station, E. I. du Pont de Nemours & Company, Wilmington, Delaware 19898

ABSTRACT

We isolated large quantities of mesophyll protoplasts from source and sink leaves of soybean plants and examined them for amino acid uptake. Accumulation of amino acids in isolated protoplasts was linear for at least 40 minutes. Uptake kinetics revealed the presence of both saturable and linear components. Increasing external pH decreases the uptake. The uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at 15 micromolar inhibited and fusicoccin at 10 micromolar stimulated amino acid uptake. Our data are consistent with a proton-cotransport mechanism for the uptake of L-glutamine and α-amino isobutyric acid into soybean mesophyll cells.

Three major obstacles in our understanding of the mechanism(s) of amino acid uptake in higher plants are: (a) the rapid intracellular metabolism of most natural amino acids (b) the influence of the medium pH on the charge species of the acid, and (c) the possible binding of the acid to components of the cell wall and membrane. Depending upon the species of amino acid and the tissue which was used, a proton-cotransport, proton-antiport, or neutral-transport (transport of the charged acid molecule) mechanism has been proposed for amino acid transport in plant tissues (6, 7, 11).

Although isolated protoplasts have been used to study amino acid uptake in higher plants (4, 5, 10, 13, 15), less research effort was made in this subject than that in sugar uptake. Isolation of protoplasts involves a high osmotic stress to plant tissue, and it is known that in some cases protein synthesis can be disturbed under these conditions (13, but see 10). The disturbance of protein syntheses could temporarily shut down amino acid incorporation into protein molecules thereby making the uptake constants reflect more of the carrier mediated process and less the influence of metabolism. The removal of the cell wall components during protoplast isolation also eliminates the apoplastic effects on the uptake processes associated with the wall.

In this investigation, we examined the feasibility of using protoplasts as a tool to study amino acid transport mechanism(s). A comparison was made of the uptake characteristics between the two protoplast populations which were isolated from source and sink soybean leaf tissues.

MATERIALS AND METHODS

Protoplast Isolation. Soybean (*Glycine max* L. cv 'Wye') plants were grown in a growth chamber under the conditions previously reported (9). Primary leaves from approximately 2-week-old plants were used as source leaves. Sink mesophyll protoplasts were isolated from young trifoliate leaves with a center leaflet less than 30 mm in length. A modified procedure of Lin (8) was used to isolate mesophyll protoplasts. In brief, the upper leaf surface was abraded with 320 grit carborundum to remove the cuticle. About 1-mm slices of leaf were cut and incubated at 30°C for 2 h, with agitation, in an enzyme mixture of 3.7% CELF cellulyase (Worthington Diagnostics Inc., NJ), 0.5% Pectolyase Y-23 (Seishin Pharmaceutical Co., Japan), 0.5 M sorbitol, 10 mM Mes, and 0.1% BSA at pH 5.8.

After filtering the digest through a 100 μm nylon mesh, the protoplasts were spun at 100*g* and washed twice. Separation of protoplasts from other components was achieved by layering on 8 ml each of a 10:20:30:40% 0.5 M metrizamide, 25 mM Mes, and 10 mM $CaCl_2$ (pH 6.0) gradient (remaining percentage of each layer was 0.5 M sorbitol buffer with 25 mM Mes, 10 mM $CaCl_2$ [pH 6.0]). After an 8 min 100*g* spin, the protoplasts were collected from the 20:30% interface. They were spun at 100*g* and washed twice in the 0.5 M sorbitol buffer to remove the metrizamide. An average of 35 and 20 μm diameter were found for protoplasts isolated from source and sink tissue, respectively. Protein content of 0.13 and 0.088 mg/10^6 protoplasts was measured for source and sink, respectively. Freshly isolated protoplasts were used for uptake studies.

Uptake Studies. Amino acid uptake was measured after 20 min incubation of protoplasts in the uptake medium at 30°C for the kinetic, pH, and chemical effects studies. Each 250 μl aliquot of uptake solution contained approximately 0.5 μCi of ^{14}C-labeled amino acid (New England Nuclear). Uptake was terminated by layering 250 μl of protoplast uptake solution mixture over a silicone oil layer of $d = 1.044$ g/ml, a 0.7 M mannitol with 25 mM Mes, 10 mM $CaCl_2$ (pH 6.0) buffer layer, and a bottom silicone layer of $d = 1.05$ g/ml, in a 400 μl microfuge tube (9). Less than 15 s were required to pellet the protoplasts. Radioactivity was measured by liquid scintillation counting, as previously described (9). Protein content was measured using the Bio-rad microprocedure (2).

All uptake solutions, except those for the pH experiments were buffered and maintained at 0.5 M sorbitol, 25 mM Mes, and 10 mM $CaCl_2$ (pH 6.0). For the pH experiments, a mix of 25 mM mes, 25 mM bicine, 25 mM Hepes, and 25 mM citrate at 10 mM $CaCl_2$ and 0.5 M sorbitol was used for the pH 4 to 8 range.

RESULTS AND DISCUSSION

Time Course. An initial characterization of the amino acid uptake by the protoplasts was examined with a time course of

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AIB[2] and L-glutamine accumulation. AIB has been used as a nonmetabolized amino acid analog in plants (3, 5, 12, 14). This was confirmed by our preliminary results with soybean MP protoplasts which showed that 91% of the AIB taken up remained in a form that co-chromatographed with a standard. L-Glutamine and AIB uptake into MP isolated from sink leaves was linear for at least 40 min (Fig. 1, A and B). Figure 1 also shows that the uptake of glutamine by the protoplasts was 10 times greater than that of AIB. This rapid uptake could be due to the cellular utilization of glutamine. The time course of source MP also followed a linear accumulation pattern (data now shown). Based on these time courses, it was assumed that uptake processes lasting 20 min were under steady state conditions.

Kinetic Measurements. The effect of exogenous glutamine concentration on glutamine uptake into both source and sink MP showed a linear and a saturable uptake kinetic (Fig. 2). Analysis of the uptake using the function $v = V_{max}S/(K_m + S) + PS$, where S is substrate concentration and P is the diffusion coefficient, gave constants that had similar K_m values, 0.96 mM, for source and sink, but differed in their V_{max}. The V_{max} of the sink MP was approximately double that of source protoplasts, (52, and 28 nmol/mg protein·h, respectively). However, since the protein content of the cell types differs, the V_{max} calculate as nmol/10^6 ppt·h results in the sink V_{max} being 25% higher than the source.

Examination of the effect of external AIB concentration on the rate of uptake (kinetic curves) indicated there was little or no saturation of uptake in the concentration range tested (1–1000 μM) (Fig. 3). A similar linear uptake (lack of saturable uptake component) for AIB into excised intact soybean cotyledons was observed previously (1). As a result, the above equation could not be used to fit the data obtained for AIB. Obviously, there was little, if any, saturable component to AIB uptake by source or sink MP. There are several possibilities for the differing kinetic response seen between glutamine and AIB. First, glutamine but not AIB can be metabolized after being taken up into the cells. Second, the two compounds may be transported by different systems. At present, our data does not enable us to differentiate between these alternatives.

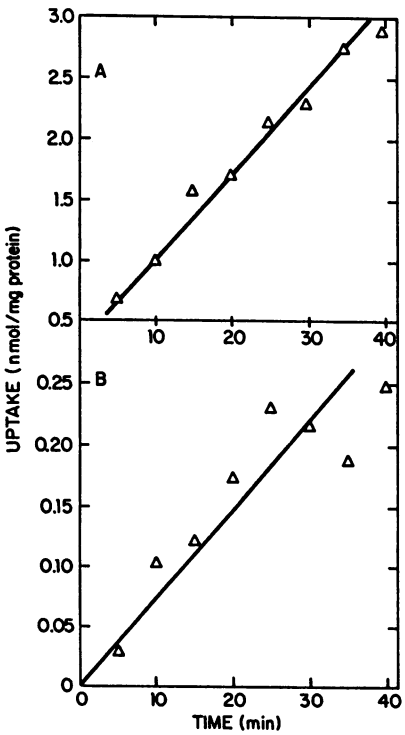

FIG. 1. Time course of amino acid (0.5 mM) accumulation into soybean sink mesophyll protoplasts. Each data point represents six replicates. A, L-Glutamine; B, AIB.

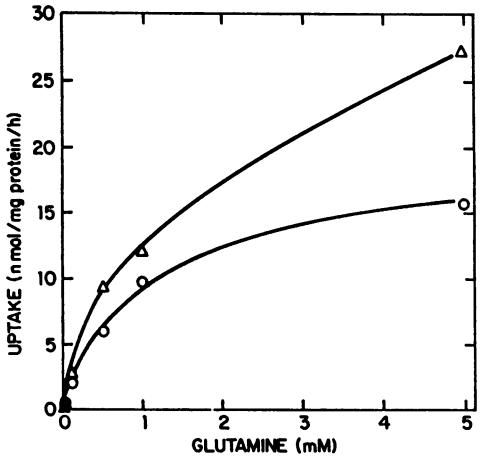

FIG. 2. Uptake of glutamine by sink and source mesophyll protoplasts of soybean. Uptake period was 20 min at 30°C. (Δ), Uptake by sink protoplasts; (O), uptake by source protoplasts and each data point was taken from a mean of b measurements; SE was less than 15%.

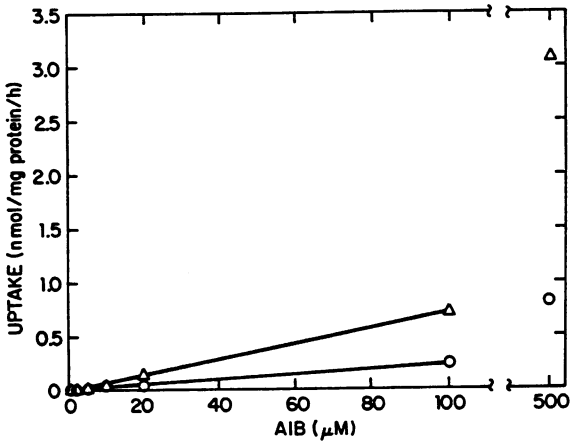

FIG. 3. Uptake of AIB by sink and source mesophyll protoplasts of soybean. Uptake period was 20 min at 30°C. (Δ), Uptake by sink protoplasts; (O), uptake by source protoplasts. No saturation was seen at 1000 μM. Each data point was taken from a mean of 6 measurements and the SE was less than 15%.

The energy fluctuations induced by light stimulation have been reported to affect amino acid uptake kinetics (12). The uptake of AIB into sink MP in 1200 μE/m²·s light, with a Mini-Lite 10 (Colortran, Burbank, CA) light source and its intensity was measured with a photometer, model LI-1905B quantum sensory (Li-Cor, Inc., Lincoln NE), showed a slight (13–23%) elevation over the rate under dark conditions. A slight light stimulation of sucrose and glucose uptake into sink soybean MP was also observed (MR Schmitt, W Lin, unpublished data).

pH Effects. It has been proposed that amino acid uptake is a proton-cotransport mediated process. Two tests used to deduce the possibility that uptake is proton mediated are the influence of pH on uptake, where enhanced uptake is expected as pH declines, and how compounds such as FC, which stimulates H^+

[2] Abbreviations; AIB, α-amino-isobutyric acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; FC, fusicoccin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MP, mesophyll protoplasts; pCMBS, *p*-chloromercuribenzene sulfonic acid.

extrusion, affect uptake (14).

To investigate the effect of protons, the uptake of amino acids was examined in the range of pH 4 to 8. A single buffer system was used to minimize buffer effects. At a 300 μM glutamine concentration, the uptake into both sink and source MP decreased with increasing external pH. L-Serine and AIB also showed an uptake inversely related to the system pH (Fig. 4, A and B). The effect of external pH on AIB uptake can be further distinguished by replotting the uptake data with the ordinate calculated as the percentage of the uptake at pH 6.0. Figure 5, A and B, shows that the three amino acids all respond in the same fashion. Comparison of the sink and source tissues shows that there is little difference between the pH response of the tissues.

Chemical Effects. The susceptibility of uptake to inhibition by chemical modifiers, which impact on the energy status of the cell, is typically used as a gauge in differentiating the active and passive transport process. Diffusional processes are not affected by chemical modifiers at the concentration tested; only the energy status of the cell is thought to be affected. The use of FC causes a stimulation in the H^+-ATPase and leads to a higher level of H^+ efflux and therefore, a stimulation of transport mediated by a proton-cotransport process. In source MP, AIB uptake was doubled by 10 μM FC, while glutamine uptake was stimulated 39% (Table I). FC did not stimulate either AIB or glutamine uptake into sink MP. In fact, the addition of FC resulted in an inexplicable decrease in uptake.

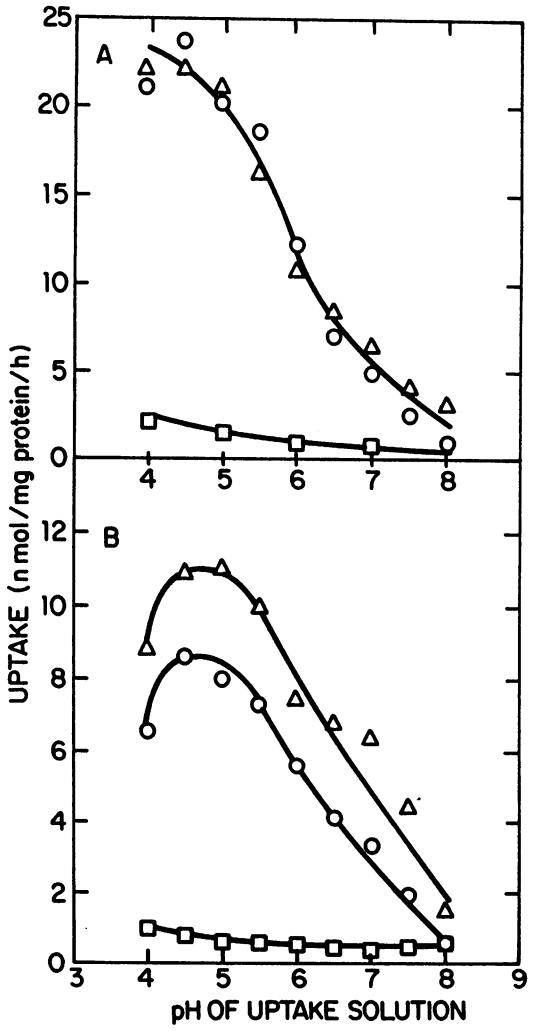

FIG. 4. The effect of exogenous pH on the uptake of L-glutamine (○), L-serine (△), and AIB (□). Uptake period was 20 min at 30°C at 0.5 mM amino acid concentration. A, Response of sink protoplasts; B, response of source protoplasts.

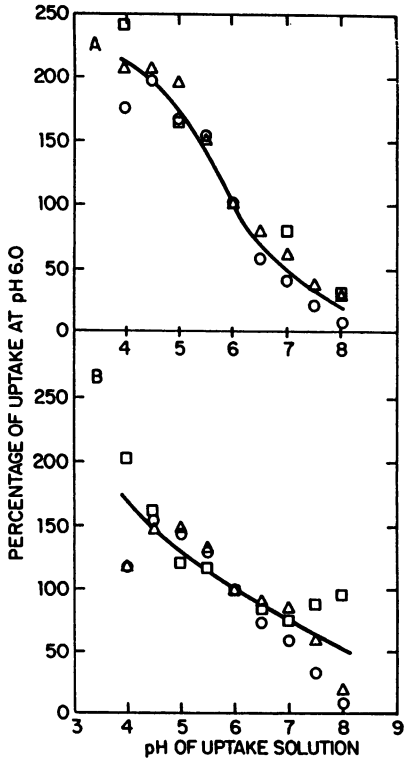

FIG. 5. Ordinate of Figure 4, A and B, calculated to show the deviation of pH from that of pH 6.0 and expressed as a percentage. L-Glutamine (○), L-serine (△), AIB (□). A, Response of sink protoplasts; B, response of source protoplasts.

Table I. *Effect of Various Chemicals on the Uptake Rate of L-Glutamine and α-Amino isobutyric Acid into Mesophyll Protoplasts Isolated from Sink and Source Tissue*

Values are percent of control.

	Sink[a]		Source[b]	
	Glutamine	AIB	Glutamine	AIB
Control	100	100	100	100
pCMBS, 0.2 mM	89	91	88	75
FCCP, 15 μM	62	75	25	13
DES, 50 μM	53	64	35	12
DCCD, 50 μM	82	77	68	37
FC, 10 μM	86	65	139	201

[a] Sink uptake rates for glutamine and AIB controls were 15.2 and 2.22 nmol/mg protein·h, respectively. [b] Source rates for glutamine and AIB were 13.7 and 1.58 nmol/mg protein·h. Amino acid concentration in uptake solutions was 0.3 mM.

Uncouplers such as FCCP and ATPase inhibitor DES collapse the H^+ gradient, the driving force for proton-cotransport mechanisms. For AIB, 15 μM FCCP or 50 μM DES caused an equal inhibition, dropping uptake to 13% in source MP. Glutamine uptake into source MP was decreased 25 to 35% by the uncouplers FCCP and DES, respectively.

In sink MP, the sulfhydryl modifier pCMBS and the H^+ channel blocker DCCD, had little effect on uptake of either acid. Source MP were more sensitive to pCMBS than DCCD. pCMBS decreased uptake of glutamine to 88% and AIB to 75%. DCCD had a greater effect, decreasing glutamine uptake to 68% and AIB uptake to 37%.

Interestingly, there was a difference in the level of effect exerted by the chemical modifiers on sink and source MP. The source

MP were much more sensitive than the sink MP. Along with this greater sensitivity there was a greater inhibition or stimulation for the nonmetabolized analog AIB than there was for glutamine. The difference may be related to greater size heterogeneity in protoplasts isolated from the sink tissue. Since the sink tissue protoplasts were more heterogeneous than the source MP, they probably represent greater diversity in cell origin and age. Since the sink population could be differing in response to an amino acid, this could be masking any difference between AIB and glutamine sensitivity to modifiers. In addition, the kinetics of glutamine uptake indicated a larger linear component for the sink MP. This could be the result of accelerated amino acid metabolism.

CONCLUSIONS

In this investigation, we have provided evidence to further substantiate the involvement of a proton-cotransport in the glutamine and AIB uptake into soybean mesophyll cells. Unlike sucrose uptake (9), the sulfhydryl modifier pCMBS had little, or no, impact on amino acid uptake. Not surprisingly, the sink tissue with its high nutritional input requirement, had a higher solute uptake than tissue from primary leaves. They did show a lack of sensitivity to a number of compounds inhibitory to amino acid uptake by source MP.

Since most natural amino acids are readily metabolized in plant cells, caution must be exercised in the interpretation of amino acid uptake data. As shown in this study, tissue which has a higher metabolic activity, *e.g.* sink tissue, exhibits a higher apparent uptake, yet this uptake is less sensitive to commonly used uptake modifiers. The impact of metabolism on the total amino acid accumulation, resulting from membrane transport plus metabolism is, therefore, greater in the sink than the source tissue.

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