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Characterization of Amino Acid Efflux from Isolated Soybean Cells[1]

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ABSTRACT

Cells from reproductive soybean (*Glycine max* [L.] Merr.) plants were isolated using a mechanical-enzymic technique that produced a high yield of uniform, physiologically active cells. Cells were incubated in a pH 6.0 buffered solution and subjected to various treatments in order to determine the nature of net amino acid efflux. Total net amino acid (ninhydrin-reactive substances) efflux was not affected by the following conditions: (a) darkness, (b) aeration, (c) K^+ concentrations of 0.1, 1.0, 10, or 100 millimolar and (d) pH 4, 5, 6, 7, or 8. The Q_{10} for net amino acid efflux between 10°C and 30°C was 1.6. Thus, it seems that net amino acid efflux requires neither current photosynthetic energy nor a pH/ion concentration gradient. Amino acid analyses of the intra- and extracellular fractions over time showed that each amino acid was exported linearly for at least 210 minutes, but that export rate was not necessarily related to internal amino acid pools. Amino acids that were exported fastest were alanine, lysine, leucine, and glycine. Addition of the inhibitor *p*-chloromercuriphenyl sulfonic acid, 3(3,4-dichlorophenyl)-1,1-dimethylurea, or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone increased the rate of total amino acid efflux but had specific effects on the efflux of certain amino acids. For example, *p*-chloromercuriphenyl sulfonic acid greatly enhanced efflux of γ-aminobutyric acid, which is not normally exported rapidly even though a high concentration normally exists within cells. The data suggest that net amino acid efflux is a selective diffusional process. Because net efflux is the result of simultaneous efflux and influx, we propose that efflux is a facilitated diffusion process whereas influx involves energy-dependent carrier proteins.

Mature soybean seeds contain about 6% N, primarily as storage protein. Approximately half of this N is remobilized (translocated) from vegetative tissue when the plant has a reduced capacity for assimilating atmospheric and soil N during the time of rapid seed growth (9). This syndrome of satisfying the N demand by degradation of leaf protein and the subsequent remobilization of N to the seeds has been referred to as the "self destruction" hypothesis by Sinclair and de Wit (21). Attempts to overcome this "self destruction" and improve soybean yields by using soil or foliar N fertilizer applications have been inconclusive (3, 4, 7, 25). This suggests an inability of the leaf to absorb and/or assimilate the applied N, or a limitation on N transport within the plant.

Because the soybean seed is so highly dependent upon leaves for its N, the leaves must be able to export large amounts of nitrogenous compounds. In fact, Schrader and Thomas (17) have postulated that one molecule of amino acid should be transported to the seed for each molecule of sucrose. To what extent these compounds originate from leaf cells or are transferred to the pod-feeding phloem directly from the leaf-feeding xylem remains unknown, although it was recently suggested that during early pod fill up to 50% of the nitrogen in the seeds was directly transferred (12). Nevertheless, it is important to know the ability and capacity of cells to export nitrogenous compounds, the nature of the export, and the identity of the substances exported.

Use of cells isolated from leaf blades provides a convenient experimental system for studying transport. Whereas in normal tissue transport can occur through both the apoplast and the symplast, in isolated cells only transport into the apoplast can occur. Thus, there are no confounding effects between the two transport systems. Furthermore, environments and treatments can be easily and precisely administered and controlled. The primary objective of this study was to determine the nature of amino acid export into the apoplast from soybean leaf cells isolated during a period when developing seeds are highly dependent upon leaf cells for N. Our interests were aimed particularly at learning whether cells have the ability to transport sufficient N to meet the high demand of seeds and to characterize this export.

MATERIALS AND METHODS

Plant Material. Individual, inoculated soybean (*Glycine max* [L.] Merr.) plants were grown in 4-L pots containing a mixture of soil:sand:peat (2:1:1, v/v/v) either outdoors or in a greenhouse. When necessary, the plants were sprayed with Plictran® (tricyclohexyltin hydroxide) to control spider mites and Orthene® (acephate [*O*, *S*-dimethyl acetylphosphoamidothioate]) to control thrips. To prevent branching, we removed axial buds as they appeared during vegetative growth.

Cell Preparation. Leaves were harvested from plants during pod filling. At each sampling, we removed a trifoliolate leaf from the midsection of the stem from each of two plants. After removing the midribs, we finely diced the leaves, pooled the leaf pieces and vacuum infiltrated them three times (15 s each) in 25 ml of 20 mM Mes-NaOH, pH 6.0, 2% (w/w) PVP-40, 1% (v/v) Glusulase (Endo Laboratories, Garden City, NY), and 3% (w/v) Macerase (Calbiochem). The addition of Glusulase increased cell yield by about 50%. The Macerase was partially purified by dissolving 0.75 g in 1.5 ml 20 mM Mes-NaOH (pH 6.0), then centrifuging it for 5 min at 250 *g*, and finally passing the supernatant fluid through a 50-ml column (2.6 × 10 cm) of Bio-Gel P-6 (Bio-Rad) equilibrated with 20 mM Mes-NaOH (pH 6.0). The eluant containing the Macerase was used as a solvent for the infiltration medium.

After vacuum infiltration, the solution containing the leaf

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pieces was poured into a maceration apparatus similar to that described by Servaites and Ogren (19), except we replaced the 430-μm mesh filter, which separates the leaf material from the cells, with a 157-μm mesh filter. The 157-μm mesh prevented the passage, and subsequent collection, of large aggregates of unseparated cells. Fifty ml of maceration medium (same as the infiltration medium except that 450 mM sorbitol was included to give a final concentration of 300 mM) were added to the apparatus after which cell collection was started. Cells collected during the 1st min were discarded; thereafter, we collected cells approximately every 15 min for 1 h. After each collection, we gently washed the cells from the 20 μm collection mesh with about 5 ml of the incubation medium containing 50 mM Mes-NaOH (pH 6.0), 200 mM sorbitol, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM K_2HPO_4, 10 mM $NaHCO_3$, and 0.1% PVP-40. The inclusion of the PVP-40 was necessary to maintain the membrane integrity of more than 95% of the cells as judged by cellular exclusion of Evans blue dye (6). The PVP-40 was shown not to interfere directly with the action of the Evans blue (data not presented). Cells were aerated and kept on ice until the final collection, after which they were washed and centrifuged three times at 40 g for 3 min. The cells were then counted using a hemocytometer and the volume adjusted to render the desired final cell concentration (usually 2×10^6 cells $\cdot$ ml^{-1}).

Experimental Conditions. Unless otherwise noted, the following conditions and techniques were employed. Cells were incubated in aerated vials (either 8 or 20 ml) in a shaking water bath at 25°C with supplemental incandescent irradiance supplying a photosynthetic photon flux density of 900 μmol s^{-1} m^{-2} at the water level. All experiments were repeated at least twice, usually with two replications per experiment. Replications within an experiment represent separate incubations (treatments) of cells rather than multiple subsampling of one incubation.

Efflux was measured as the release of substances from cells into the incubation (bathing) medium. This efflux is net efflux, which is the result of concurrent uptake and export. Most experiments were started by adding one volume of cells to four volumes of incubation medium. At certain intervals during the incubation period, 500-μl aliquots were removed from the vials and centrifuged at 3000 g for 20 s. The supernatant fluid was then carefully removed using a Pasteur pipette and frozen until analyzed for amino acids (usually within 24 h). For those experiments in which intracellular contents were analyzed, we dissolved the pellet in 500 μl of a solution containing methanol:chloroform:water (12:5:3, v/v/v), heated at 40°C for 1 h, and then centrifuged for 1 min at 10,000 g. The supernatant fluid was then added to a test tube containing 400 μl H_2O and 500 μl $CHCl_3$. Following a low force centrifugation (250 g for 10 min), the phases were allowed to separate overnight at 4°C. The clear, aqueous phase was collected, dried at 40°C *in vacuo* or lyophilized, and the residue was dissolved in 400 μl of incubation medium, after which aliquots were removed for analysis.

Procedural modifications were necessary in experiments in which amino acids were identified. Larger aliquots (usually 2 ml) were removed from the incubation solution and processed by centrifugation and extraction as described above. The efflux and cellular samples were passed through a 2 ml Dowex 50-X8 (200–400 mesh) column, equilibrated as described by Atkins and Canvin (2). Before the samples were passed through the column, 20 μl of 250 μM norleucine were added to each sample as an internal standard so we could calculate the efficiency of amino acid recovery during sample preparation and analysis. After a sample was added to the Dowex column, the resin was washed twice with 5 ml H_2O, after which the amino acids were eluted by washing the column three times with 5 ml 2 N NH_4OH. The eluted amino acids in the 15 ml NH_4OH were freeze-dried and then dissolved in 100 μl Li citrate sample dilution buffer (Pierce Chemical) before being acidified with 1 μl 5% sulfosalicylic acid, centrifuged for 5 min at 6000 g, and sampled for amino acid analysis. It is important to note that for these experiments the cell incubation medium was buffered with 5 mM, instead of 50 mM, Mes-NaOH. The higher concentration of Mes-NaOH interfered with the ion exchange chromatography.

Ninhydrin-reactive compounds (primarily amino acids) were assayed according to the method of Lee and Takahashi (13). Amino acid analyses were performed with a Dionex amino acid analyzer using a five-step pH/ion concentration gradient elution system. At the end of each experiment, aliquots of cells were removed from the vials and Chl extracted in 80% ethanol and determined by the method of Wintermans and deMots (27).

The cells were bathed in an incubation medium at pH 6.0, a pH that closely resembles that of the apoplast (26). This pH, however, presents a problem with regard to the amount of CO_2 in solution (Fig. 1). Thus, even though we added 10 mM $NaHCO_3$ to the solution, we aerated the cells to ensure that a constant CO_2 concentration would be maintained. The fact that a lowering of one pH unit results in a 10-fold loss of CO_2 in solution (23) is a major consideration when conducting experiments with varying pH.

Cell viability was judged by four methods (data not shown). The cells did not take up Evans blue dye. Second, the cells were photosynthetically active. At saturating levels of $NaH^{14}CO_3$ (20 mM, 1.7 kBq $\cdot$ nmol^{-1}), the cells incorporated ^{14}C linearly for at least 60 s. In addition, at pH 7.8, the cells showed CO_2-dependent O_2 evolution when incubated in an O_2 electrode apparatus (Hansatech, Kings Lynn, U.K.). Third, the cells were able to incorporate very low concentrations of [^{35}S]methionine into TCA-precipitable compounds. Last, the cells excluded [^{14}C]sorbitol.

RESULTS

Net amino acid efflux rates were calculated from the regression of amount of amino acids in the incubation solution over time. The y intercept represents extracellular amino acids transferred with the cells to the incubation solution at time 0.

The rate of net amino acid efflux was linearly related to cell concentration. Cells at three concentrations (1×10^6, 3×10^6, and 6×10^6 cells $\cdot$ ml^{-1}) were incubated for 150 min and sampled at 30, 90, and 150 min. The net efflux rates were 15.0, 13.3, and 13.3 nmol amino-N $\cdot$ h^{-1} $\cdot$ 10^{-6} cells, indicating that the rate of net efflux is proportional to cell concentration. For most experiments we used a cell concentration close to 2×10^6 cells $\cdot$ ml^{-1}.

Several experiments were conducted to determine the importance of current photosynthetic energy on efflux. Cells were

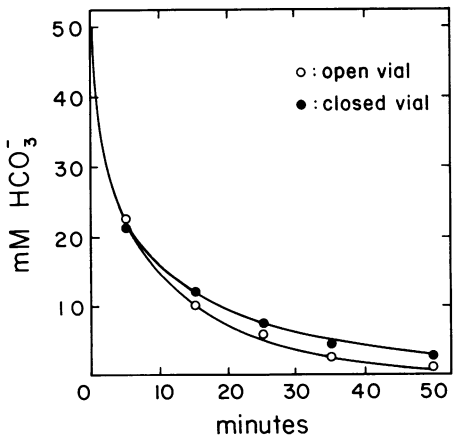

FIG. 1. Loss of CO_2 from pH 6.0 solution. Radioactivity was determined in aliquots from open and closed vials containing 50 mM $NaH^{14}CO_3$ (0.7 Bq $\cdot$ nmol^{-1}) in 50 mM Mes-NaOH (pH 6.0).

incubated in light or dark, as well as with or without aeration. Neither aeration nor light affected net amino acid efflux rates (Fig. 2). The rate of net amino acid efflux was 8.4 ± 0.7 nmol amino-N·h^{-1}·10^{-6} cells for aerated cells *versus* 7.2 ± 0.5 nmol amino-N·h^{-1}·10^{-6} cells for nonaerated cells. Net amino acid efflux rates for aerated light and dark treatments were 16.6 ± 3.9 and 15.8 ± 5.1 nmol amino-N·h^{-1}·10^{-6} cells, respectively. Thus, the data indicate that current photosynthesis is not a requirement for net amino acid efflux. Because aeration had no effect on efflux, we discontinued aerating the cells during incubation, although we continued to illuminate them.

To determine the effect of pH on net efflux, we buffered the cell incubation medium with a mixture containing 20 mM each of Mes, Hepes, and Tris and then adjusted the pH with either HCl or NaOH such that the desired pH would be obtained after the cells were added to the solution. As shown in Figure 3, pH had no significant effect on net amino acid efflux.

The effect of external K^+ concentration was tested by incubating cells in solutions containing 0, 0.5, 5.0, and 50 mM K_2SO_4 and replacing the K_2HPO_4 in the incubation solution with Na_2HPO_4. The differences in efflux rates among the treatments were small (Fig. 4) and the results of other experiments indicated that K^+ had no consistent effect, suggesting that K^+ concentration does not affect net amino acid efflux.

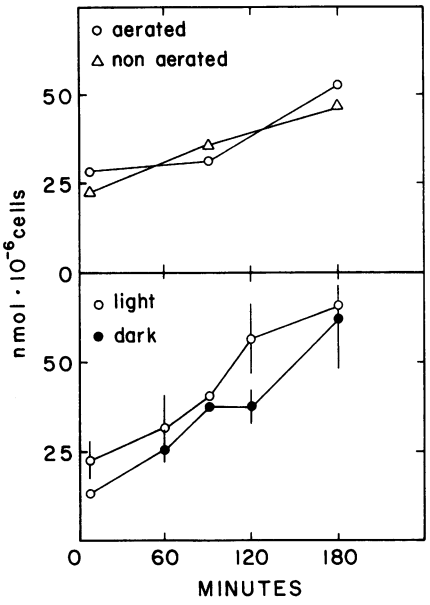

FIG. 2. Effect of aeration and darkness on net amino acid efflux. Values are means ± SE from one experiment (two replications) for the aeration/nonaeration data and from two experiments (two replications per experiment) for the light/dark data. Chl content was 40.3 μg·10^{-6} cells for the aeration/nonaeration experiment and averaged 47.9 ± 1.9 μg·10^{-6} cells for the light/dark experiments.

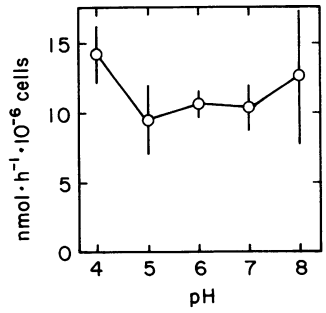

FIG. 3. Effect of pH on the rate of efflux of net amino acids. Rates were determined from aliquots sampled after 30, 90, and 150 min of incubation. Each value is the mean ± SE of two experiments (two replications per experiment). Mean Chl content was 42.0 ± 2.0 μg·10^{-6} cells.

The influence of temperature was studied by incubating cells at 10, 20, 30, and 40°C for 210 min and sampling at 30, 90, 150, and 210 min. Net amino acid efflux was linear for 210 min; efflux rates are presented in Table I. The Q_{10} values varied from 1.4 for the 10 to 20°C range to 2.7 for the 30 to 40°C range; however, the Q_{10} from 10 to 30°C, the physiological range for soybean plants, was 1.6. When the data were used in an Arrhenius equation, an activation energy of 11.6 kcal·mol^{-1} was calculated for the flux of amino acids through the plasma membrane.

To further characterize amino acid efflux, we subjected the cells to incubation media containing different inhibitors. As shown in Table II, 50 μM FCCP[3] has the greatest effect on increasing efflux followed by 1 mM PCMBS. The effect of 1 mM DCMU was variable; in some experiments it increased net amino acid efflux, but never as much as did 1 mM PCMBS, whereas in other experiments its effect was negligible compared to the control.

Changes in both the intra- and extracellular amounts of each amino acid after 0, 30, 90, 150, and 210 min of incubation were determined by amino acid analyses (Fig. 5). It is important to note the different scales on the ordinate in Figure 5; for example, the efflux rate of γ-aminobutyric acid was similar to that for

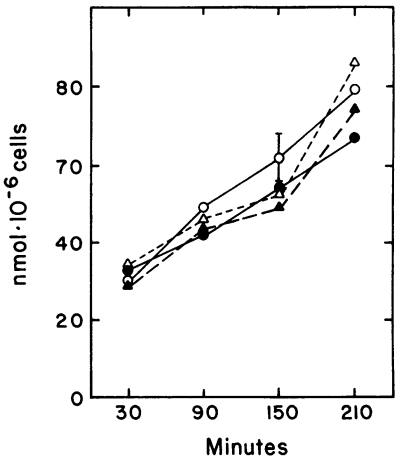

FIG. 4. Effect of K^+ concentration on net amino acid efflux. Cells were prepared and washed in K^+-free solutions and then placed in incubation media containing K_2SO_4 to make a final K^+ concentration of 0 (○), 1.0, (●), 10 (△), or 100 (▲) mM. Each value represents the mean from two experiments. Cells were not aerated. Mean Chl content was 35.0 ± 1.7 μg·10^{-6} cells.

Table I. *Effect of Temperature on Net Amino Acid Efflux*

Cells (34.9 μg Chl·10^{-6} cells) were sampled after 30, 90, 150, and 210 min of incubation. Values are means ± SE from one experiment with two replicates.

Temperature	Net Efflux Rate
°C	*nmol·h^{-1}·10^{-6} cells*
10	9.1 ± 1.7
20	12.6 ± 0.8
30	24.6 ± 5.2
40	65.8 ± 9.8

[3] Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; PCMBS, *p*-chloromercuriphenyl sulfonic acid; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

Table II. *Effect of Various Inhibitor Treatments on Net Amino Acid Efflux*

Cells were placed in an incubation medium containing the inhibitor and sampled after 30, 90, and 150 min. Each value is a mean ± SE from one of two experiments. Each experiment had two replications. Chl content of the two experiments was 31.1 ± 1.0 $\mu g \cdot 10^{-6}$ cells.

Treatment	Experiment	Net Efflux Rate
		nmol·h⁻¹·10⁻⁶ cells
Control	1	17.4 ± 2.3
50 μM FCCP	1	95.5 ± 3.1
100 μM PCMBS	1	18.6 ± 1.1
1 mM PCMBS	2	38.6 ± 2.2
1 mM DCMU	1, 2	18.7 ± 0.9
0.5% Ethanol	1	17.5[a]

[a] No SE available.

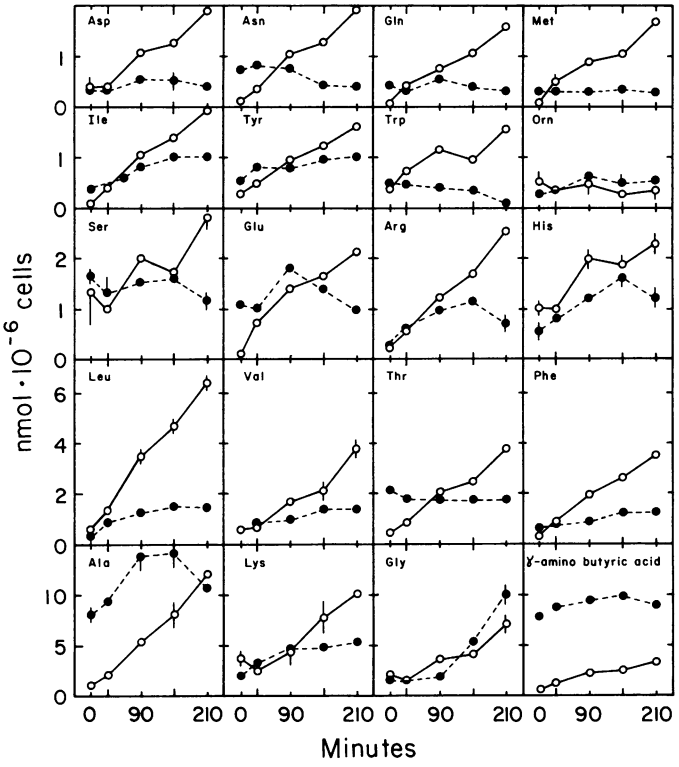

FIG. 5. Changes in intracellular (●) and extracellular (○) amino acid content during a nonaerated 210-min incubation period. Aliquots were removed at 0, 30, 90, 150, and 210 min and processed as described in Materials and Methods. Values are means ± SE of two replicates from an experiment. Cell concentration was 1.5 × 10^6 cells·ml^{-1} with a Chl content of 41.2 $\mu \cdot^{-6}$ cells.

arginine. Efflux patterns for all amino acids except ornithine were generally linear, but with varying rates. The fastest rates (nmol·h^{-1}·10^{-6} cells) were exhibited by alanine (3.20), lysine (2.09), leucine (1.57), and glycine (1.50). Not all the amino acids began to be exported from time 0; for example, aspartic acid, serine, lysine, glycine, valine, and histidine all seemed to have a lag period before linear export commenced. Whether this represents changes in the transport mechanism (activation of export system, inactivation of uptake system), or metabolic changes is unknown.

Intracellular amino acid pools (Fig. 5) showed more individual trends than did efflux. This can be partly explained by intracellular metabolic conversions occurring within cells during incubation. The observation that internal pools of some amino acids did not change greatly during incubation may be due to regulation of amino acid metabolism, differential efflux, or protein degradation. Some amino acid pools displayed temporal changes. The intracellular amounts of arginine, histidine, and alanine increased and then declined, wheres glycine began to show a rapid linear increase after 90 min. Although it is tempting to conjecture about the changes in and among various pool sizes, the complex interrelationships among amino acid metabolic pathways preclude any definitive statement regarding the causes for observed changes in pool sizes. The primary purpose of measuring the intracellular content of each amino acid was to compare it with the rate of efflux. In this experiment, extracellular amounts of most amino acids became higher than intracellular amounts. However, when converted to a volume basis (10^6 cells ≃ 70 μl packed volume; thus, 1 ml of cell suspension of 10^6 cells·ml^{-1} contains 0.070 ml cells and 0.930 ml buffer), the concentration of each amino acid was greater within cells, resulting in an outward concentration gradient. Large differences existed among amino acids with regard to distribution between internal and external pools. For example, after 210 min of incubation, 82% of the total amount of leucine was outside cells, whereas only 26% of the γ-aminobutyric acid was outside cells. Thus, even though a concentration gradient existed toward the outside, efflux seemed to be a controlled phenomenon. This suggests that a selective transport (uptake or export) system is operative or availability for transport differs among amino acids.

To compare the transport characteristics of each amino acid, we subjected cells to three inhibitors for 210 min and then identified the amino acids in the intra- and extracellular fractions (Table III). Regardless of their specific modes of action, all inhibitors caused a decline, but to varying degrees, in the internal pools of all amino acids except γ-aminobutyric acid, tryptophan, ornithine, lysine, and asparagine. The decline in internal pools may have resulted from an increase in export, a reduction in amino acid synthesis, or a rapid conversion to another molecule. Declines in the internal pools were not necessarily related to increases in the external pool sizes. In fact, the effect of an inhibitor on an external pool depended very much on the amino acid. For example, whereas PCMBS greatly increased the net efflux of alanine and γ-aminobutyric acid, it had almost no effect on glutamic acid or glutamine relative to the control treatment. An important consideration, though, is that changes in efflux may result from changes in internal pools of each amino acid, which may have been affected by the inhibitors. In other words, the inhibitors may have an indirect effect on efflux through changes within the cell. Perhaps a more meaningful way of examining the efflux data would be in terms of the external partitioning of each amino acid relative to its total amount (Table III).

The addition of FCCP, an uncoupler of phosphorylation, DCMU, an inhibitor of photosynthetic electron transport, or PCMBS, which inactivates proteins containing sulfhydryl groups, generally increased the external partitioning of amino acids but had differential effects on the actual pool sizes. For example, PCMBS caused a large increase in the external partitioning of glutamine, alanine, and γ-aminobutyric acid. There was little change in the total amount of alanine; it accumulated outside the cell at the expense of the internal pool. On the other hand, unlike alanine, the increase in the export of γ-aminobutyric acid was accompanied by an increase in its internal pool. This could suggest that the export of γ-aminobutyric acid is sensitive to intracellular amounts available for export, whereas alanine export is independent of internal pools and its net export rate may depend on another factor such as a balance between an uptake and export mechanism. Although the data do not prove this, it is suggested by the action of PCMBS, which is believed to inactivate sulfhydryl-dependent, membrane-bound carriers. Ad-

Table III. *Effects of Various Inhibitors on Intra- and Extracellular Amino Acid Content*

Cells were incubated for 210 min in 50 μM FCCP, 1 mM DCMU, 1 mM PCMBS, or buffer (control) and then processed as described in Materials and Methods. Cell concentration was 1.43×10^6 cells·ml^{-1} with a Chl content of 46.5 μg·10^{-6} cells.

Amino acid	Intracellular				Extracellular				Percentage Outside Cells[a]			
	Control	FCCP	DCMU	PCMBS	Control	FCCP	DCMU	PCMBS	Control	FCCP	DCMU	PCMBS
	nmol·10^{-6} cells								%			
Asp	1.08	0.41	0.58	0.40	0.88	1.01	1.10	1.06	45	71	65	73
Thr	2.38	1.76	1.56	1.56	1.41	2.18	1.76	2.61	37	55	53	63
Ser	3.07	1.58	2.04	2.06	1.93	2.08	2.39	2.88	39	57	54	58
Asn	0.64	0.94	1.15	1.24	0.94	1.35	1.09	1.41	59	59	49	53
Glu	3.79	1.13	0.63	1.24	1.29	1.97	1.88	1.21	25	64	75	49
Gln	3.43	2.75	0.86	0.43	1.11	1.03	1.06	1.13	24	27	55	72
Gly	10.26	1.91	2.17	2.27	7.28	5.01	4.81	4.37	42	72	69	66
Ala	16.47	14.93	9.37	4.33	5.82	13.43	12.18	19.00	29	47	57	81
Val	2.35	0.96	0.93	0.93	2.00	2.14	2.01	1.57	46	69	68	63
Met	0.13	0.06	0.10	0.12	0.39	0.62	0.57	0.17	75	91	85	60
Ile	1.50	0.56	0.65	0.43	1.12	1.16	1.06	0.82	43	67	62	66
Leu	2.51	0.80	0.87	0.74	3.14	3.60	3.29	1.77	56	82	79	71
Tyr	1.01	0.65	0.50	0.53	0.96	2.09	0.94	0.67	55	76	65	56
Phe	1.71	0.67	0.68	0.69	1.52	2.81	1.69	1.18	50	81	71	63
Gaba[b]	8.45	9.55	6.61	14.14	2.27	3.43	3.36	10.83	21	26	34	43
Trp	0.22	0.38	0.49	0.56	0.85	1.26	0.95	1.68	79	76	63	73
Orn	1.38	1.62	1.79	2.01	0.85	1.40	0.99	1.50	38	46	36	43
Lys	4.62	5.21	4.35	4.39	6.00	9.14	6.45	7.07	56	64	60	62
His	2.88	2.09	2.35	2.08	3.08	3.26	2.57	2.52	52	61	52	55
Arg	0.94	0.64	0.78	0.63	1.73	1.94	1.71	1.20	65	75	69	66
Total	68.62	47.25	38.46	40.78	44.57	60.91	51.86	64.64	39[c]	56	57	61

[a] $\frac{\text{nmol outside}}{\text{nmol inside + nmol outside}} \times 100$

[b] γ-Aminobutyric acid.

[c] Calculated from total amino acids.

ditional support that alanine export may be independent of its internal pool is shown by addition of FCCP, which increased export without affecting the internal pool.

DISCUSSION

We have demonstrated that isolated soybean leaf cells have the ability to export amino acids into a bathing medium void of these compounds. We observed that the efflux rates varied among days, suggesting that effects associated with plant age and/or environmental conditions may influence efflux.

Because isolated cells lack symplastic connections and are not associated with vascular tissue, export of compounds from the cells must occur apoplastically. We observed that net amino acid efflux was generally about 0.250 nmol amino-N·min^{-1}·10^{-6} cells, which converts approximately to a loss of soluble leaf protein at the rate of 100 μg protein·day^{-1}·cm^{-2} leaf area (10^6 cells ≃ 35 μg Chl; 50 μg Chl ≃ cm^2 leaf area). This rate of protein loss compares favorably with reported values for soybean leaves (15, 18, 20, 28). Thus, our experimental system seems to be physiologically relevant to whole-plant studies. Furthermore, our results indicate that amino acid export into the apoplast can account for soluble protein loss in leaves.

The data suggest that amino acids are exported from soybean cells via a selective diffusional process. Several lines of evidence point to this conclusion. First, treatments that decreased or eliminated photosynthesis did not significantly change the rate of efflux. Second, pH and K^+ concentration did not influence efflux, suggesting that an energy-requiring proton/ion pumping mechanism is not essential for amino acid efflux. A third line of evidence is that temperature effects produced a Q_{10} indicating a passive process. Although the Arrhenius equation showed an energy of activation suggesting metabolic energy dependence for efflux, an equally valid interpretation is that the process must overcome a barrier, which in this case is the plasma membrane (14).

The amino acid analyses of the intra- and extracellular fractions over time (Fig. 5) revealed that amino acids were exported at different rates. Whereas we observed that alanine, lysine, leucine, and glycine were exported at the fastest rates, other researchers have reported somewhat different results. A summary of the $^{14}CO_2$ leaf feeding experiments of Housley *et al.* (11) and the petiole phloem sap amino acid analyses by Layzell and LaRue (12) indicates that the amino acids in greatest concentration in the petioles of nodulated soybean plants are asparagine, glutamic acid, serine, alanine, and aspartic acid. The differences between this report and earlier reports may be due to differences in plant age (we used older plants), preferential uptake of amino acids by phloem, metabolic conversions within the phloem, or differences among experimental systems (cells *versus* leaves). The differences in sap composition between petiole and fruit tip phloem in the data of Layzell and LaRue (12) suggest that conversions or transfers occur within the petiole or that developing seeds selectively withdraw amino acids from fruit phloem. In addition, there is evidence that enzymes are present in phloem sap (5, 29). Selectivity of amino acid loading into the phloem was studied by Housley *et al.* (10), who concluded that no barrier existed to prevent entry into the phloem of leucine, lysine, or serine when they were applied to an abraded spot on a soybean leaflet. Thus, if phloem uptake is nonselective and petiole phloem exudate composition is different than what is exported from leaf cells, it seems as though xylem to phloem transfer and/or metabolic conversions within the phloem are important factors determining the final composition of phloem sap.

Intracellular concentration was not necessarily an indicator or

a direct determinant of the rate of efflux. As mentioned in the Results section, even though more of an amino acid was outside the cell, its concentration was greater within the cell. In fact, in some experiments (Table III), intracellular amounts were greater. In Figure 5, one can see that no overall relationship existed between the rate of efflux and the intracellular pools. A good example is γ-aminobutyric acid, which always constituted a major portion of the total intracellular amino acid pool, yet always had a slow net export rate. When compared with γ-aminobutyric acid, alanine, which had a fast export rate and a large intracellular pool, seemed to have a different export control mechanism. That there was no direct relationship between intracellular amino acid concentration and export, particularly among amino acids with similar chemical and physical properties, rules out simple diffusion through the membrane. Possibly, some amino acids are sequestered within the cell and are not available for export.

Results of the inhibitor studies (Tables II and III) suggest that carriers are involved in net amino acid efflux. The inhibitors increased the rate of net efflux, but not equally among the amino acids. Had the inhibitors injured the integrity of the membranes, one would have observed large losses in intracellular amino acid pools accompanied by equally large extracellular increases. If amino acid efflux were solely dependent on carrier proteins with sulfhydryl groups, the addition of PCMBS would have decreased, not increased, the rate of amino acid efflux. In studies using leaf discs, PCMBS has been shown to increase the release of valine (24), but to decrease sucrose release (1). There is considerable evidence in the literature that amino acid uptake occurs via a proton-mediated carrier system (8, 16, 22, 24); thus it seems that the increased net efflux observed with inhibitors may have resulted from decreased uptake of amino acids.

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