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Energy-dependent Loading of Amino Acids and Sucrose into the Phloem of Soybean[1]

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ABSTRACT

Radioactive sucrose, L-leucine, L-glutamate, and γ-aminobutyrate were applied exogenously to abraded areas of soybean leaves. The three amino acids were translocated with similar velocities and mass transfer rates on a molar basis, although they were metabolized differently in the sink tissue. The concentration dependence of leucine translocation showed a triphasic saturation response, while sucrose translocation showed a biphasic saturation response to increasing concentration. Apparent K_m and V_{max} for leucine and sucrose loading in the phloem differed. Both leucine and sucrose translocation were inhibited by uncouplers, high K^+, and *p*-chloromercuribenzenesulfonic acid. Treatment with 0.8 M sorbitol had little effect on sucrose translocation but stimulated leucine translocation, indicating an apoplastic route of loading for leucine. No effect on mass transfer rates was observed when sucrose and amino acids were applied exogenously together. These data provide evidence that phloem loading of amino acids and sucrose is mediated by different and separate carriers, both being dependent on an energy-requiring mechanism.

Although soybean, *Glycine max* (L.) Merr., leaves synthesize many compounds, relatively few are found in the phloem (9, 13). The principal photosynthetic product found in the phloem of most plants is sucrose, but smaller amounts of amino and organic acids are also present (15). Sucrose is actively and selectively loaded into the phloem (3). A specific carrier is believed to mediate this phloem-loading process (14). It is not known if other compounds found in the phloem such as amino acids are actively loaded or move into the phloem by facilitated or simple diffusion, or if certain amino acids are selectively loaded while others diffuse. Unlike sucrose (2), certain amino acids are in lower concentration in the phloem than in the mesophyll cells (9), suggesting that their movement into the phloem may be restricted. A previous report from this laboratory (8) showed that three amino acids having different functional groups, when applied exogenously to an abraded area on the leaf, were transported in the phloem with the same velocity as sucrose, but mass transfer rates on a gram-carbon-atom basis for amino acids were two to three orders of magnitude lower than for that of sucrose. Amino acids were applied to the abraded leaf at a lower concentration than was sucrose. Hence, it was not possible to surmise whether the lower mass transfer rates for amino acids were due to lower availability of amino acids as compared to sucrose, or whether plants had a lower capacity for amino acid transport.

The phloem loading of sucrose from the free space is sensitive to metabolic (3, 11, 14) and sulfhydryl group inhibitors (4), supporting the premise of active loading of sucrose into the phloem. It is of interest to know if amino acid movement into the phloem has a similar energy requirement and mechanism for loading. We examined the concentration dependence of leucine and sucrose translocation in the soybean and its response to certain metabolic inhibitors. Our results suggest that amino acids are loaded into the phloem in a manner similar to sucrose (6).

MATERIALS AND METHODS

Soybean plants were grown in coarse, acid-washed sand in environmental chambers having a 14-h photoperiod and a 30 C day and 20 C night temperature. Relative humidity in the chamber was maintained at 45 ± 5% during the day. The photosynthetic photon flux density was 45 nE cm^{-2} s^{-1} as measured with a quantum sensor (Lambda model LI-190S and a model LI-185 meter). Plants were subirrigated with a modified Hoagland solution (8). The day before the experiment, a 30-day-old plant was simplified to a single source leaflet or a pair of opposing leaflets (usually on the fourth node) and a single sink trifoliolate leaf (usually on the eighth node) by removing all other leaves and petioles. Using a small cork and a paste of Alundum 600[3] (Edmund Scientific Co., Barrington, N.J.) and 5 mM K-phosphate buffer (pH 6.5), a 0.5-cm^2 circular area on the adaxial surface of the source leaflet was abraded as described earlier (8). Abrasion was stopped when two criteria were met: (*a*) the abraded surface noticeably reflected less light than the unabraded portion; and (*b*) when viewed from the abaxial surface of the leaf, appreciably more light was transmitted through the abraded area as compared with the unabraded area. Preliminary work (both here and 8) showed that with insufficient abrasion of the fed area, the solution was taken up slowly or not at all and translocation velocities were slower and variable, but once a threshold amount of abrasion was done, as established by the aforementioned criteria, rates of solution uptake, about 50 μl in 30 min, and translocation velocities, about 1 cm/min, were constant. After abrasion, the leaflet was washed with phosphate buffer. A thin strip of petrolatum was applied to the perimeter of the abraded area and the area was moistened with phosphate buffer. After 1 h, 50 μl of L-[3,4,5-^{3}H(N)]leucine, L-^{14}C(U)-amino-acid, or [^{14}C(U)]sucrose concentrations as indicated, in 5 mM phosphate buffer (pH 6.5) was applied together to a single source leaflet or separately to a pair of opposing source leaflets. After the labeled solution was taken up into the leaf, the area was kept moist with phosphate buffer.

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Arrival of the label at the sink leaf was monitored with a GM tube positioned against the sink leaf (8).

After 2 h, the plant was divided into parts and each part was homogenized separately in 25 ml of ethanol-H_2O-formic acid (88:7:2) and filtered through Celite. Alternately, the entire plant minus source leaf was homogenized twice in 50 ml of methanol-chloroform-H_2O (12:5:3), filtered, and separated into lipid-soluble, water-soluble, and insoluble fractions. Separation of radioactive compounds from the soluble fraction was by ion exchange and TLC as described previously (8). The insoluble fraction was treated with 20 ml of 0.04% (w/v) protease (Sigma, type IV) in 50 mM Tris-Cl buffer (pH 7.4) for 48 h at 37 C and then filtered to obtain a protein hydrolysate fraction. Residual ^{14}C remaining in the residue from the protease treatment was determined by wet combustion of the residue, trapping the evolved $^{14}CO_2$ in ethanolamine and counting by scintillation spectroscopy (10). From this analysis, it was determined that the protease treatment released about 97% of ^{14}C from the insoluble fraction. Aliquots of the various fractions were dried and decolorized to reduce quenching by adding 1 ml of 10% (v/v) H_2O_2 and heating for 2 h at 80 C. Radioactivity was determined by scintillation spectroscopy (1) and radioactivity present as ^3H or ^{14}C was calculated using the internal standard technique (7). Because movement of label from the source leaf was rapid (about 1 cm/min) and linear over the time course of the experiment, total nmol of amino acid or sucrose translocated out of the source leaf cm^{-2} of abraded leaf area h^{-1} was used as an estimate of mass transfer rate.

We observed as had Sovonick *et al.* (14) from autoradiographs of the source leaves that the radioactivity moved from the spot of application during the course of the experiment (Fig. 1). This movement of the labeled solution from the abraded area makes the exact concentration of the substrates and inhibitors in the free space of the leaf difficult to determine. Because duration of the experiment, location of the abraded area on the leaf, leaf size, and environmental conditions surrounding the leaf were kept constant, we believe that relative differences in rates resulting from this dilution were minimized.

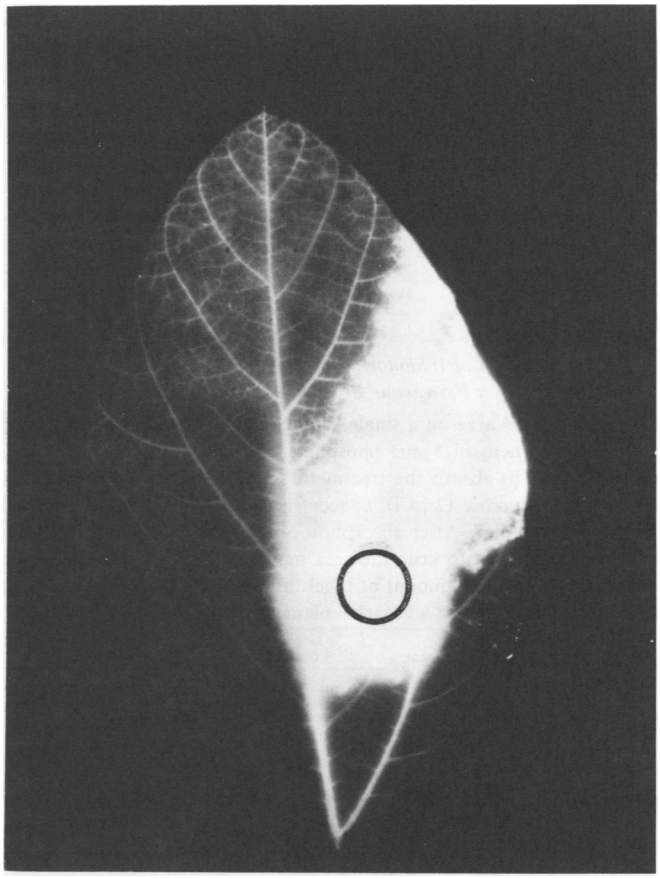

FIG. 1. Autoradiograph of source leaf after application of [^{14}C]leucine to an abraded area. A source leaflet was abraded as described in the text and 10 mM [^{14}C]leucine (10 μCi) was applied to the abraded area. After 2 h the leaflet was removed from the plant, pressed between sheets of paper, and dried in an oven at 80 C for 24 h. The dried leaflet was then exposed to x-ray film (Kodak Blue Brand) for 72 h. White areas show presence of radioactivity. Circle identifies perimeter of abraded area.

RESULTS

Specificity. Housley *et al.* (8) showed that three amino acids (serine, lysine, and leucine) applied to an abraded spot were translocated with the same velocity as sucrose in the phloem of soybeans. We compared leucine to two other amino acids, glutamate and GABA,[4] which are both predominant amino acids in the phloem of soybean (9, 13). Using [^3H]leucine as a control on a source leaflet, [^{14}C]glutamate, [^{14}C]GABA, and [^{14}C]leucine were applied to the opposing leaflet. All three amino acids showed comparable translocation velocities and mass transfer rates but were metabolized quite differently in the plant (Table I). Whereas glutamate and GABA were rapidly converted to acidic and neutral compounds, leucine was not. About one-half of the labeled leucine in the sink region of the plant was recovered in the protein hydrolysate fraction, but only 15 and 10% of the total label from glutamate and GABA was incorporated into protein. Larson and Beevers (12) also found about one-half of the exogenous leucine applied to pea cotyledons incorporated into protein, but only 5% for glutamate. Glutamate was actively metabolized to CO_2, organic acids, and other amino acids. In spite of the extensive metabolism of GABA and glutamate, we believe that both amino acids were loaded directly into the phloem because a significant portion of the soluble label in the sink tissue was recovered as the free amino acid that was applied (GABA, 23%; glutamate, 17%). Because leucine recovery was much higher than glutamate or GABA, we used leucine in further experiments.

Metabolic Dependence. Metabolic inhibitors were applied to the abraded areas of source leaves before, during, and after the addition of labeled sucrose and leucine. The uncouplers, DNP and CCCP, effectively inhibited the translocation of 30 and 100 mM leucine and sucrose, but sucrose export was more sensitive than leucine export to inhibition by DNP (Table II).

PCMBS complexes with sulfhydryl groups and has been shown to be a specific and potent inhibitor of sucrose phloem loading and translocation (4). PCMBS at 1 mM inhibited sucrose translocation by about 90% and leucine translocation by about 65% (Table II).

The application of 0.8 M mannitol to sugar beet leaves resulted in partial plasmolysis of mesophyll cells without disturbing the translocation of exogenous sucrose (14). We found that 0.8 M sorbitol inhibited sucrose translocation slightly, but stimulated leucine translocation.

High K^+ concentrations inhibited sucrose phloem loading in sugar beet (5) and castor bean (11), presumably by depolarizing the proton gradient between phloem and free space. We found that 100 mM K^+ decreased sucrose and leucine translocation by about 50% (Table II).

Concentration Dependence. We examined the effect of leucine and sucrose concentration to determine if their concentration in the free space could be limiting translocation. Leucine translocation showed a triphasic saturation response to concentration (Fig. 2). The first phase was from 0 to 4 mM, the second from 4 to 40 mM, and the third from 40 to 100 mM. The latter concentration

[4] Abbreviations: GABA: γ-aminobutyric acid; DNP: 2,4-dinitrophenol; CCCP: carbonylcyanide-*m*-chlorophenylhydrazone; PCMBS: *p*-chloromercuribenzenesulfonic acid.

Table I. *Translocation of Exogenous Leucine, Glutamate, and GABA*

[^{3}H]Leucine (500 nmol, 30 μCi) was applied to an abraded area on a single source leaflet (leaf A) to act as a control. To an abraded area on the opposite leaflet (leaf B) was applied the indicated ^{14}C-amino-acid (500 nmol, 10 μCi). After 2 h, the source leaflets were removed and label in the remainder of the plant was determined. Labeled compounds were separated into basic (B), acidic (A), neutral (N), and protease-extractable label (P) fractions as described in the text.

Amino Acid	Transport Velocity	Mass Transfer Rate		% Total ^{14}C in				% Total ^{14}C Recovered
		Leaf A	Leaf B	B	A	N	P	
	cm/min	nmol cm^{-2} h^{-1}						
Leucine	1.3	21	22	54	2	0	44	90
Glutamate	1.2	30	24	39	28	18	15	76
GABA	1.0	23	28	28	53	9	10	81

Table II. *Effect of Inhibitor Treatments on Mass Transfer Rate of Exogenous Leucine and Sucrose*

To an abraded area on a single source leaflet was applied 50 μl of the indicated treatment in 5 mM phosphate buffer (pH 6.5). After 30 min, sufficient time to absorb the treatment solution, 50 μl of [^{3}H]leucine (6 μCi) and [^{14}C]sucrose (2 μCi), concentrations as indicated, in treatment solution was applied. After absorption of the labeled solution, treatment solution was applied to keep the area moist. After 2 h, the source leaflet was removed and the amount of label in the remainder of the plant was determined. Control was a separate plant.

Treatment	Experiment No.	Leucine[a] % of control		Sucrose[a] % of control	
		30 mM	100 mM	30 mM	100 mM
Control (no addition)	1	100		100	
	2	100		100	
	3		100		100
PCMBS, 1 mM	1	23		10	
	2	37		11	
	3		39		7
CCCP, 0.1 mM	1	51		47	
Sorbitol, 0.8 M	1	222		74	
	2	117		80	
KCl, 0.1 M	1	51		46	
DNP, 5 mM	1	40		5	
	2	22		11	
	3		56		12

[a] Control rates (nmol cm^{-2} h^{-1}) for leucine, experiments 1–3 were 42, 51, and 91, respectively, and for sucrose, experiments 1–3 and 91, 104, and 248, respectively.

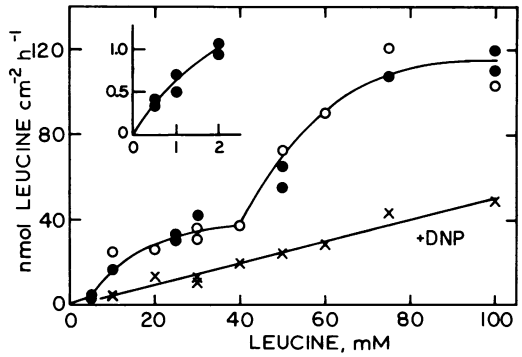

FIG. 2. Concentration dependence of mass transfer rate of exogenous leucine. One group of plants (n = 14, group I, ●) had two source leaflets. [^{14}C]Leucine (concentration as indicated) was applied to an abraded area on source leaf B (experimental leaf). To the opposite leaflet, source leaf A (control leaf), was applied 10 mM [^{3}H]leucine. After 2 h, source leaves were removed and label transported to the plant was determined. Control rate for [^{3}H]leucine (10 mM) transport was 22 ± 5 nmol leucine cm^{-2} h^{-1}. Another group of plants (n = 18, group II, ○) were used to measure leucine and sucrose transport simultaneously. These plants had one source leaf to which was applied varying concentrations of [^{3}H]leucine and [^{14}C]sucrose. Sucrose concentration was always four times the concentration of leucine. One half of these plants were pretreated for 30 min with 50 μl of 5 mM DNP (×).

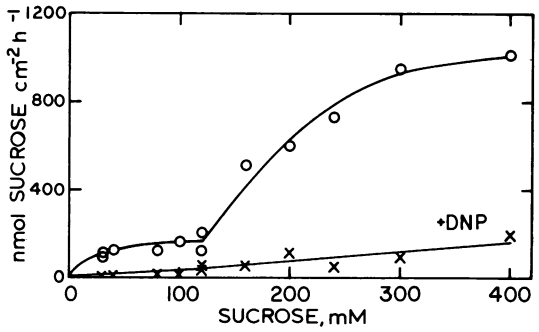

FIG. 3. Concentration dependence of mass transfer rate of exogenous sucrose. From plants of Figure 2 (group II and Table II) was determined the mass transfer rate of sucrose transport for the indicated concentration for control (○) and DNP-treated plants (×).

approaches the limits of solubility of leucine in H_2O at 25 C. The presence of many saturated phases in the concentration response curve is evidence that transport is mediated by carriers.

We found a biphasic concentration curve for sucrose translocation in soybean (Fig. 3) similar to that reported (14) for sugar beet. The first phase extended from 0 to 110 mM sucrose and had a K_m of about 35 mM (Table III), which is similar to the first phase K_m reported for sucrose loading into the phloem of sugar beet leaves (14) and leaf discs (4). The second phase extended from 110 to 400 mM with a K_m of 169 mM.

The concentration dependence of leucine incorporation into protein in the source (Fig. 4) differed markedly from the curve for translocation (Fig. 2). There is no evidence for more than one phase, and the K_m was 14 mM (Table III).

The application of DNP at 5 mM inhibited sucrose transport (Fig. 3) more than leucine transport (Fig. 2). All phases were inhibited by DNP.

DISCUSSION

These data further support the suggestion (8) that there is no barrier to the loading and translocation of most amino acids in the phloem when applied exogenously to the free space. Of the different amino acids applied to abraded leaves (8; Table I), all were translocated with similar velocities and mass transfer rates, when expressed on a gram-mole basis. We found no difference even though the amino acids differed significantly on the basis of charge, position of the amino group (α *versus* γ), and relative natural abundance in the phloem. There may be only one general carrier system or mechanism for loading of amino acids into the phloem. The possibility also exists that there may be a specific carrier for each amino acid. Although there is an over-all similarity between leucine and sucrose transport in terms of a multiphasic concentration dependence, a metabolic requirement, and similar translocation velocities, the kinetic data of vein loading are quite

Table III. *Kinetic Parameters of Sucrose Translocation, and Leucine Translocation and Incorporation into Protein*

	Concentration Range	K_m	V_{max}
	mM	mM	nmol cm^{-2} h^{-1}
Leucine translocation[a]	0 to 4	3	2
	4 to 40	21	64
	40 to 100	52	140
Leucine incorporation into protein[b]	0 to 100	14	77
Sucrose translocation[c]	0 to 110	35	213
	110 to 400	169	1273

Calculated from double reciprocal plots of data in:
[a] Figure 2.
[b] Figure 4.
[c] Figure 3.

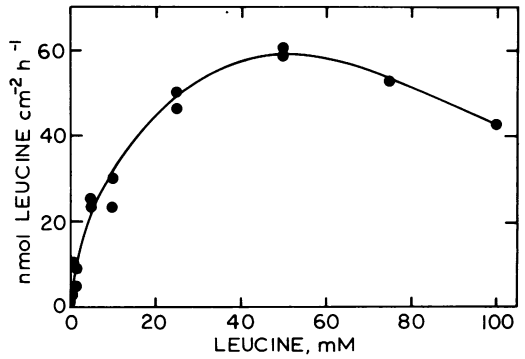

FIG. 4. Concentration dependence of [^{14}C]leucine incorporation into protein in the source leaf. Experimental source leaves (source leaf B) from plants used in Figure 2 (group I) were analyzed for protease-extractable label.

different, indicating that different carrier systems are involved.

The soybean plant has the ability to translocate exogenous leucine and other amino acids at relatively low concentrations (4–40 mM) at rates on a mole basis only two to three times lower than sucrose. However, leucine is not one of the amino acids found in high concentration in the phloem (9, 12) in soybean. These data may help to explain this apparent anomaly. Generally amino acids which are in high concentration in the leaf are also of high concentration in the phloem. The exception to this is GABA which is of significantly higher concentration in the petiole than in the leaf, compared with other amino acids (9, 13). At very low concentrations of leucine, 0 to 4 mM, the phloem-loading system for leucine has a very low capacity; the capacity for leucine incorporation into protein is much higher (Table III). Therefore, most of the leucine synthesized in the mesophyll leaf cells fulfills the protein synthesis requirement. When leucine concentration increases above 4 mM, the transport system can compete favorably with protein synthesis for leucine (Table III). This situation may occur during leaf senescence when higher concentrations of amino acids are present because of extensive protein hydrolysis.

Inhibition of sucrose translocation by uncouplers (3, 5, 11, 14) has been used as evidence that the first phase of sucrose loading into the phloem is entirely an energy-requiring process. Sovonick *et al.* (14) found that 4 mM DNP inhibited translocation of exogenous sucrose by about 80% and this inhibition was reversed by the addition of ATP. At 8 mM, DNP irreversibly inhibited sucrose transport. We found that 5 mM DNP inhibited the translocation of sucrose by 80 to 90% and leucine transport by 50 to 80% over the entire range of concentrations used (Figs. 2 and 3). Since all phases of transport were inhibited, we conclude that all phases of sucrose and leucine transport are energy-dependent or sensitive to compounds which dissipate proton gradients across membranes. Giaquinta (4) showed that PCMBS, a sulfhydryl group inhibitor that does not penetrate membranes, is a potent inhibitor of vein loading of sucrose in sugar beet. He concluded that sucrose was actively accumulated in the phloem from the apoplast and that membrane sulfhydryl groups were involved in the process. Because PCMBS inhibited both sucrose and leucine transport in this study, sulfhydryl groups may be important for amino acid loading as well. Sovonick *et al.* (14) showed that 0.8 M mannitol severely inhibited photosynthesis and photosynthate translocation but had little effect on the transport of exogenous sucrose. We found that 0.8 M sorbitol inhibited sucrose transport slightly, but stimulated leucine transport. The reason for this stimulation is not known. It is possible that the high osmoticum plasmolyzed mesophyll cells permitting a faster penetration of the leucine into the free space. Regardless of the reason for this stimulation, these data support the role of the apoplast as the primary route for amino acid loading.

Sucrose and leucine translocation show many similarities in their response to inhibitors. These inhibitors could be affecting amino acid translocation by inhibiting some process common to both amino acid and sucrose loading or they may inhibit amino acid transport indirectly by inhibiting sucrose translocation directly. In the latter case, amino acid transport could be dependent on or limited by sucrose loading to provide the driving force for solute movement in the phloem (8). We found that the mass transfer rate of leucine translocation was the same whether measured with or without exogenous sucrose at four times the amino acid concentration (Fig. 2). In another experiment, exogenous sucrose at 100 mM had no effect on the translocation of 10 mM leucine (data not shown). If amino acid transport were dependent on sucrose transport, then some stimulation of amino acid transport should have been apparent at higher sucrose concentrations. Factors other than sucrose translocation must be limiting amino acid transport. It seems probable that availability or an amino acid-loading system or both regulate amino acid transport.

These data are consistent with a model of phloem loading of sucrose driven by an ATPase-generated proton gradient across the sieve tube plasmalemma (6). According to this model, sucrose movement into the phloem is mediated by a specific carrier and occurs with simultaneous co-transport of a proton which partially neutralizes the proton gradient across the sieve tube plasmalemma. To reestablish the proton gradient, ATP is hydrolyzed by a plasmalemma ATPase and protons are extruded from the sieve tube into the free space. We propose that separate carrier-mediated loading systems exist for sucrose and amino acid loading into the phloem. These carrier systems show some specificity for their respective substrates and have a different K_m and V_{max}. Because each carrier system works independently of the other, rates of loading are dependent on the specific kinetic parameters of the carrier and the concentration of substrate in the free space. Ultimately the driving force for loading of sucrose and amino acids is dependent on the membrane-bound ATPase which generates the proton gradient across the sieve tube plasmalemma. This model is consistent with the different kinetic parameters measured for leucine and sucrose translocation (Table III). Loading of both leucine and sucrose is sensitive to sulfhydryl group inhibitors which could inhibit the plasmalemma ATPase and uncouplers which could neutralize the proton gradient across the plasmalemma. Further experiments are needed to confirm the specificity of the amino acid carrier system, its dependence on the ATPase-generated proton gradient, and the accumulation of amino acids against a concentration gradient.

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