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CROSS-POLARIZATION NMR OF N-15 LABELED SOYBEANS

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SUMMARY: Cross-polarization ^{15}N nmr spectra of ^{15}N-labeled soybean seeds, pods, and leaves have been obtained at 9.12 MHz both with and without high-speed sample rotation at the magic angle. Spectral resolution is sufficient to permit a determination of the relative concentrations of amide and amine nitrogens, as well as of a few specific amino acid residues of proteins in the solid, intact samples. Utilization by soybean of nitrogen from labeled fertilizer in the presence of dinitrogen fixation can be determined from these spectra. A double-cross polarization ^{13}C nmr spectrum of a spinning, ^{15}N-labeled seed has been obtained in which resonances are observed only from these carbons directly bonded to nitrogens. This technique leads to a qualitative estimate of amino-acid composition of the protein which is complementary to that obtained directly from the ^{15}N nmr spectrum.

INTRODUCTION: The absence of a relatively long-lived radioisotope of nitrogen has limited research of the transport and metabolism of nitrogen in plants. The 10-minute half life of ^{13}N prohibits experiments lasting much more than two hours (1). During this time, the radioisotope label must be prepared, administered, extracted, and counted (2). Characterization of the details of complicated protein synthesis by this procedure appears difficult. Stable-isotope ^{15}N labeling experiments have been performed in the past with nitrogen-containing parts of the plant degraded, extracted, chromatographically separated, reduced to N_2, and the label detected by mass spectrometry (3). This destructive analysis runs the risk of chemically altering the sample (losing information about amide nitrogens, for example) and depends on the efficacy of an established chromatographic procedure.

We have labeled soybeans with 99% ^{15}N-enriched ammonium nitrate fertilizer, and report here the non-destructive detection of label in parts of the plants by solid-state cross-polarization nmr (4).

ABBREVIATIONS: nmr, nuclear magnetic resonance; CP, cross polarization; DCP, double-cross polarization; H_1, applied radio-frequency magnetic field; γ, gyromagnetic ratio; T_1, nmr spin-lattice relaxation time; $T_{1\rho}$, lifetime of the nmr magnetization spin-locked along H_1.

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MATERIALS AND METHODS: Glycine max L. (cv Elf) were grown in chambers having interior dimensions of approximately 1x1.5x2 meters. The growth chambers were fitted with four ceiling-mounted 1000-watt metal halide discharge lamps and 24 40-watt 1.2 meter white fluorescent lamps mounted on the walls. The plants were grown at 30°C (day) and 20°C (night) with a photoperiod of 14 hours. Daytime CO_2 concentration was controlled at 325 ppm. The plants were grown (two each) in 22.5-cm diameter pots filled with a mixture of vermiculite and sterilized soil fertilized with 1-5 g ^{15}N-labeled ammonium nitrate. Seeds were innoculated with Rhizobium japonicum.

Cross-polarization (CP) nmr spectra were obtained at 9.12 MHz using matched spin-lock transfers (4) with 1-msec single contacts and 25-kHz H_1's. The samples were lyophilized and examined as powders, except for the seeds which remained intact. Each sample, contained in an ordinary 10-mm diameter nmr tube, had a dry weight of 100-200 mg with about 2% total nitrogen from which a satisfactory spectrum could be obtained in less than an hour. Magic-angle CP nmr spectra (5) were each obtained in 15 minutes with the samples contained in a Beams-Andrew 420-µl hollow rotor spinning at 1.7 kHz at the magic angle. Double-cross polarization (DCP) experiments were performed using consecutive matched spin-lock CP transfers either from ^1H to ^{13}C, and then from ^{13}C to ^{15}N; or, from ^1H to ^{15}N, and then from ^{15}N to ^{13}C. Spectra from either ^{15}N or ^{13}C could be detected providing information about nitrogen-carbon coupling with greater sensitivity than can be achieved by single CP transfers between those low-γ, long-T_1 nuclei. Technical details have been presented elsewhere (6).

RESULTS: As shown in Figure 1, CP ^{15}N nmr spectra of stationary soybean samples are dominated by the chemical shift anisotropy of nitrogens in storage and enzymatic proteins. With one exception (Figure 1, middle, top),

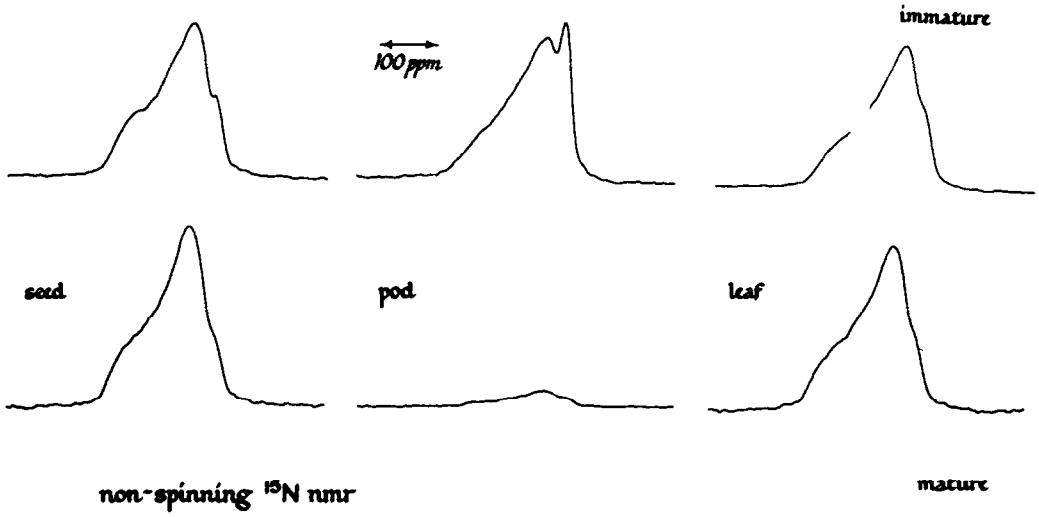

Figure 1. Cross-polarization 9.12-MHz ^{15}N nmr spectra of various parts of a uniformly-labeled ^{15}N soybean plant removed at different points of development. Spectra of immature, dried seeds (or ovules), pods, and leaves not fully expanded are shown in the top row, and of the corresponding mature plant parts in the bottom row.

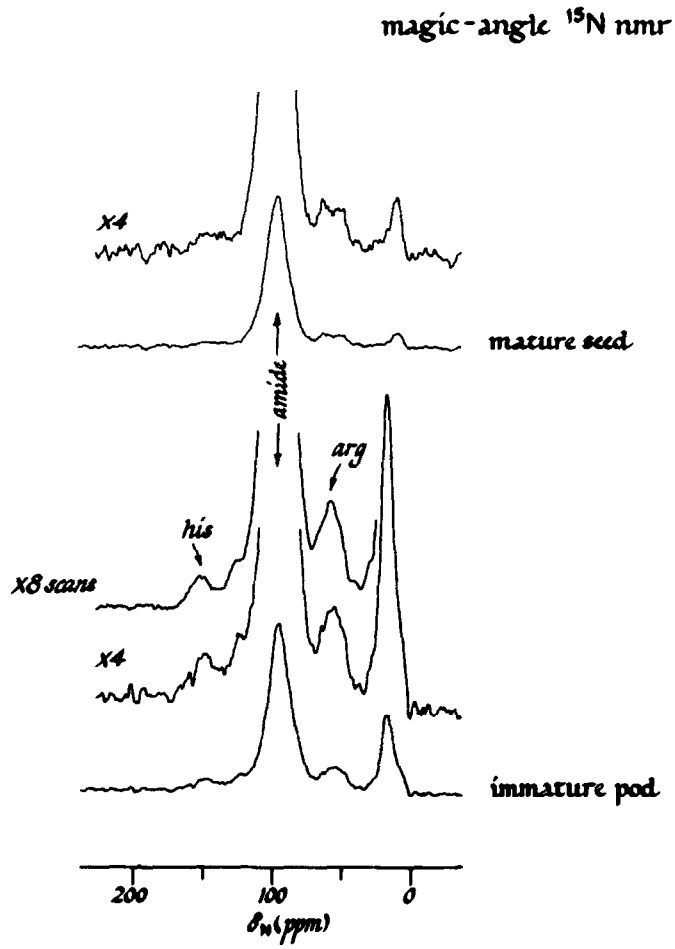

Figure 2. Cross-polarization 9.12-MHz ^{15}N nmr spectra of two of the samples of Figure 1, obtained with magic-angle spinning at 1.7 kHz. The scale is in ppm from solid ammonium sulfate.

shift differences between different chemical species are hard to see. Nevertheless, these spectra can be used to determine non-destructively the level of incorporation of label. Thus, the transfer of nitrogen out of a maturing pod is reflected in the drastic reduction of total intensity of the ^{15}N spectrum of a mature pod, relative to that of a pod containing an immature seed. (Figure 1, middle, bottom and top).

Line broadening due to chemical shift anisotropy in a CP experiment can be eliminated by high-speed sample rotation at the magic-angle (5). As shown in Figure 2, the resolution achieved by magic-angle spinning is sufficient to

permit a determination of the relative concentrations of amide and amine nitrogens in the seed and pod of a soybean plant at various stages of development. In addition, a few chemically different amine nitrogens can be identified. For example, the low-field side of the intense protein main-chain amide-nitrogen line of the mature seed spectrum (Figure 2, top) can be assigned to histidine ring nitrogens (7), while the high-field side can be assigned, for the most part, to the guanidine NH_2 nitrogens of arginine residues (7). (The side-chain arginine NH resonance may fall under the tail of the protein main-chain amide-nitrogen line.) The high-field amine-nitrogen resonance observed in the spectrum of the immature pod (Figure 2, middle) is probably due to high concentrations of free amino acids, presumably the transport species asparagine and glutamine.

A comparison of CP and DCP nmr is shown in Figure 3. The natural-abundance magic-angle CP ^{13}C nmr spectrum (collected in 16 hours) of a single, unlabeled, intact soybean is complicated (Figure 3, top). The soybean consists of approximately 40% protein and 20% lipids, with the remainder in the form of $-CH_2O-$ (starch, sugars, etc.) The tallest peak in this spectrum is due to starch; the major low-field peak is due to carbonyl carbons in all kinds of chemical environments (most of which arise from proteins). The natural-abundance ^{13}C nmr spectrum of a single, uniformly-labeled ^{15}N soybean is similar (Figure 3, middle), perhaps the most significant difference being a narrowing of the rigid-protein carbonyl-carbon line. The DCP spectrum (Figure 3, bottom) was obtained by a polarization transfer scheme of $^{1}H \rightarrow {}^{15}N \rightarrow {}^{13}C$. Thus, resonances are observed only from those carbons directly bonded to nitrogen. This explains the relative simplicity and resolution of the DCP spectrum.

DISCUSSION: By using $^{15}NH_4{}^{14}NO_3$, $^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$ as fertilizer, by varying the concentration of fertilizer in the soil, and by inhibiting

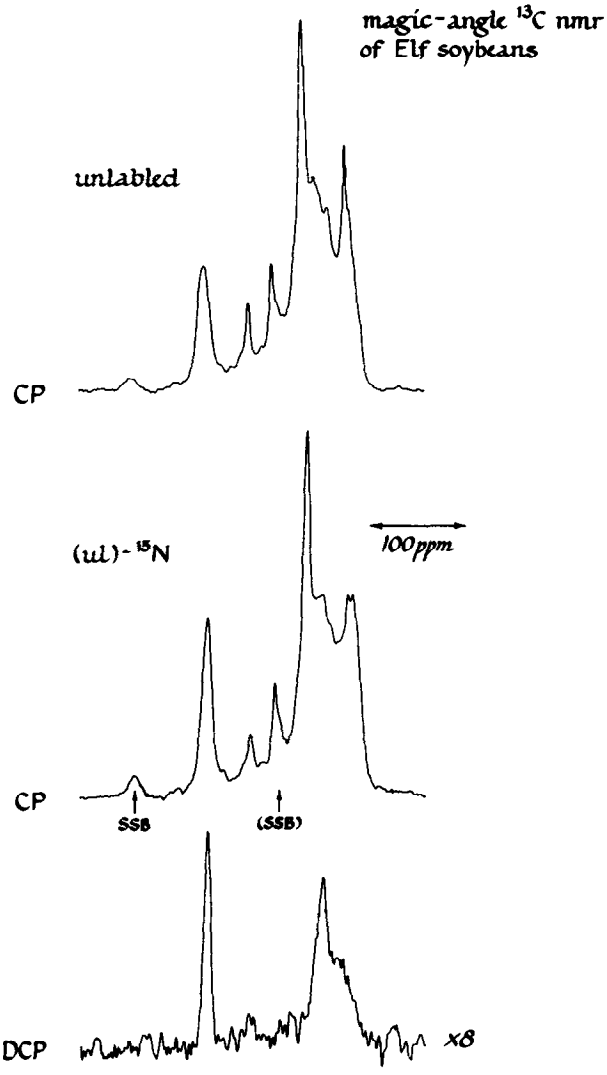

Figure 3. Cross-polarization 22.6-MHz magic-angle ^{13}C nmr spectra of a single, intact unlabeled soybean (top) and of a uniformly-labeled ^{15}N soybean (middle). The double-cross polarization ^{13}C nmr spectrum of the same labeled soybean is shown at the bottom of the figure. In the double-cross experiment, polarization is first transferred from protons to nitrogens (1-msec contacts) and then from nitrogens to carbons (5-msec contacts). Thus, signals arise only from those carbons directly bonded to ^{15}N. The double transfer is about 20% as efficient as a direct spin-lock transfer from protons to carbons, with losses due primarily to short $T_{1\rho}$'s at 25 kHz.

or encouraging the fixation of $^{14}N_2$ by Rhizobium attached to the soybean root system, we have found that the relative intensities of CP ^{15}N nmr spectra such as those shown in Figures 1 and 2 provide a simple, fast, and direct confirmation (8) that (a) under normal soil conditions both

nitrogens of ammonium nitrate are used equally in protein synthesis; (b) high levels of fertilizer (5 grams of ammonium nitrate per 2.5 liters of soil) will inhibit N_2 fixation, and will also inhibit nitrogen transport from senescing leaves; and (c) low levels of fertilizer (1 gram of ammonium nitrate per 2.5 liters of soil innoculated with Rhizobium) will result in 80% of the nitrogen of the mature seed arising from $^{14}N_2$, so long as optimum photosynthetic conditions are maintained in the growth chamber used to grow the plants.

Magic-angle spinning easily removes the 1-kHz static dipolar coupling between ^{15}N and ^{13}C (6). Since the carbonyl-carbon line of the massively ^{15}N-labeled proteins of the soybean is actually narrower than that of the ^{14}N material (Figure 3, middle), the resolution of the spectrum of the unlabeled proteins may be degraded by second-order ^{14}N-^{13}C quadrupolar effects which cannot be removed by magic-angle spinning (9). (This result has implications for magic-angle experiments on rigid crystalline proteins designed to compare isotropic solid-state and solution ^{13}C chemical shifts.)

The high-field aliphatic-carbon region is more intense than the carbonyl-carbon region in the DCP spectrum of Figure 3. Thus, soybean storage proteins must have higher concentrations of arginine and lysine than of glutamine and asparagine. Naturally, the use of detailed line assignments for individual amino-acid residues should allow comparisons between predicted and observed DCP spectra, and so ultimately more quantitative conclusions than reached here.

The kind of ^{13}C DCP experiment illustrated in Figure 3 can also be performed on ^{15}N specifically labeled materials. In fact, the sensitivity and favorable resolution achieved by all the CP and DCP measurements described above on non-specifically, uniformly-labeled materials suggest the practicality of a variety of new experiments including (a) monitoring the metabolism of specific ^{15}N labels fed to organ or cell cultures; (b) determining the

relative concentrations of ^{15}N-^{13}C and ^{15}N-^{12}C pairs in nitrogen-carbon double-label experiments, and (c) identifying the chemical structure of carbons directly coupled to nitrogens in ^{15}N single-labeled materials. The information resulting from these experiments should be of value in unraveling nitrogen metabolic pathways.

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