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^{15}N- and [^{13}C]NMR Determination of Utilization of Glycine for Synthesis of Storage Protein in the Presence of Glutamine in Developing Cotyledons of Soybean

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ABSTRACT

Solid-state ^{15}N- and [^{13}C] NMR have been used to measure quantitatively the utilization of glycine in the presence of glutamine for the synthesis of storage protein in immature cotyledons of soybean (*Glycine max* L. cv. Elf) in culture. The presence of an equal molar amount of glycine in the medium causes a decrease in the use of glutamine-amide nitrogen. Glycine nitrogen is incorporated extensively into peptide bonds (in amounts greater than what would be expected if it appeared solely in glycine residues), but is used sparingly for synthesis of histidine ring residues, guanidino nitrogen residues of arginine, and lysine residues. The modest use of glycine carbon in protein synthesis does not parallel the use of glycine nitrogen.

Developing seeds are dependent upon other parts of the plant to obtain the nitrogen required for synthesis of storage protein. A large fraction of the amino acid nitrogen in the translocation stream of many plants is present as asparagine or glutamine, but varying amounts of most of the other protein amino acids are also present (4, 6, 10, 12). Although asparagine, glutamine, and ureides are believed to be the major sources of nitrogen for protein in the developing seed, little is known about the utilization of other amino acids present in the translocation stream or what effect they have on the metabolism of asparagine and glutamine. Glycine is found in relatively small amounts in the translocation stream of soybean (12) even though glycine nitrogen represents approximately 12% of the nitrogen of soybean seed protein (2).

In this paper, we report the effect of glycine on glutamine metabolism in developing soybean cotyledons (as determined by solid-state [^{15}N]NMR) and establish the extent of glycine utilization for protein synthesis in the presence of glutamine.

MATERIALS AND METHODS

Growth of Cotyledons in Culture. Growth of plants (*Glycine max* L. cv. Elf), selection and preparation of cotyledons for culture, culture medium, and conditions for culture were as previously described (9, 11, 13).

Preparation of Cotyledons for NMR Analysis. After incubation, the cotyledons were rinsed with distilled H_2O, blotted, weighed, frozen in liquid nitrogen and lyophilized. After lyophilization, the cotyledons were subjected to solid-state magic-angle cross-polarization [^{15}N]NMR analysis (7, 8). When only the nitrogen present in protein was to be observed, the cotyledons were extracted with 80% ethanol to remove free amino acids (11).

Determination of Protein Content. Protein content of the cotyledons was determined by the method of Lowry *et al.* (5), with BSA as a standard. Total nitrogen was determined by pyrolysis and gas chromatography.

Stable Isotopes. All ^{15}N (98 atom %) and ^{13}C-labeled (90 atom %) amino acids were obtained from Merck (Montreal, Canada).

NMR. Magic-angle [^{15}N]NMR spectra were obtained at 9.12 MHz using matched spin-lock cross-polarization transfers with 1-ms single contacts and 25 kHz H_1s with the dried samples contained in a Beams-Andrew 420 µl hollow rotor spinning at 1.5 kHz (7). Technical details of the spinning and cross-polarization procedures have been reported elsewhere (8). Fast cross-polarization rates for protonated nitrogens, long proton rotating-frame lifetimes, and high concentrations of protons in these samples ensure representiative NMR intensities for all types of nitrogens discussed here (7, 8, 11).

The amount of ^{15}N present in the protein of the ethanol-extracted cotyledons was calculated from the NMR spectra using a natural abundance [^{15}N]NMR spectrum of a soybean seed as a standard. The amount of ^{15}N represented by the soybean seed spectrum was calculated by multiplying the natural abundance of ^{15}N (0.00365) times the amount of protein nitrogen present in the samples as determined by protein and total nitrogen assays.

Magic-angle [^{13}C]NMR spectra were obtained at 15.1 MHz using matched spin-lock cross-polarization transfers with 2-ms single contacts and 50 kHz H_1s with the dried samples contained in a KEL-F Beams-Andrew 700 µl hollow rotor spinning at 2.0 kHz.

RESULTS

Growth of Cotyledons on Glutamine and Glycine. Immature soybean cotyledons in culture grew well on 30 mM glutamine as the nitrogen source. Average increases in fresh weight, dry weight, and protein of 10-fold were observed after 8 days. When 30 mM glycine was given along with the glutamine, growth was similar with minor increases observed in fresh weight, dry weight, and protein (Table I). When 30 mM glycine was given as the sole nitrogen source, growth was considerably less, with protein production only 22% of that observed with glutamine (Table I).

[^{15}N]NMR. [^{15}N]NMR spectra of cotyledons grown on 30 mM [^{15}N]amide glutamine, and on 30 mM [^{15}N]amide glutamine plus 30 mM natural abundance [^{15}N]glycine are shown in Figure 1. Line assignments for spectra such as these have been given before

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Table I. *Growth of Soybean Cotyledons on Glutamine and Glycine*

Cotyledons were grown on medium containing glutamine and/or glycine as the nitrogen source. After 8 days in culture, they were harvested and their increase in fresh weight, dry weight, and protein was determined. The increase in growth is expressed as the percentage (± SE) of that observed when the cotyledons were grown on 30 mM glutamine. The cotyledons grown on 30 mM glutamine had an average initial fresh weight of 20 mg and an average, final fresh weight, dry weight, and protein content of 210, 45, and 8 mg, respectively.

Nitrogen Source	Fresh Weight	Dry Weight	Protein
		% of control	
30 mM Glutamine	100 ± 11	100 ± 8	100 ± 8
30 mM Glutamine + 30 mM glycine	104 ± 11	102 ± 10	107 ± 10
30 mM Glycine	42 ± 5	52 ± 6	22 ± 5

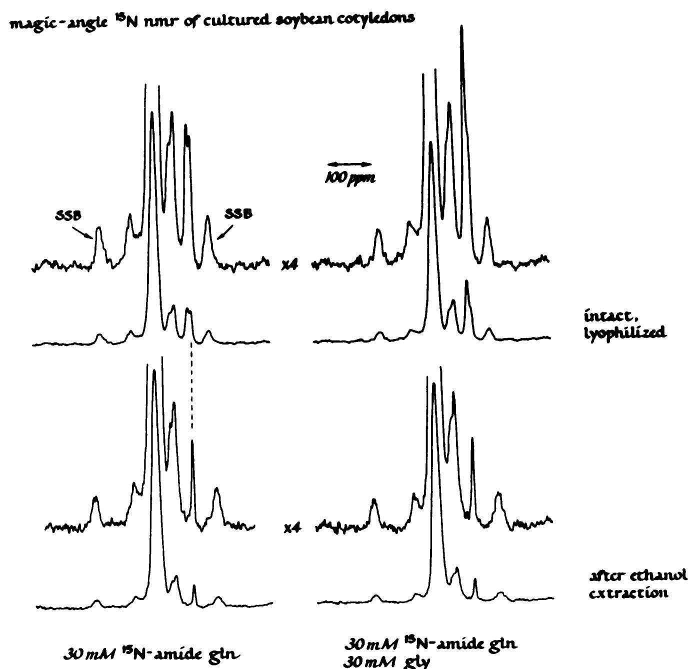

FIG. 1. Magic-angle cross-polarization 9.12 MHz [^{15}N]NMR spectra of five lyophilized cotyledons cultured 7 days on a medium containing 30 mM [^{15}N]amide glutamine (left), or a combination of [^{15}N]amide glutamine and natural-abundance [^{15}N]glycine (right). The bottom spectra are of cotyledons following an extraction with 80% ethanol. The large central peak of the spectra of unextracted tissue represents ^{15}N present as amide nitrogen (peptide nitrogen of protein plus amide nitrogen of glutamine and asparagine). This peak appears about 100 ppm downfield from that of solid ammonium sulfate as an external reference. The small peak at the left of the amide peak arises from nitrogen of histidine rings. The double peak immediately to the right of the amide peak represents the guanidino nitrogens of arginine. The two peaks immediately to the right, or high-field side of the arginine peak, represent primarily the amino nitrogen of free amino acids and the ϵ nitrogen of lysine, respectively. The remaining two peaks at the high- and low-field extremes of the spectrum appear as a result of the mechanical spinning procedure and are called spinning sidebands (SSB).

(7, 11). Spectra for cotyledons grown on media containing glutamine and glutamine plus glycine are similar, except that the free amino acid peak is slightly enhanced for the glutamine plus glycine sample.

The corresponding spectra of the two 80% ethanol-extracted samples (Fig. 1, lower spectra) are also similar. In these spectra, the ^{15}N of free amino acids is no longer observed and the ϵ nitrogens of protein lysine are represented by a sharp peak. The presence of glycine in the medium does not have a qualitative effect on the appearance of ^{15}N from glutamine amide into various nitrogen compounds, with the possible exception of incorporation into the free amino acid pool.

The [^{15}N]NMR spectrum of cotyledons fed 30 mM glutamine (natural abundance) plus [^{15}N]glycine (Fig. 2, left) shows that the ^{15}N of glycine is extensively incorporated into protein as amide nitrogen. However, comparison of this spectrum of the extracted cotyledons with the corresponding spectrum arising from cotyledons fed labeled glutamine and natural abundance glycine (with vertical scales adjusted so that it has a comparable peptide-nitrogen intensity; Fig. 2, right) shows that considerably less ^{15}N is incorporated as ring nitrogen of histidine, arginine, guanidino nitrogen, and ϵ-amino nitrogen.

The ^{15}N in the protein of the 80% ethanol-extracted cotyledons was calculated using the [^{15}N]NMR spectra and the natural abundance [^{15}N]NMR spectrum of a soybean seed as a standard. The ^{15}N in protein is presented in Table II as the percentage of total protein nitrogen. Cotyledons grown on [^{15}N]amide glutamine incorporated about 49% of their total nitrogen as ^{15}N. In the presence of glycine, less nitrogen was incorporated as ^{15}N (32%, Table II). Although a large fraction of nitrogen was incorporated as ^{14}N when the cotyledons were grown on glutamine plus [^{15}N]glycine, 33% still came from the ^{15}N of glycine (Table II).

[^{13}C]NMR. The [^{13}C]NMR spectra of cotyledons grown on natural abundance 30 mM glutamine plus 30 mM glycine and on 30 mM glutamine plus 30 mM 1-[^{13}C]glycine are shown in Figure 3. The major peaks represent carbonyl and carboxyl carbons in protein (180 ppm), polysaccharides (80–105 ppm) and methyl and methylene carbons in protein and structural lipid (~20–30 ppm). There is no significant ^{13}C-enrichment of any particular carbons in the cotyledons given the [^{13}C]glycine along with the glutamine (Figure 3, bottom spectrum). Assuming that most of the carbonyl and carboxyl carbons are in protein, and using the carbon natural abundance spectrum as a standard to calculate the amount of ^{13}C incorporated as carbonyl and carboxyl carbons in the cotyledons fed glutamine plus [^{13}C]glycine, about 2% of the carbonyl and carboxyl carbons of the protein in these cotyledons are ^{13}C. This is only twice the amount expected in the cotyledons fed natural abundance glutamine plus glycine (^{13}C natural abundance is

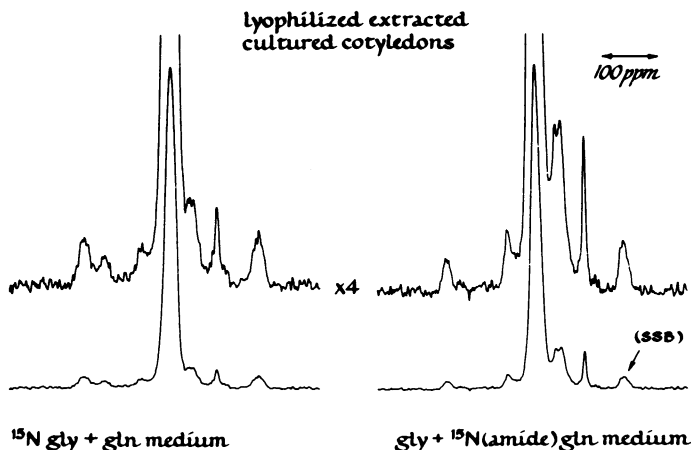

FIG. 2. Magic-angle cross-polarization 9.12 MHz [^{15}N]NMR of lyophilized ethanol-extracted cotyledons cultured 7 days on a medium containing 30 mM glycine and 30 mM glutamine in which the glycine nitrogen was ^{15}N-labeled (left) or the glutamine amide nitrogen was ^{15}N-labeled (right). The verticle scales of the two spectra have been adjusted so that the intense peptide-nitrogen peaks are equal in intensity.

Table II. *Incorporation of* ^{15}N *into Cotyledon Protein*

Five cotyledons were grown on medium containing 30 mM [^{15}N]amide glutamine, 30 mM [^{15}N]amide glutamine plus 30 mM glycine (natural abundance) or 30 mM glutamine (natural abundance) plus 30 mM [^{15}N]-glycine. After 8 days in culture, they were lyophilized, pooled together as one sample, extracted with 80% ethanol, and observed with NMR. The amount of ^{15}N present in protein was quantitatively determined as described in the text and is presented as the percentage of total protein nitrogen.

Nitrogen Source	Percentage of Total Protein Nitrogen		
	Present at start of expt	Composed of ^{15}N	Composed of ^{14}N (incorporated during expt)
[^{15}N]amide glutamine[a]	10	49	41
[^{15}N]amide glutamine plus glycine[a]	8	32	60
Glutamine plus [^{15}N]glycine	7	33	60

[a] Average of two replicates.

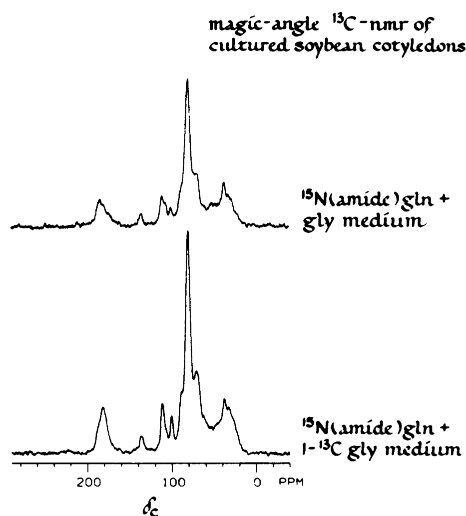

FIG. 3. Magic-angle cross-polarization 15.1 MHz [^{13}C]NMR spectra of five intact lyophilized cotyledons cultured 7 days on a medium containing 30 mM [^{15}N]amide glutamine and 30 mM glycine in which both amino acids are natural abundance in carbon (top) or the C_1 carbon of glycine is labeled (bottom). The major peaks represent carbonyl and carboxyl carbons in protein (180 ppm), polysaccharides (80 and 105 ppm), and methyl and methylene carbons in protein and structural lipid (~20–30 ppm). The C_1 carbon of glycine is not used extensively or selectively in protein synthesis.

1.1%). The same low level of incorporation is observed when the 2-carbon of glycine is ^{13}C-labeled.

DISCUSSION

The presence of glycine in the culture medium directly influences the utilization of glutamine amide nitrogen for synthesis of storage protein in a developing cotyledon. Considerably less glutamine amide nitrogen is incorporated into protein (Table II) when glycine is present. Conceivably, glycine could have either accelerated the use of glutamine amino nitrogen, or glycine nitrogen could have been used instead of glutamine amide nitrogen. The incorporation of ^{15}N into protein when glutamine plus [^{15}N]glycine was fed to cotyledons shows that a significant amount of nitrogen from glycine is, in fact, incorporated into protein (Table II).

The [^{15}N]NMR results also indicate that the nitrogen of glycine is used selectively in the synthesis of protein in developing cotyledons of soybean. Far more [^{15}N]nitrogen was incorporated into protein (33%, Table II) than could have been due to sole incorporation of [^{15}N]glycine (only 12% of the protein nitrogen of soybean seed is glycine nitrogen). Nevertheless, relatively little of this glycine nitrogen is used to synthesize ring nitrogen of histidine, and even less appears in ϵ nitrogen of lysine or guanidino nitrogen of arginine. In short, the glycine nitrogen does not enter a nitrogen pool common to that of the amide nitrogen of glutamine, but rather is used discriminately by the cotyledon.

An amino acid metabolism scheme (3) shows that both nitrogen and carbon of glycine are precursors for cysteine and serine. However, the results presented here indicate that little of the carbon of glycine is incorporated into protein ([^{13}C]NMR results), whereas a substantial amount of the nitrogen of glycine is. Therefore, the use of glycine nitrogen via transamination to make various amino acids may be an important function of glycine in the synthesis of protein. This is consistent with the notion that most exogenous amino acids are poor direct precursors for protein (1), and that most protein amino acids (including glycine) are susceptible to transamination reactions in plant tissues (14).

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