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Derepression of Amino Acid-H^+ Cotransport in Developing Soybean Embryos[1]

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ABSTRACT

The uptake of the unnatural amino acid α-aminoisobutyric acid (AIB) and glutamine by developing soybean (*Glycine max* Merr. cv Chippewa 64) embryos was investigated. In freshly excised embryos, the accumulation ratio (cytoplasmic concentration/external concentration) of AIB did not exceed 1.0. After an 18-hour preincubation in nitrogen-free medium the accumulation ratio of AIB exceeded 4.5 at an external AIB concentration of 10 micromolar. This indicates the derepression of an active amino acid uptake mechanism operative at low external amino acid concentration. The presence of sucrose, NH_4NO_3, or glutamine during a 21-hour preincubation prior to measuring glutamine uptake inhibited the enhancement of uptake by 43%, 51%, and 96%, respectively. The time course of the decline in free amino acids and the time course of enhancement of amino acid uptake was not consistent with enhanced uptake resulting from relief of transinhibition, but suggested instead the derepression of synthesis of new carriers. The time course of enhancement of amino acid uptake was paralleled by an increase in glutamine-induced depolarization of the membrane potential. The kinetics of glutamine uptake indicated the presence of a saturable and a nonsaturable component of uptake. The saturable component of uptake is attributed to a mechanism of amino acid-H^+ cotransport which is derepressed by nitrogen and/or carbon starvation. At physiological concentrations of amino acids, uptake through the saturable system in freshly excised embryos is negligible. Thus, uptake through the nonsaturable system is of primary importance in the nitrogen nutrition of developing soybean embryos.

Partitioning of assimilates is generally recognized as an important determinant of yield in soybeans (3). The complexity of source-sink interactions and translocation processes, however, has hindered our understanding of the physiological processes regulating assimilate partitioning. A report that varietal differences in soybean seed growth rates were maintained when cotyledons were cultured *in vitro* (2) indicated that the physiological factors limiting soybean seed growth rates reside in the developing cotyledon and not the maternal plant. Other work has also suggested that processes localized in sink tissues may be important in regulating the partitioning of assimilates to storage tissues (22, 23).

The translocation of nitrogenous compounds, primarily amino acids, to the developing soybean embryo is of major importance in determining soybean seed yield and nutritional quality. Quantitatively, amino acids are required in a 1:1 M ratio with sucrose for normal soybean seed development (17). In agreement with this calculated amino acid requirement, we have found similar free space concentrations of sucrose and total amino acids, ranging from 5 to 25 mM, in soybean seedcoats and developing embryos (4). Consequently, an understanding of amino acid transport into developing soybean embryos is necessary to evaluate this step in the regulation and possible limitation of assimilate movement to the developing embryo. This study characterizes the mechanism of uptake of the neutral amino acids AIB[2] and glutamine by developing soybean embryos.

MATERIALS AND METHODS

Plant Material. Soybeans (*Glycine max* Merr. cv Chippewa 64) were grown in the greenhouse with supplemental lighting. Pods with three developing seeds were selected at an embryo (cotyledons and embryonic axis) FW of 75 to 150 mg which occurred early in the linear phase of seed growth. Seed coats were excised using sterile technique and the embryos placed in flasks of autoclaved standard salt solution (0.5 mM KCl, 0.5 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 1 mM Mes adjusted to pH 6.0 with NaOH) and aerated. Organic compounds were added to these solutions after autoclaving the standard salt solution.

Flux Measurements. After preincubation in sterile solutions, three embryos were blotted, weighed, and transferred to 4 ml of uptake medium consisting of the same standard salt solution described above with various concentrations of glutamine or AIB and either [^{14}C]glutamine or [^{3}H]AIB (New England Nuclear). Inhibitors, when used, were present 30 min before labeled substrate was added. The uptake medium was aerated, illuminated, and maintained at 27°C. After the uptake period (generally 20 min), embryos were removed from the uptake medium by filtration and transferred to unlabeled standard salt solution for 18 min to remove free space label. Embryos were then allowed to dry overnight and ^{14}C or ^{3}H collected after combustion of the sample in a Packard Tri-Carb B306 sample oxidizer. Radioactivity was measured with a Beckman LS-100C scintillation counter and uptake calculated and expressed as nmol or μmol g^{-1}(FW) h^{-1}.

Electrophysiological Measurements. Electrophysiological measurements were made as previously described (10), with the exception that sorbitol was not present in the standard salt solution.

Amino Acid Determinations. Uncombined amino acids were determined by a modified ninhydrin reaction (16) after extraction of 300 to 400 mg soybean embryos in hot 5% TCA. Glycine was used as standard. Glutamine and AIB were separated and identified by one-dimensional TLC on cellulose plates (Brinkman Instruments) with isopropanol:formic acid:water (80:4:20, v/v/v).

ATP Assay. Following treatments, 300 to 400 mg FW soybean embryos were homogenized in ice-cold 5% TCA with 0.1% EDTA.

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[2] Abbreviations: AIB, α-aminoisobutyric acid; FW, fresh weight; FC, fusicoccin; SHAM, salicylhydroxamic acid.

After centrifugation, the supernatant was saved and the pellet reextracted. ATP levels in the combined supernatants were determined with a luciferin-luciferase assay as previously described (6).

Microscopy and Stereology. Soybean embryos (approx. 100 mg FW) were fixed in 4% paraformaldehyde, 5% glutaraldehyde, and 0.1 M K_2HPO_4 at pH 7.2 (5) and postfixed in 1% osmium tetroxide. Fixed tissue was dehydrated in a graded acetone series and embedded in epoxy resin (19). Thick sections (1 μM) were taken on a Sorvall MT-1 microtome equipped with glass knives. Sections were stained with 0.05% toluidine blue in 0.1 M $NaHCO_3$ and 0.05 M Na_2CO_3 (pH 9.5). Cells were photographed and cellular and vacuolar dimensions were estimated by measuring surface areas with an electronic planimeter. The ratio of vacuolar surface area/cell surface area was taken to be equivalent to the ratio of vacuolar volume/cell volume (20). The proportion of cellular volume occupied by cytoplasm was calculated as 1 − (vacuolar volume/cell volume) and was found to be 39.1 ± 3.5% SE. Tissue water was determined to be 0.8 ml g^{-1} (FW), of which approximately 0.03 ml was estimated by compartmental analysis of efflux kinetics to be apoplastic. Using these values, a cytoplasmic volume of 0.301 ml g^{-1} (FW) was calculated.

RESULTS AND DISCUSSION

Metabolism of AIB. The unnatural amino acid, AIB, has been used often to study amino acid transport mechanisms without interference from metabolism of the transported substrate (1, 15, 18). We were interested in calculating accumulation ratios (cytoplasmic concentration/external concentration) of a transported amino acid and so used AIB here. More than 97% of the [^{3}H]AIB was TCA soluble and cochromatographed with AIB standards after a 2-h labeling period, whereas less than 30% of the [^{14}C] glutamine was identified as either glutamine or glutamate under the same conditions (Table I). These results confirmed our expectation that AIB was not metabolized and so could be used to estimate accumulation ratios without corrections for loss of internal AIB by metabolic conversion or incorporation.

Time Course of Amino Acid Uptake. The concentration dependence of sucrose uptake by soybean embryos and other tissues are nonsaturating at high concentrations of substrate (11, 13, 21). Inasmuch as such kinetics could be generated artifactually by incomplete removal of free space label during rinsing, it was important to determine that the rate of amino acid uptake was constant over a given time period and that a plot of the data passed through the origin. Figure 1 shows the results of AIB uptake over a 25-min period with either a 6- or 18-min rinse following uptake. From this curve, we determined that an 18-min rinse was required to clear the free space of labeled AIB.

AIB Uptake and Enhancement of Uptake. Measurements of amino acids in the embryo apoplast indicated that concentrations are above 1 mM for most amino acids (Hsu, Bennett, and Spanswick, unpublished). The kinetics of AIB uptake over what was felt to be a physiological concentration range were nonsaturating (Fig. 2). These results are similar to those reported for alanine uptake by soybean root suspension cells (7). King and Oleniuk (7) also reported that transfer of soybean suspension cells to nitrogen-free media resulted in the appearance of an active component of alanine uptake operative at low alanine concentrations. A similar result was obtained when soybean embryos were incubated in nitrogen-free standard salt solution before measuring uptake. Table II shows the accumulation ratio of AIB calculated after a 30-min uptake period. The accumulation ratio in freshly excised embryos was 0.26 at either 10 μM or 1 mM external AIB concentration. After incubation in standard salt solution for 18 h, total uptake of AIB was enhanced and the accumulation ratio at low (10 μM) external AIB concentration exceeded 4.5. This enhanced uptake was strongly inhibited by the metabolic inhibitor NaCN. At high (1 mM) external AIB concentration, the accumulation ratio after preincubation in standard salt solution was close to 1. These results indicate that during nitrogen starvation an active uptake system responding to low (<1 mM) AIB concentra-

Table I. *Metabolic Incorporation and Conversion of AIB and Glutamine*

Developing soybean embryos were incubated for 2 h in either 1 mM [^{3}H]AIB or 1 mM [^{14}C]glutamine and extracted in hot 5% TCA. TCA-soluble amino acids were separated by TLC and the percentage of radioactivity associated with originally labeled amino acid calculated. For glutamine-treated embryos, radioactivity associated with glutamine and glutamate (Glx) was summed to avoid an overestimate of metabolic conversion due to nonenzymic deamination of glutamine during extraction.

Labeled Amino Acid	TCA-Soluble Label	TCA-Soluble Label Co-Chromatographed as Original Amino Acid	Metabolic Conversion of Labeled Amino Acid
		%	
AIB	99.7	97.4	2.9
Glutamine	66.8	41.2	72.5

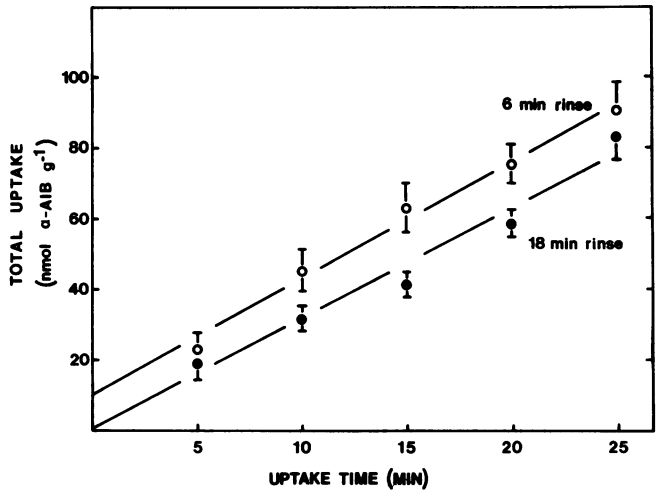

FIG. 1. Time course of uptake of AIB. Embryos were incubated in 1 mM AIB for the indicated time, rinsed in unlabeled solution for either 6 (○) or 18 min (●) and the total uptake determined. Points represent the mean ± SE of three experiments.

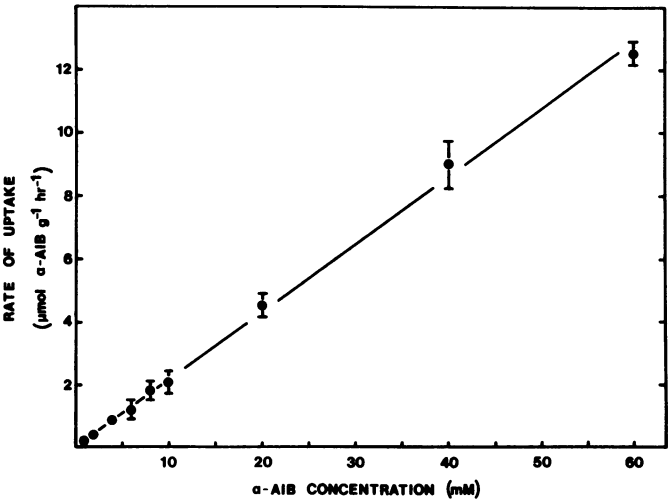

FIG. 2. Concentration dependence of AIB uptake between 1 and 60 mM. Points represent the mean ± SE of three experiments.

Table II. *Accumulation Ratios of AIB in Developing Soybean Embryos*

Total uptake of AIB was measured in either freshly excised embryos, or embryos preincubated for 18 h in nitrogen-free standard salt solutions. Compartmental analysis of efflux kinetics indicated that 81% of AIB taken up in 30 min was localized in the cytoplasm. A cytoplasmic volume of 0.301 ml g^{-1} FW was determined by stereological analysis of light micrographs (see "Materials and Methods" for details). Using these values, the cytoplasmic concentration of AIB could be calculated by:

$$[AIB]_{cyt} = \frac{\text{Total uptake} \times 0.81}{301 \times 10^{-6}}$$

$[AIB]_{out}$	Total Uptake	$[AIB]_{cyt}$	Accumulation $[AIB]_{cyt}/[AIB]_{out}$
	nmol g^{-1}	*μM*	*ratio*
Fresh			
1 mM	97.0	262.8	0.26
10 μM	0.95	2.6	0.26
18 h			
1 mM	434.3	1177.0	1.17
10 μM	16.0	45.7	4.57
10 μM + 0.5 mM NaCN	4.2	11.3	1.13

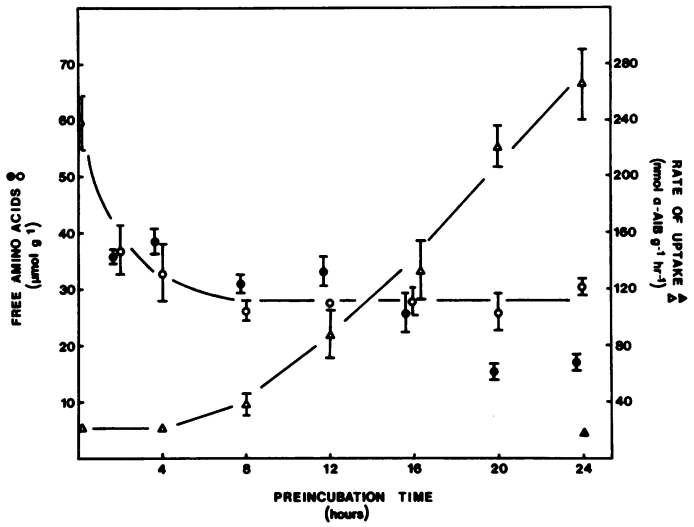

FIG. 3. Time course of the enhancement of AIB uptake and of the decline in free amino acids during preincubation in nitrogen-free standard salt solution. AIB uptake was measured at an external concentration of 100 μM after the indicated time of preincubation (△). One point at 24 h is the uptake rate measured when cycloheximide (5 μg/ml) was present during preincubation (▲). Uncombined amino acids were measured in embryos preincubated in either the absence (○) or presence (●) of cycloheximide (5 μg/ml).

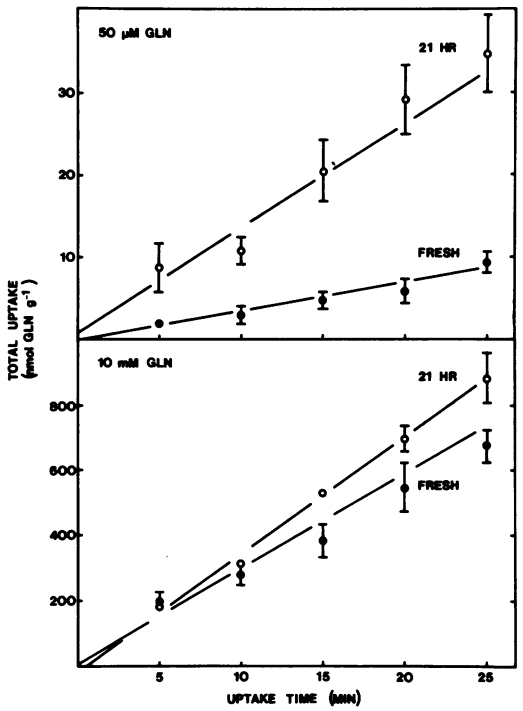

FIG. 4. Time course of glutamine uptake. Embryos were incubated in 50 μM (upper panel) or 10 mM (lower panel) glutamine for the indicated time, rinsed for 18 min, and the total uptake determined. Freshly excised (●) or 21-h nitrogen-starved (○) embryos were used. Points represent the mean ± SD of three to six determinations.

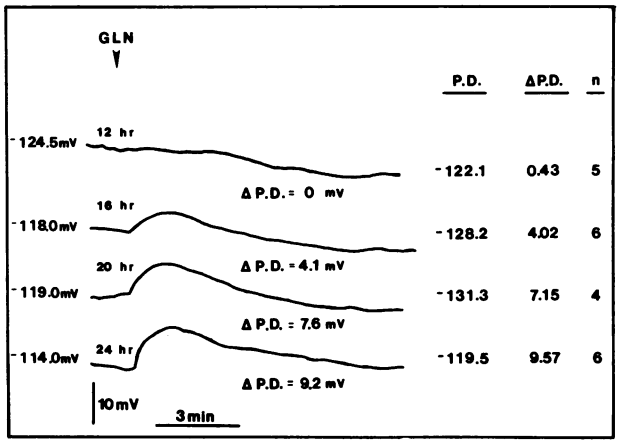

FIG. 5. Glutamine-induced depolarization of the membrane potential during preincubation in standard salt solution. Glutamine (1 mM) was added at the indicated point to embryos preincubated for 12, 16, 20, or 24 h as indicated on the individual traces. Adjacent to each trace is the mean resting membrane potential (P.D.), mean glutamine-induced depolarization (ΔP.D.), and the number of observations at each time.

tions becomes operative.

The time course of enhancement of AIB uptake during incubation in standard salt solution is shown in Figure 3. Inasmuch as in other cell types high internal substrate concentrations have been shown to inhibit uptake of the substrate (trans-inhibition) (15), we measured the decline in uncombined amino acids over the same period that enhanced uptake was observed (Fig. 3). Free amino acid levels in excised embryos declined rapidly and reached their lowest level before rates of AIB uptake began to increase. Rates of AIB uptake continued to increase while free amino acid levels were low and unchanging. This is inconsistent with the possibility that enhanced uptake rates result from a relief of trans-inhibition but instead suggests that after a decline in free amino acids, the synthesis of new carriers is triggered, which continues while free amino acid levels stay low. The inclusion of cycloheximide (5 μg/ml) completely abolished enhancement of AIB uptake (Fig. 3) but had little effect on the decline in uncombined amino acid levels (Fig. 3), again indicating that enhanced uptake is not simply related to relief from trans-inhibition.

Enhancement of Glutamine Uptake. Figure 4 shows the time course of glutamine uptake in soybean embryos either freshly excised or preincubated for 21 h in standard salt solution. The enhancement of glutamine uptake is apparent only when meas-

Table III. *Effect of Nutritional Substrates on the Enhancement of Glutamine Uptake*

Glutamine uptake was measured at an external concentration of 50 μM in either freshly excised soybean embryos or in embryos preincubated for 21 h in standard salt solution with the indicated additions. Values are the mean ± SE of six determinations.

Treatment	Rate of Uptake	Inhibition of Enhancement
	nmol Gln g^{-1} h^{-1}	%
Fresh	15.47 ± 1.8	
21 h	72.32 ± 9.2	0
+ PEG-400 (50 mM)	62.30 ± 5.2	17.6
+ Sucrose (50 mM)	48.13 ± 4.8	42.6
+ NH_4NO_3 (25 mM)	43.27 ± 10.6	51.1
+ NH_4NO_3 (25 mM) Sucrose (50 mM)	20.64 ± 2.0	90.9
+ Gln (50 mM)	17.49 ± 4.5	96.4

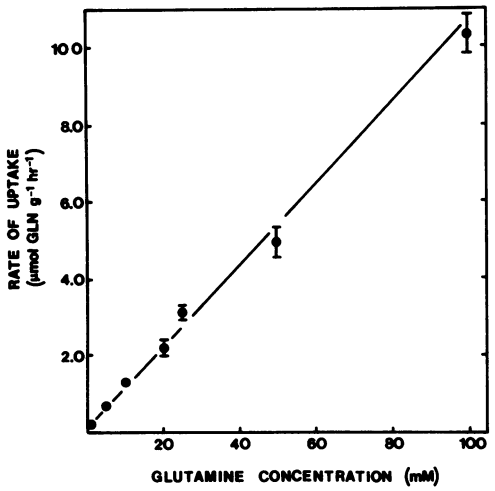

FIG. 6. Concentration dependence of glutamine uptake at high concentrations of glutamine. Uptake was measured with freshly excised embryos. Points represent the mean ± SE of three experiments.

ured at low (50 μM) external glutamine concentrations (Fig. 4). Uptake rates of glutamine were enhanced during 24 h of preincubation with a time dependence similar to the enhancement of AIB uptake rates but to a lesser extent (not shown). The uptake rate of AIB (measured at 100 μM AIB) was typically enhanced about 10-fold over a 24-h period while glutamine uptake rates (measured at 100 μM glutamine) were enhanced about 5-fold over the same time period. Because AIB inhibits glutamine uptake (Table IV), it is likely that they are taken up by the same carrier and that differences in enhanced uptake reflect a greater affinity of the uptake system for AIB. Subsequent experiments were performed with glutamine as substrate since both AIB and glutamine showed qualitatively similar uptake behavior and results with glutamine are of greater physiological relevance.

Amino acid uptake in many plant tissues has been suggested to be coupled with H^+ influx (9, 12). Coupled amino acid-H^+ influx results in the movement of positive charge across the membrane causing a transient depolarization of the membrane potential (9). Attempts to measure amino acid-induced depolarization of the membrane potential of freshly excised soybean cotyledons were unsuccessful, although sucrose-induced depolarizations were routinely observed. Following preincubation in standard salt solution for times up to 24 h, the addition of glutamine to the solution bathing excised soybean cotyledons resulted in depolarizations of the membrane potential (Fig. 5). After 12 h of preincubation, the increases in the magnitude of glutamine-induced depolarizations

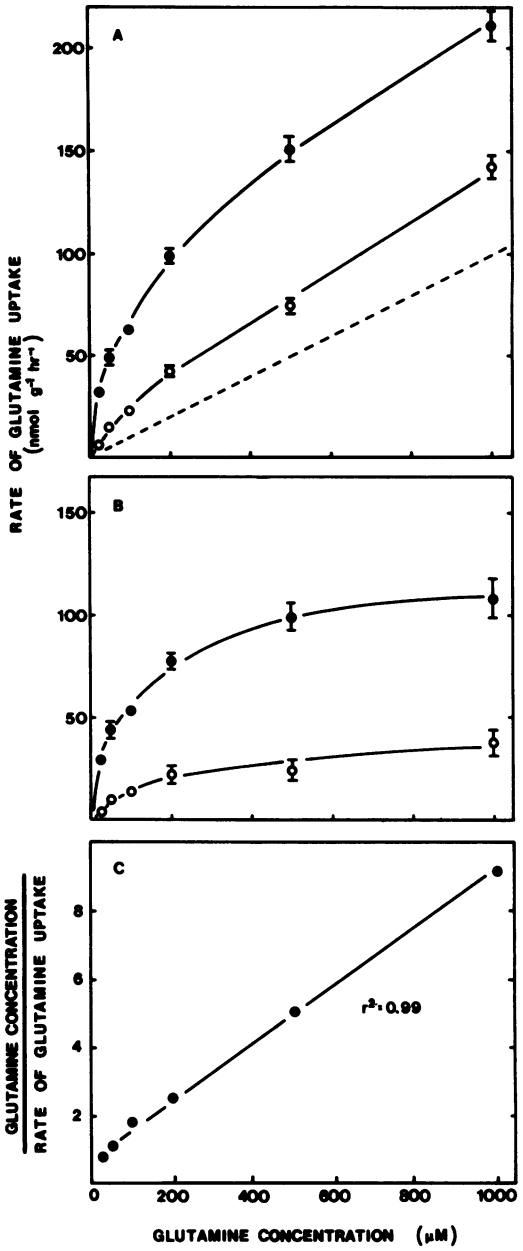

FIG. 7. A, Concentration dependence of glutamine uptake at low concentrations of glutamine. Uptake was measured in freshly excised embryos (○) or embryos preincubated 21 h in nitrogen-free standard salt solution (●). Dashed line is the slope of the nonsaturable component of uptake determined from the data in Figure 6. B, Concentration dependence of the saturable component of glutamine uptake. Data from 'A' replotted after subtraction of the nonsaturable component of uptake (dashed line in 'A'). Points represent the mean ± SE of three experiments. C, Hanes-Woolf plot of the data shown in 'B' (●), for glutamine uptake after derepression of the saturable uptake system.

are correlated with the enhancement of uptake rates. This result suggests that enhanced rates of amino acid uptake result from amino acid-H^+ cotransport which becomes electrophysiologically detectable only after derepression of this transport system. The threshold level of activity of the uptake system for electrophysiological detection is apparently not reached until after 12 h of incubation in standard salt solution.

King and Oleniuk proposed (7) that enhanced amino acid uptake in cultured soybean root cells resulted from nitrogen starvation. The enhanced uptake by soybean embryos may have

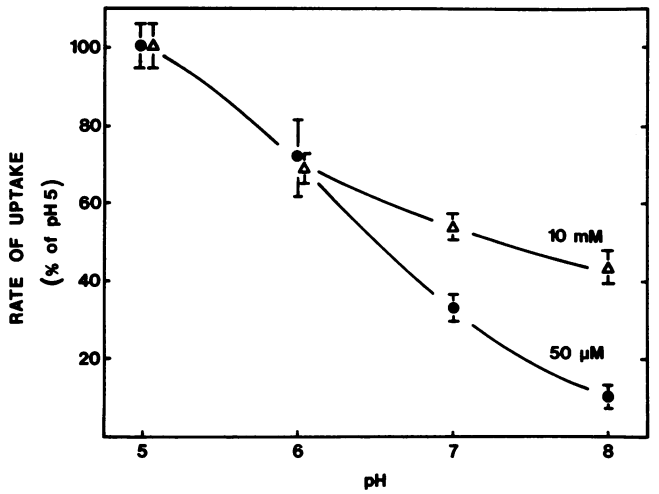

FIG. 8. pH dependence of glutamine uptake. Embryos were preincubated 21 h in nitrogen-free solution at pH 6 and transferred to appropriate pH solutions 30 min prior to measuring glutamine uptake at either 50 μM (●) or 10 mM (△) external glutamine concentration. Points represent the mean ± SD of three to six determinations.

Table IV. *Effect of Various Compounds on Membrane Potential (PD), Cellular ATP Level, and Glutamine Uptake*

All experiments were performed as indicated using soybean embryos preincubated 21 h in nitrogen-free standard salt solution. Concentrations of AIB or glutamate were 10 times that of glutamine (*i.e.* either 500 μM or 100 mM). Control values were 133.2 mv (PD), 197.0 nmol ATP g^{-1} FW (ATP), 66.4 nmol g^{-1} h^{-1} (50 μM Gln uptake), and 1.97 μmol g^{-1} h^{-1} (10 mM Gln uptake). Values not determined are noted (ND).

Treatment	PD	ATP	Glutamine Uptake	
			50 μM	10 mM
			%	
Control	100	100	100	100
NaCN (0.5 mM) + SHAM (1 mM)	64.7	66.7	41.1	52.1
FC (10 μM)	125.0	98.3	130.0	101.4
AIB (500 μM or 100 mM)	ND	ND	44.1	92.7
Glu (500 μM)	ND	ND	86.8	ND

resulted from a less specific aging phenomenon associated with removal of the embryo from the pod and seed coat. To test this, excised embryos were incubated for 21 h in standard salt solution with various nutritional substrates added. Embryos were transferred to solutions without nutritional substrate for 30 min prior to measuring uptake. Preincubation in the presence of either sucrose of NH_4NO_3 decreased the enhancement of glutamine uptake rates and, when added together, almost completely abolished the enhancement of uptake (Table III). Glutamine alone almost completely abolished enhancement of uptake, whereas polyethylene glycol-400 (osmotic control) had very little effect on the enhancement of glutamine uptake rates. These results indicate that deprivation of either a carbon or nitrogen source results in the derepression of an amino acid transport system. This result is similar to that reported recently by McDaniel and Wozniak (14), and indicates that the response is related to the nutritional status of the embryo.

Kinetics of Glutamine Uptake. The kinetics of glutamine uptake at high concentrations of glutamine (Fig. 6) are nonsaturating and similar to those shown for AIB (Fig. 2). A saturable component of uptake is apparent at low glutamine concentrations (Fig. 7a). After 21 h preincubation in standard salt solution, the saturable component of uptake is greatly enhanced while the nonsaturable component is unaffected. Subtraction of the nonsaturable component (Fig. 7A, dashed line) from both curves in Figure 7A shows the enhancement of the saturable component more clearly (Fig. 7B). A Hanes-Woolf plot of the saturable component of glutamine uptake after derepression (Fig. 7C) indicates a K_m for this component of 97 μM.

Glutamine uptake through the saturable or nonsaturable system could be estimated independently by measuring uptake at either 50 μM or 10 mM external glutamine concentration in embryos nutrient starved for 21 h. Under these conditions, uptake was either 90% through the saturable system (50 μM) or 90% through the nonsaturable system (10 mM). Effects of pH and inhibitors on each uptake system were examined independently using these conditions.

Effects of pH and Inhibitors. It was suggested above that the saturable component of uptake represented the activity of an amino acid-H^+ cotransport system. If this were the case, uptake through the saturable system should be stimulated by low external pH. In the presence of 0.1 mM $CaCl_2$, the effects of pH were small and somewhat erratic. When the $CaCl_2$ concentration was raised to 0.5 mM, a strong pH dependence of glutamine uptake was observed (Fig. 8). High pH reduced uptake through the saturable system nearly 90% and through the nonsaturable system 50%. The strong pH dependence at low external glutamine concentration is consistent with the operation of an amino acid-H^+ cotransport system in this concentration range. The pH dependence of the nonsaturable system was unexpected, but in agreement with the effects of pH on a nonsaturable component of sucrose uptake into sugar beet leaf discs (13).

The effects of metabolic inhibitors, FC, AIB, and glutamate, on glutamine uptake by both the saturable and nonsaturable uptake system are shown in Table IV. Cyanide and SHAM reduced cellular ATP, depolarized the membrane potential, and inhibited both the saturable and nonsaturable component of glutamine uptake approximately 50%. FC hyperpolarized the membrane potential but only stimulated glutamine uptake by the high affinity, saturable uptake system. This is consistent with the operation of an amino acid-H^+ cotransport system in the low concentration range (12). Either AIB or glutamate was included in the uptake medium at a concentration 10-fold greater than glutamine to test for competitive inhibition of glutamine uptake. AIB strongly inhibited glutamine uptake through the saturable system but had no effect on the nonsaturable system of uptake. Glutamate had only a slight effect on glutamine uptake through the saturable system, suggesting that AIB but not glutamate competes for the carrier responsible for the saturable component of glutamine uptake. The effect of glutamate on nonsaturable uptake was not determined because of the large pH change associated with large glutamate additions.

CONCLUSIONS

We have shown that amino acid uptake by developing soybean embryos is characterized by a saturable and a nonsaturable component of influx. The saturable component of amino acid uptake is attributed to an amino acid-H^+ cotransport system, although electrophysiological evidence for such a system was only obtainable after derepression of the system by nitrogen and/or carbon starvation. The high affinity and low capacity of the amino acid-H^+ cotransport system indicate that influx through the system is insignificant at physiologically meaningful amino acid concentrations (1–10 mM). The maximal capacity of the saturable system accounts for only 15% of glutamine uptake measured at an external concentration of 5 mM even when derepressed by 21 h of nutrient starvation. Similar studies of sucrose uptake by developing soybean embryos indicate the existence of saturable and nonsaturable components of sucrose uptake operative in parallel (11, 21). The

saturable component is attributed to the operation of a constitutive sucrose-H^+ cotransport system (10) and, in contrast to the situation for amino acid uptake, is estimated to account for 50 to 80% of sucrose uptake at physiological sucrose concentrations (21).

These results force us to recognize the importance of the non-saturable system of amino acid uptake. Although the kinetics of uptake suggest a diffusion-like process, the pH (Fig. 8) and energy (Table IV) dependence of the process suggest that it is not passive diffusion. A kinetically similar amino acid uptake system in mouse brain slices has been suggested to represent a concentrative uptake mechanism (1). Consideration of the surface area required to support the rates of uptake observed also suggest that this uptake system is not diffusive.

In this study, we have only addressed the general characteristics of uptake of one naturally occurring amino acid. Although we feel the results presented here apply qualitatively to all neutral amino acids, mechanisms may differ for the acidic and basic amino acids (8).

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