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Rapid Accumulation of γ-Aminobutyric Acid and Alanine in Soybean Leaves in Response to an Abrupt Transfer to Lower Temperature, Darkness, or Mechanical Manipulation[1]

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ABSTRACT

Soybean (*Glycine max* [L.] Merr) leaves contain a low level (0.05 micromole per gram fresh weight) of γ-aminobutyric acid (Gaba) but the concentration of this non-protein amino acid increased to 1 to 2 micromoles per gram fresh weight within 5 minutes after transfer of plants or detached leaves from 33°C to 22°C or lower temperatures. A parallel decrease occurred in the concentration of glutamate. Accumulation of Gaba was also triggered by mechanical damage to the soybean leaves, but in plants subjected to a gradual reduction in temperature (2°C per minute) only a small increase in Gaba occurred. A rapid increase in the concentration of alanine and decrease in glycine occurred upon transfer of the soybean plants to darkness and was not influenced by temperature. When plants were returned to normal growing conditions, all changes in amino acid concentrations were fully reversed in 1 hour.

In soybean leaf discs incubated with [^{14}C]glutamate, a rapid accumulation of [^{14}C]Gaba was detected, and glutamate decarboxylase activity of the soybean leaf considerably exceeded (>30-fold) that of Gaba pyruvate transaminase. Part of the transaminase was localized in the mitochondria, but glutamate decarboxylase was not associated with any organelle or membrane component of the leaf cell. We consider that Gaba accumulation results from some change in intracellular compartmentation of the cell triggered by low temperature shock or mechanical damage. The accumulation of alanine due to a light-dark transition could be accounted for by transamination. [^{14}C]Alanine formation was demonstrated when soybean leaf extracts were incubated with glutamate, aspartate, or serine and [^{14}C]pyruvate.

The changes in amino acid concentrations described for soybean leaves were demonstrated for all the vegetative tissues of the soybean plant and at variable rates in the leaves of a range of plant species. The response in detached tomato (*Lycopersicon esculentum* Mill.) leaves was of a similar magnitude to soybean. Thus, precautions are necessary to minimize changes in amino acid composition induced by manipulation and extraction of plant material.

Secor and Schrader (17) reported that Gaba[4] is one of the major constituents of the amino acid pools in isolated soybean leaf cells. While this decarboxylation product of glutamate was given considerable prominence in early literature on nitrogen metabolism in plants (20), there is some evidence that it only accumulates in plants under some stress, such as prolonged periods of anoxia (for review, see 21).

The major objective of this study was to characterize the accumulation of Gaba in soybean leaves. Our results indicate that rapid changes can be induced in the content of Gaba and certain other amino acids in soybean and other species in response to relatively common experimental procedures such as rapid transfer of plants or detached leaves to lower temperatures.

MATERIALS AND METHODS

Plant Material. The main study was undertaken on nodulated soybean (*Glycine max* [L.] Merr. cv Corsoy 79) with cv Wells II being checked for comparison. These were grown 45 to 55 d as described previously (17) either outdoors or in a greenhouse. Experiments were mainly undertaken in the middle of the day when the plants were in sunlight and the air temperature was 30 to 35°C. The following nodulated legumes were grown in the greenhouse conditions described above: cowpea (*Vigna unguiculata* [L.] Walp. cv Queen Anne Black-eye); bean (*Phaseolus vulgaris* L. cv Tender green); pea (*Pisum sativum* L. cv Sprite); and alfalfa (*Medicago sativa* L. cv Saranac). Radish (*Raphanus sativus* L. cv Champion) and barley (*Hordeum vulgare* L. cv Morex) were grown under the same conditions as the legumes but supplied with a complete nutrient solution (3). Maize (*Zea mays* L. cv W64A X W182E) was grown in a growth room (1) while tomato (*Lycopersicon esculentum* Mill. unreleased line), yellow foxtail (*Setaria lutescens* Weigel), purslane (*Portulaca oleracea* L.), and pigweed (*Amaranthus retroflexus* L.) were grown outdoors.

Amino Acid Extraction and Analysis. All samples (usually stored in liquid N_2 for less than 1 h) were placed in a mortar, covered with liquid N_2, and pulverized to a fine powder. They were then extracted with cold 80% (v/v) ethanol (5 vol with 300 nmol norleucine added g^{-1} fresh weight). Extracts were centrifuged at 25,000 *g* for 10 min and the supernatants then stored at −20°C until analysis with a Dionex amino acid analyzer (17). Norleucine was used as an internal standard to adjust for any variation in the extraction and amino acid analysis procedure. In each experiment, leaflets of approximately the same area were selected and, for those harvested directly into liquid N_2, their fresh weight was estimated by reference to that of other leaflets. All amino acid analyses are representative of at least two separate experiments.

To determine the ^{14}C content in individual amino acids, the ethanol extracts were dried at 40°C *in vacuo* and then partitioned

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[4] Abbreviation: Gaba, γ-aminobutyric acid.

between petroleum ether and H_2O, the aqueous phase finally being concentrated by lyophilization. Fractions of the eluant from the amino acid analyzer (0.5 ml) were collected, and 1 ml H_2O and 8 ml complete counting cocktail (Research Products International Corp., product 3a70B) were then added. Radioactivity was determined and dpm were computed after appropriate reference to external standard data.

Enzyme Assays. Extraction procedures are described in the legends to appropriate tables and, unless stated, were undertaken at 0 to 5°C. Glutamate decarboxylase (EC 4.1.1.15) and Gaba-pyruvate transaminase (EC 2.6.1.19) were assayed at 30°C by the utilization of [^{14}C]glutamic acid and [^{14}C]Gaba, respectively, as described by Streeter and Thompson (23). Fumarase (EC 4.2.1.2) activity was determined by the procedure followed by Duke and Kakefuda (5) and protein was estimated by the Bradford procedure (2). All enzymatic studies represent duplicate assays of each extract and all experiments were repeated at least once.

RESULTS

Changes in Amino Acid Composition of Soybean Leaflets Due to Detachment. Gaba, present at a very low level in soybean leaflets detached directly into liquid N_2, increased rapidly in leaflets detached and maintained in the dark for a few min before freezing (Fig. 1). Maximum accumulation of Gaba was nearly attained only 2 min after detaching leaflets. A similar increase occurred in alanine with a maximum level being attained at 5 min. By contrast, a rapid decrease occurred in glycine concentration, largely in the 1st min after detachment, whereas glutamate showed a progressive decrease over 5 min. In another experiment conducted for 30 min, no further change occurred in the amount of these amino acids. Except for aspartate, which was present at a lower level than glutamate and decreased with leaf detachment, there was no change in the concentration of other amino acids (data not shown). Even when the soybean leaves were placed in liquid N_2 before detachment, a low level of Gaba was always detected (approximately 0.05 μmol g^{-1} fresh weight).

Alanine increased and glycine decreased in detached leaflets independently of temperature (Table I), but the accumulation of Gaba and disappearance of glutamate were enhanced by transferring the detached leaflets to lower temperatures. Some loss of aspartate was observed in each treatment.

Leaflets isolated and maintained at 33°C for 10 min in sunlight (floating on water) showed no accumulation of alanine and only 30% of the glycine decrease measured in the dark (data not shown). In detached leaflets, a smaller increase in Gaba also occurred in the light (accumulation about 70% of dark value).

Effect of Transferring Soybean Plants to Darkness and Low Temperature on Amino Acid Composition. The rapid increase in alanine and decrease in glycine described above (Fig. 1; Table I) for detached soybean leaflets were also observed in intact plants (Table II). The changes were primarily a response to darkness and occurred in all dark treatments including those plants not subjected to a temperature change (*i.e.* 33°C for 5 min). Serine,

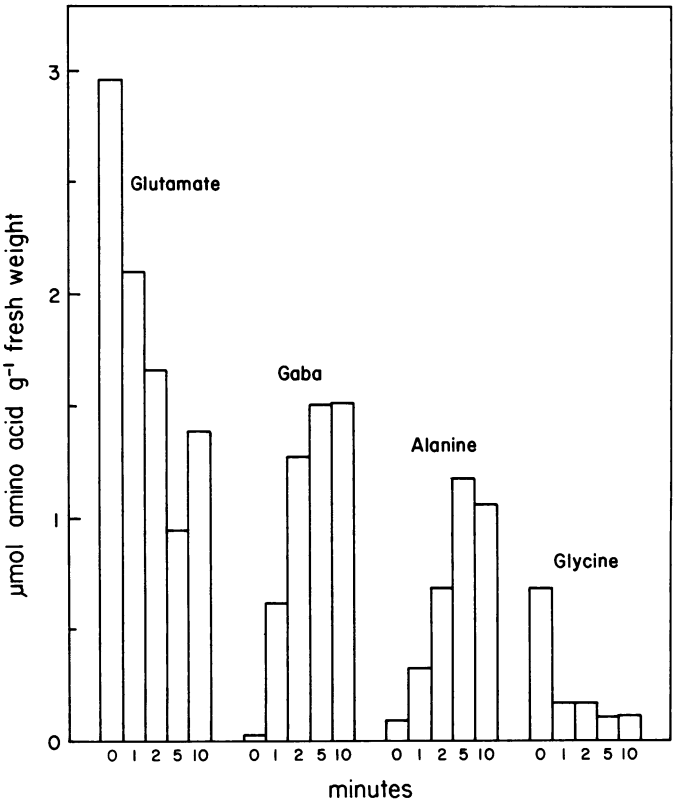

FIG. 1. Changes in amino acid composition of detached soybean leaflets with time. Leaflets were detached and either collected directly into liquid N_2 or placed in the dark at 25°C (petiolule in water). At the time indicated, samples were taken and frozen in liquid N_2. Extraction with cold 80% (v/v) ethanol and analysis of amino acids are described in "Materials and Methods."

Table I. *Influence of Temperature on Changes in Amino Acid Composition in Detached Soybean Leaflets*

Leaflets were sampled from glasshouse-grown plants at 35°C and after detachment were either immediately frozen in liquid N_2 or placed in the dark (petiolule in H_2O) for 5 min at the indicated temperature before freezing. Data are means of three experiments.

Amino Acid	Immediate Harvest	Treatment		
		35°C	22°C	6°C
	μmol g^{-1} fresh wt			
Asp	0.17 ± 0.05[a]	0.13 ± 0.03	0.08 ± 0.02	0.11 ± 0.02
Glu	2.31 ± 0.19	2.13 ± 0.47	1.06 ± 0.39	1.39 ± 0.55
Gly	0.62 ± 0.17	0.24 ± 0.09	0.18 ± 0.07	0.18 ± 0.08
Ala	0.08 ± 0.02	1.00 ± 0.24	0.96 ± 0.23	0.85 ± 0.11
Gaba	0.05 ± 0.03	0.45 ± 0.52	1.16 ± 0.40	1.18 ± 0.26

[a] Mean ± SE.

Table II. *Effect of Transferring Soybean Plants to Darkness and Low Temperature on Amino Acid Composition of Leaves*

At the start of the experiment, the plants (grown outdoors) were at 33°C in full sunlight. For treatment at this temperature, plants were covered with a box for 5 min. For other treatments, plants were transferred during a 1-min period to the dark at the temperature indicated; changes in amino acid composition were measured in similar leaflets at 0 and 5 min after transfer. For the 33° to 22°C and 33° to 6°C study, plants were transferred to a growth cabinet (dark) at the higher temperature and the growth cabinet controls were set to effect maximum cooling. Change in amino acid concentration over the period of treatment is shown. Initial concentrations of Asp, Ser, Glu, Gly, Ala, and Gaba were 0.52, 0.50, 3.30, 0.77, 0.18, and 0.04 μmol g^{-1} fresh weight.

Amino Acid	Change in Amino Acid Concn.					
	33°C, 5 min	29°C, 5 min	22°C, 5 min	6°C, 5 min	33–22°C, 5 min	33–6°C, 15 min
	μmol g^{-1} fresh wt					
Asp	−0.26	−0.32	−0.16	−0.55	−0.48	−0.22
Ser	+0.09	+0.09	−0.13	−0.12	−0.05	−0.09
Glu	+0.25	+0.50	−1.83	−1.66	−0.82	−0.39
Gly	−0.61	−0.26	−0.40	−0.07	−0.27	−0.30
Ala	+1.17	+1.23	+1.55	+0.86	+1.40	+0.98
Gaba	+0.10	+0.02	+1.45	+1.77	+0.25	+0.19

which is derived from glycine during photorespiration, showed a relatively minor fluctuation in concentration (initial level 0.5 μmol g^{-1} fresh weight). On the other hand, increased Gaba was only detected when the plants were subjected for 5 min to a marked, abrupt temperature decrease (*e.g.* abruptly to 22°C or 6°C). Plants exposed to a gradual reduction in temperature (*e.g.* 33°C to 6°C in 15 min or 33°C to 22°C in 5 min) did not show the same accumulation of Gaba. Soybean leaflets sampled at 3 AM (6 h of darkness; 20°C at sampling time) also contained only a trace of Gaba (0.04 μmol g^{-1} fresh weight) and a low level of alanine (0.2 μmol g^{-1} fresh weight).

To determine the reversibility of the observed changes in amino acid composition, plants were returned to their original growing conditions after a low temperature treatment in the dark (Table III). As expected, a marked accumulation of alanine and Gaba as well as a decrease in glutamate, glycine, and aspartate were apparent after 5 min at 6°C in darkness. There was no additional accumulation of alanine or Gaba in leaflets sampled after 10 and 20 min at the lower temperature (data not shown). When the plants were returned outdoors (full sunlight, 33°C) there was a rapid rise in the amount of glycine but a slower readjustment of the levels of alanine, Gaba, glutamate, and aspartate. At 1 h after being returned to 33°C, the leaflets had approximately the same amount of these amino acids as at the start of the experiment (initial values at 6°C in Table III represent plants sampled after transfer from 33°C when some change in amino acid concentration would already have occurred; *e.g.* increase in alanine and decrease in glycine).

Table III. *Changes in Amino Acid Composition in Soybean Leaflets after Returning Plants to Original Growing Conditions following Low Temperature Treatment in Dark*

Plants (outdoor grown) at 33°C in sunlight were transferred to 6°C in dark and subsequently transferred back to 33°C in sunlight at 8 min. Each transfer required 1 min. Leaflets were collected in liquid N_2 at the times indicated and analyzed for amino acid concentration.

Temp	Time at Temp Indicated	Asp	Glu	Gly	Ala	Gaba
°C	min	μmol g^{-1} fresh wt				
Transfer to 6°C, dark						
6	0	1.35	7.46	0.79	0.99	0.23
6	5	0.44	4.76	0.32	2.23	1.94
Return to 33°C, light						
33	0	0.59	4.90	0.99	3.04	2.28
33	5	0.42	5.19	1.02	2.07	2.10
33	30	0.91	7.26	1.45	0.98	0.82
33	60	1.34	9.21	1.56	0.39	0.29

Table IV. *Influence of a Rolling Procedure and Other Experimental Treatments after Detachment on the Amino Acid Composition of Soybean Leaflets*

Leaflets from greenhouse-grown plants (30°C) were either collected in liquid N_2 (immediate harvest) or kept on ice for 15 min in darkness. Part of the sample collected in liquid N_2 was allowed to thaw (2 min) and then kept for a further 15 min on ice or at 22°C. Some leaflets were rolled up in a coil (six times before detaching) or crushed with a mortar and pestle; all treated leaflets were finally collected in liquid N_2.

Amino Acid	Immediate Harvest	15 min on Ice	Preliminary Freeze in Liquid N_2		Leaflets Rolled	Leaflets Crushed
			15 min on ice	15 min at 22°C		
	μmol g^{-1} fresh wt					
Asp	0.38	0.29	0.16	0.26	0.18	0.36
Glu	2.85	1.54	0.79	0.28	1.89	0.76
Gly	1.07	0.17	0.82	0.92	0.51	0.85
Ala	0.14	0.93	0.14	0.23	0.66	0.60
Gaba	0.04	0.71	1.01	2.54	1.44	1.74

Table V. *[^{14}C]Glutamate Conversion to Gaba in Soybean Leaf Discs*

Thirty leaf discs (2.14 cm^2) were vacuum infiltrated with 25 ml of 5 mM $CaCl_2$ and 1 mM $MgCl_2$ containing 370 kBq L-[U-^{14}C]glutamic acid (10.5 MBq μmol^{-1}). The discs were incubated at 25°C in light (450 μmol m^{-2} s^{-1}) and five discs (0.16 g fresh wt) were removed at the time indicated. These were washed with cold H_2O, pulverized in liquid N_2, and extracted with 5 ml cold 80% ethanol. Amino acid separation and analysis of their concentration and ^{14}C content are described in "Materials and Methods." Data are expressed per g leaf fresh weight.

Time	Glutamate		Gaba	
min	Bq	μmol	Bq	μmol
1	1407	2.03	107	1.66
5	1934	2.84	147	1.71
10	1483	2.77	268	1.81

Influence of Leaf Damage and Other Experimental Procedures on the Amino Acid Composition of Soybean Leaflets. When a detached soybean leaflet was kept for 15 min on ice (dark), changes in amino acid concentrations similar to those attributed above to low temperature shock and darkness were observed (Table IV). Glutamate, aspartate, and glycine decreased while alanine and Gaba increased. When the leaflets were first frozen in liquid N_2, the accumulation of Gaba and disappearance of glutamate were accelerated especially when the leaflets were subsequently maintained at 22°C. After the initial freezing, however, negligible changes in glycine and in alanine were observed.

A gentle rolling of the soybean leaflets before detachment also triggered the accumulation of Gaba and alanine and decreased the other amino acids described above (Table IV). An increase in Gaba and alanine was detected after only rolling the leaflets twice while after more extensive rolling (six times) of two leaflets of the trifoliolate leaf, the remaining leaflet showed no change in amino acid composition (data not shown). Alanine and Gaba concentrations also increased in leaflets crushed with a mortar and pestle (Table IV).

Utilization of [^{14}C]Glutamate and [^{14}C]Aspartate by Soybean Leaf Discs. In the 5-min period required to isolate leaf discs and vacuum infiltrate them with the ^{14}C medium, almost maximum accumulation of Gaba could have occurred. However, in the subsequent incubation period (Table V) conversion of [^{14}C] glutamate to Gaba continued and the specific activity of the Gaba increased with time. No ^{14}C label from glutamate was detected in any other amino acid at the end of the 10-min incubation period, but by 30 min significant ^{14}C was detected in aspartate and alanine (data not shown).

Soybean leaf discs were incubated with [^{14}C]aspartate using the same procedure as described in Table V. After 10 min, 363 Bq were recovered in 1.2 μmol aspartate (g^{-1} fresh weight). Glutamate was the only other ^{14}C-amino acid detected (57 Bq g^{-1} fresh weight).

Glutamate Decarboxylase and Gaba-Pyruvate Transaminase in Soybean Leaves. In this study of the enzymes involved in Gaba biosynthesis and degradation, the extraction and assay procedures optimized for radish (23) were followed. In soybean, as in radish, there is a considerable excess of glutamate decarboxylase activity relative to Gaba-pyruvate transaminase (Table VI). With the latter, only a low activity (10%) was detected with α-ketoglutarate instead of pyruvate. Both enzymes had reduced activity at lower temperature (Table VI) but the ratio of glutamate decarboxylase to Gaba-pyruvate transaminase activity increased as the temperature was reduced. In soybean leaf extracts prepared at 22°C, lower glutamate decarboxylase activity was

Table VI. *Influence of Temperature on the In Vitro Activity of Glutamate Decarboxylase and Gaba-Pyruvate Transaminase*

Soybean leaf samples (midrib removed) were extracted in 0.1 M K-phosphate (pH 7.3) containing 0.2 mM EDTA, 1 mM mercaptoethanol, and 1% (v/v) Triton X-100. Enzyme activity was measured in the 20,000*g* supernatant after passage through a Sephadex G-25 column equilibrated with 10 mM K-phosphate (pH 7.3) containing 1 mM mercaptoethanol.

Assay Temperature	Glutamate Decarboxylase	Gaba-Pyruvate Transaminase
°C	*μmol product h^{-1} g^{-1} fresh wt*	
30	49.5	1.50
20	36.1	0.86
6	14.4	0.15

recovered (37% of that at approximately 2°C). However, the decarboxylase was rather labile *in vitro* with 15% loss of activity h^{-1} on ice. Extracts prepared at 22°C and transferred to 2°C showed no activation of the decarboxylase.

Glutamate decarboxylase was not associated with any organelle or membrane fraction of the soybean leaf cell (Table VII) whereas part of the Gaba-pyruvate transaminase was found in the chloroplast-enriched 2,500*g* pellet (16%) and 9,500*g* pelleted mitochondrial fraction (37%). Considerable chloroplast disruption had occurred as judged by the low protein concentration of the 2,500*g* pellets (Table VII) and the occurrence of ribulose-1,5-bisphosphate carboxylase in the 100,000*g* supernatant (data not shown). Fumarase was largely detected in the 9,500*g* pellet (90%) and the remainder in the 2,500*g* pellet indicating minimal disruption of the mitochondria.

Biosynthesis of Alanine by Transamination. To investigate if the increase in alanine, after transfer of soybean plants to the dark, could be accounted for by transamination, we measured [^{14}C]alanine formation when a desalted soybean leaf extract was incubated with [^{14}C]pyruvate (Table VIII). In the absence of any added amino acid, 0.29 μmol of alanine accumulated in 2 h at 30°C, but only 0.11 μmol was derived from the labeled pyruvate. The remainder of the alanine was apparently contributed by proteolysis and/or transamination of unlabeled pyruvate; proteolysis indeed occurred because the level of all the protein amino acids increased during the incubation period (data not shown).

At pH 7.5, glutamate was the most effective amino N donor for transamination of pyruvate; a high level of alanine was also formed with aspartate and serine (Table VIII). When glycine was supplied, alanine formation and [^{14}C]pyruvate incorporation were only slightly enhanced over the control (none added). In each treatment, the final specific activity of alanine was approximately the same.

Formation of Gaba and Alanine in Detached Leaves; a Comparison of Soybean with Other Plants. High levels of alanine and Gaba accumulated in all ages of soybean leaves (and in two cultivars and a wide range of growth conditions) when they were detached, stored at 22°C for 15 min, and chopped before extraction (see Table IX for representative data). Similar treatment of the petiole (Table IX) and root (data not shown) also resulted in Gaba accumulation. The petiole and especially the root had a relatively low concentration of glycine; alanine did not increase in the root after its isolation. In the other legumes tested (cowpea, common bean, pea, and alfalfa) there was some accumulation of Gaba and alanine in detached leaves (about 25% and 50%, respectively, of equivalent values for soybean).

Tomato most closely resembled soybean in showing a high level of Gaba and alanine formation after leaf isolation (Table IX). The other species to give a relatively high accumulation of Gaba was yellow foxtail. Radish, barley, maize, purslane, and pigweed leaf samples accumulated a much lower level of Gaba, but in each case there was a several-fold increase from the amount in the leaf sample collected immediately in liquid N_2. Foxtail and maize (C_4 monocots) leaves had a high initial concentration of alanine (and glycine) and did not show any change in alanine level after detachment. For purslane and pigweed (C_4 dicots), an increase in alanine was found.

The soybean and tomato leaf samples which showed the greatest accumulation of Gaba had the highest glutamate decarboxylase activity and greatest excess of it over Gaba-pyruvate transaminase activity (Table IX). Yellow foxtail also had relatively high decarboxylase activity. Lower glutamate decarboxylase activity was detected in the other plants tested and its ratio to that of the transaminase activity was considerably smaller. Although the soybean petiole had a high glutamate decarboxylase activity, its relatively small accumulation of Gaba is probably due to its lower glutamate concentration.

DISCUSSION

This study demonstrated that when soybean plants are placed in the dark there is a rapid decrease in glycine and increase in alanine concentration. If the plants are transferred rapidly to a lower temperature, a dramatic increase in Gaba is accompanied by a decrease in glutamate within 5 min. When leaflets are detached, a Gaba increase is triggered, especially when they are placed at a lower temperature. If the detached leaflets are kept in the dark or relatively low light, alanine also increases and glycine decreases.

Table VII. *Intracellular Distribution of Glutamate Decarboxylase and Gaba-Pyruvate Transaminase in the Soybean Leaf*

Sliced leaf tissue (2.5 g) was macerated in 10 ml of extraction medium for 3 × 5 s at 30,000 rpm in a VirTis homogenizer. The extraction medium was 50 mM Hepes (pH 7.5), containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM mercaptoethanol, and 0.1% (w/v) BSA. Pellet fractions were resuspended in the same medium with sorbitol omitted, 50 mM K-phosphate instead of Hepes, and 1% (v/v) Triton X-100 included.

Fraction	Glutamate Decarboxylase	Gaba-Pyruvate Transaminase	Protein
	μmol product h^{-1} g^{-1} fresh wt		*mg g^{-1} fresh wt*
2,500*g* (1 min) pellet	0.59	0.09	9.6
9,500*g* (15 min) pellet	0.48	0.21	6.0
100,000*g* (60 min) pellet	0.64	0.03	5.5
100,000*g* (60 min) supernatant	21.03	0.24	19.6

Table VIII. *Transamination to Alanine in Soybean Leaves*

A mature leaf sample was macerated in 0.1 M Hepes (pH 7.5) containing 1 mM mercaptoethanol and passed through a Sephadex G-25 column equilibrated with 0.05 M Hepes (pH 7.5), and 0.5 mM mercaptoethanol. Desalted enzyme sample (0.5 ml, 5 mg protein) was incubated with 0.05 ml 10 mM Na pyruvate containing 37 kBq [3-^{14}C]pyruvate, 0.02 ml 0.5 mM pyridoxal phosphate, and 0.05 ml 0.1 M amino acid as indicated. After 2 h at 30°C, 0.5 ml 5% (w/v) 5-sulfosalicylic acid was added, the precipitate was removed, and the supernatant analyzed for alanine and ^{14}C incorporation into this amino acid (see "Materials and Methods"). The desalted enzyme sample contained 0.01 μmol alanine.

Amino Acid	Alanine	
	μmol	*kBq*
None	0.29	7.48
Glu	0.74	20.55
Asp	0.59	15.77
Gly	0.31	8.58
Ser	0.54	14.00

We have confirmed, as previously demonstrated for radish (23), that [^{14}C]glutamate is rapidly converted to Gaba in soybean leaves. The soybean leaf has a relatively high level of glutamate decarboxylase activity; at 30°C the activity was approximately 30- to 70-fold that of Gaba-pyruvate transaminase. Part of the transaminase appears to be localized in the mitochondria (Table VII; 25) separated from the soluble decarboxylase. Even at 6°C, the activity of glutamate decarboxylase measured *in vitro* (approximately 0.3 μmol Gaba produced min^{-1} g^{-1} fresh weight) could account for the *in vivo* rate of Gaba accumulation.

We propose that accumulation of Gaba in response either to a low temperature shock or damage to the soybean leaf results from an altered intracellular compartmentation of glutamate or some effector molecule of glutamate decarboxylase activity. However, because of the apparent localization of glutamate decarboxylase in the vacuolar or cytosolic region of the leaf cell, where glutamate is also found (11, 12), it is unlikely that the decarboxylase is sequestered away from its substrate. Alternatively, a change in the distribution of some other metabolite or ion, *e.g.* Ca^{2+} (28), could trigger activation of glutamate decarboxylase. No evidence was obtained for low temperature activation of glutamate decarboxylase. It has a decreased *in vitro* activity at lower temperature and a gradual reduction in the temperature of the plant does not promote the same accumulation of Gaba. This correlates with measurements on the membrane potential of corn coleoptile cells (14) which were affected markedly by a rapid temperature decrease but only slightly influenced by slowly decreasing the temperature.

An alternative explanation for the accumulation of Gaba was suggested by Streeter and Thompson (22) to explain the same phenomenon in radish leaves incubated under anaerobic conditions, but for longer periods of time. They proposed that intracellular damage and a lowering of protoplasmic pH would favor the glutamate decarboxylase (pH optimum 5.9) *versus* the transaminase, which utilizes Gaba (pH optimum 8.9). In our low temperature treatments, minimal damage must occur because the effects were reversed within 1 h when the plants were returned to normal growing conditions.

The decrease in glycine in the dark is most likely linked to the cessation of photorespiration (26) and its further oxidation to serine in the mitochondria. No change in the level of serine was detected. Hitz and Stewart (7) also have shown that the serine pool in soybean leaves was not altered under conditions which increased the glycine pool and flux through the decarboxylation reaction.

Our data indicate that at least part of the rapid accumulation of alanine in plants transferred to darkness could be linked to the decrease in glycine and a subsequent transamination reaction

Table IX. *A Comparison of the Influence of Leaf Detachment on the Alanine and Gaba Concentration and In Vitro Activities of Glutamate Decarboxylase and Gaba-Pyruvate Transaminase in Soybeans and Other Species*

Leaves were either collected directly into liquid N_2 or detached, transferred to 22°C for 15 min, and chopped before being frozen in liquid N_2. For enzyme analysis, samples of the same leaf material were collected a few min before extraction and placed on ice. Enzymes were extracted as described in Table VI (Sephadex step omitted) and enzyme activity measured as described in "Materials and Methods." Specific activity (mg^{-1} protein) is shown in parentheses.

Plant Sample	Amino Acid Concn.					Glutamate Decarboxylase	Gaba-Pyruvate Transaminase
	Immediately frozen			Increase after 15 min at 22°C			
	Glu	Ala	Gaba	Ala	Gaba		
	μmol g^{-1} fresh wt					*μmol product h^{-1} g^{-1} fresh wt*	
Soybean—1st trifoliolate	4.97	0.86	0.19	2.45	2.92	76.9 (2.19)	1.15 (0.032)
Soybean—mature trifoliolate	2.90	0.16	0.13	1.95	2.51	54.5 (1.74)	1.51 (0.038)
Soybean—petiole	0.98	0.33	0.07	0.23	0.99	78.3 (7.34)	1.57 (0.117)
Cowpea—1st trifoliolate	4.85	0.09	0.03	1.29	0.50	5.4 (0.2)	0.74 (0.027)
Bean—1st trifoliolate	4.41	0.36	0.04	1.10	0.41		
Pea—mature leaf	3.89	0.75	0.09	0.69	0.48		
Alfalfa—young leaves	8.89	1.13	0.05	1.50	0.56	19.3 (0.28)	0.86 (0.013)
Tomato—mature leaf	6.38	1.23	0.29	2.60	2.12	49.1 (1.29)	0.80 (0.02)
Radish—mature leaf	1.33	0.49	0.03	1.10	0.15	20.2 (0.76)	1.69 (0.064)
Barley—1st leaf	5.55	1.64	0.06	1.43	0.15	15.3 (0.69)	1.16 (0.052)
Yellow foxtail—mature leaf	2.43	3.15	0.03	0	0.76	37.4 (1.47)	1.43 (0.056)
Maize—mature leaf	3.01	3.90	0.04	0	0.16		
Purslane—young shoot	2.38	2.15	0.02	1.40	0.10		
Pigweed—leaves	1.13	0.65	0.03	1.80	0.13		

from serine. Glutamate, aspartate, and also Gaba could act as amino N donors but the reason for the accelerated biosynthesis of alanine after a light/dark transition is not apparent. We also observed that the enhanced biosynthesis of alanine does not occur after a freeze thaw treatment of the soybean leaf, indicating the requirement of normal cell integrity for the provision of pyruvate or to support the transamination reaction. While demonstrated in a range of C_3 plants and two C_4 dicot species, enhanced alanine biosynthesis was not observed in the C_4 monocots maize and yellow foxtail. The lack of significant direct transamination of pyruvate with glycine (Table VIII) is in agreement with the recent report (13) that the alanine:glyoxylate aminotransferase reaction in spinach leaf peroxisomes is irreversible. Serine:pyruvate aminotransferase activity has been reported in kidney bean (19) and spinach leaves (16). No evidence was found for a direct decarboxylation of aspartic acid to alanine.

Similar changes in the amino acid composition of detached leaf tissue, as described in this investigation, have been reported by others. There are several reports of an increase in alanine and Gaba and decrease in glutamate and aspartate when leaves were incubated under anaerobic conditions (6, 22, 24). Unlike the rapid changes we have demonstrated, a treatment of at least 1 h under anoxia was necessary to give the same accumulation of Gaba and alanine in radish leaves (22). The radish leaf sample we tested had a lower glutamate decarboxylase activity than did soybean (slightly lower activity than reported by Streeter and Thompson [23]), and showed only a small accumulation of Gaba when detached for 15 min. Mills and Joy (12) compared pea chloroplasts isolated by a rapid mechanical method (<5 min) with those obtained by a slower protoplast preparation procedure and found Gaba and alanine were increased in the latter. We confirmed that Gaba and alanine do accumulate in detached pea leaves.

There are interesting reports in the literature that Gaba content increases with leaf age (10), molybdenum deficiency (15), or in response to viral attack (4). The latter two observations were made with tomato and it has been proposed for this plant that Gaba is a temporary storage product for protein amino acids (18). It will, however, be necessary to confirm that the Gaba levels measured did not accumulate during the preparation of the plant samples for analysis. We have shown that tomato leaves, like soybean, accumulate high levels of Gaba after short periods of leaf detachment. Some Gaba accumulation was detected in detached leaves of all plants tested and although some caution is necessary with comparative data on enzyme levels in different plants it appears that a high level of Gaba accumulation is correlated with a high ratio of glutamate decarboxylase:Gaba-pyruvate transaminase. In cultured rice cells, Gaba increases in response to ammonium and glutamine nutrition (9), while in the early stages of the growth of callus cells derived from the soybean cotyledon, the activity of glutamate decarboxylase and Gaba-pyruvate transaminase increased 10-fold (25).

It is likely that insects feeding on leaves would trigger the induced accumulation of Gaba and this could have a profound effect on their feeding habits. Because Gaba is a neurotransmitter in insects (27) the induced formation of Gaba, observed in our studies, provides an interesting area for investigation in plant-insect interactions.

Finally, what precautions should be taken to minimize changes in amino acid composition during the sampling of plant material? When possible, samples should be collected directly into liquid N_2, pulverized in liquid N_2, and extracted with an organic solvent such as cold 80% ethanol to inactivate enzymes. Kennedy and Williams (8) also demonstrated the importance of a liquid N_2 kill in minimizing changes in the distribution of ^{14}C label among early photosynthetic products in two C_4 plants. When some manipulation of detached plant material is necessary, this should be at the temperature at which the plant is growing, cause minimum structural damage, and be undertaken in high light and in the shortest time possible.

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