The metabolism of the broad-spectrum herbicide, glyphosate (N-phosphonomethylglycine) in a soil Pseudomonas sp. PG2982 has been determined by cross-polarization magic-angle spinning $^{15}$N and $^{13}$C NMR of intact lyophilized cultures. Using samples grown on $^{15}$C- and $^{15}$N-labeled glyphosate, we find that PG2982 does not metabolize glyphosate to aminomethylphosphonate as has been reported for mixed cultures of soil microbes. Rather, the phosphonomethyl carbon-nitrogen bond in glyphosate is cleaved, releasing glycine. Solid-state NMR analysis reveals that 20% of this glycine is used in the synthesis of purines, 35% is incorporated into protein as glycyl residues, with an additional 35% incorporated as seryl residues. The phosphonomethyl carbon of glyphosate is ultimately incorporated into a number of sites, including the C-2 and C-8 positions of the purine rings of nucleic acids, methyl groups of methionine and thymidine, and the methylene group of serine. The pattern of phosphonomethyl carbon incorporation indicates the involvement of tetrahydrofolate, a coenzyme which facilitates single-carbon transfers.

This is the first complete determination of the metabolism of glyphosate in a pure culture, and the first bacterial metabolic study using both single and double cross-polarization solid-state NMR.

The metabolism of glyphosate by PG2982 leads to aminomethylphosphonic acid (Structure 2)

\[
\begin{align*}
\text{O} & \quad \text{I} \\
\text{O-P-CH}_2\text{NHCH}_2\text{CO}_2\text{H} & \quad \text{(1)} \\
\text{O} & \quad \text{I}
\end{align*}
\]

as one of the products of the reaction. Aminomethylphosphonic acid is then transported into the soil where other microorganisms with the ability to metabolize carbon-phosphorus bonds are active (5-7). Thus, the complete elimination of glyphosate from the soil results from co-metabolism (4).

Recently, Moore et al. (8) reported the isolation of a pure pseudomonad culture of undetermined species, referred to as Pseudomonas sp. PG2982, which could utilize glyphosate as the sole source of phosphorus. The ability of this microorganism to use glyphosate as a source of phosphorus indicates that at some stage the carbon-phosphorus bond was broken. However, it was not known whether glyphosate was first degraded to aminomethylphosphonic acid, and then further metabolized releasing phosphorus, or if some other pathway was involved.

To determine the details of glyphosate metabolism in this organism, we have performed solid-state $^{13}$C and $^{15}$N NMR experiments on samples of Pseudomonas PG2982 grown on $^{13}$C- and $^{15}$N-labeled glyphosate. The application of solid-state NMR to the study of metabolism offers unique capabilities (9). Intact, lyophilized materials are examined directly in solid-state NMR experiments, eliminating the need for handling of fragile samples. Labeled peptide bonds, for example, are not exposed to hydrolysis by endogenous proteolytic enzymes. Since there are no extractions, the loss of material to cellular debris is avoided. The solid state NMR experiment produces a spectrum arising from all the components of the sample, proteins as well as nucleic acids. The labeling patterns are often simple and specific. Such experiments have led to an identification of the complete pathway in PG2982 for glyphosate degradation via glycine, a pathway different from that reported earlier for mixed soil microbes.

**MATERIALS AND METHODS**

Chemicals—N-Phosphonomethylglycine (99.7% purity, commonly referred to as glyphosate) was provided as the free acid form by Monsanto Agricultural Products, St. Louis, Mo. N-Phosphono[2-$^{13}$C,$^{15}$N]methylglycine (99 atom % $^{13}$C; 99 atom % $^{15}$N), N-phosphono[3-$^{13}$C,$^{15}$N]methylglycine (99 atom % $^{13}$C; 99 atom % $^{15}$N), and N-Phosphono[1-$^{15}$C]methylglycine (99 atom % $^{15}$C) were obtained from Merck, Canada. These compounds were tested for herbicidal activity by direct application to plants and found to give results indistinguishable from those of natural abundance glyphosate. Activity of the labeled materials was further confirmed by measuring their ability to inhibit the EPSP synthase reaction, a known property of glyphosate.

The abbreviations used are: glyphosate, N-phosphonomethylglycine; CPMAS, cross-polarization magic-angle spinning; DCPMAS, double cross-polarization magic-angle spinning; EPSP synthase, 3-phosphoshikimate-1-carboxyvinyl transferase; FT, Fourier transform; $T_2^*_{c}$, spin-lock transfer time constant; HPLC, high performance liquid chromatography; $H_1$, radiofrequency-field amplitude.
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glyphosate (1). The labeled materials had the same activity as that of natural abundance glyphosate.

Culture Methods—The glyphosate-degrading bacterium used in this study, Pseudomonas PG2982, was a gift from Dr. Alworth Larson, Louisiana State University. The organism was originally isolated by subculturing Pseudomonas aeruginosa ATCC 9027 in a medium containing glyphosate, and was identified as a Pseudomonas sp. based on its biochemical activities and the guanine plus cytosine content of its DNA. The synthetic medium of Moore et al. (8) was used for all growth experiments. This medium consisted of a Dworkin-Foster salt mixture (10) minus the inorganic phosphate, plus 1% potassium gluconate as a carbon source. The medium also contained thiamine- HCl (5 μg/ml). Glyphosate or inorganic phosphate was added as a source of phosphorus to give “glyphosate” or “Pi,” medium, respectively. Glyphosate was added to the medium by filter sterilization. Media were adjusted to pH 7.0 before autoclaving.

Stock cultures of PG2982 were made by aseptically transferring about 1 ml of a culture of cells grown on glyphosate medium into a sterile glass vial containing about 10 sterile 1/4 inch concentration discs (Difco). The contents of the vial were then frozen, lyophilized, aseptically sealed, and stored at -10°C until needed. To initiate a growth experiment, a disc from one of the vials was aseptically transferred to 10 ml of glyphosate medium contained in a 50-ml Erlenmeyer flask. After approximately 2 days at 30°C on a rotary shaker at 200 rpm, the culture was ready to be used as an inoculum. Growth on glyphosate media inoculated with these cultures showed virtually no lag phase. Pseudomonas PG2982 had a doubling time of about 6 hrs, and reached a stationary-phase absorbance (660 nm) of about 4.5 when grown on glyphosate medium containing 1 mM glyphosate. During this time the concentration of glyphosate in the medium dropped from an initial value of 1.0 mM to a stationary-phase value of 40 μM. No aminomethylphosphonic acid was detected in the medium before or after growth.

PG2982 grown in media containing initial concentrations of glyphosate ranging from 0.5 to 10 mM did not show shortened doubling times. The stationary-phase absorbance reached a maximum of about 4.5 in all cases. Glyphosate was totally depleted only from the culture with a 0.5 mM starting concentration. Cultures grown on media with 1, 2, 5, and 10 mM starting glyphosate concentrations led to 0.02, 1.0, 4.1, and 8.9 mM glyphosate concentrations, respectively, after growth had ceased.

PG2982 was also grown on glyphosate media containing 1 mM concentrations of [3-13C,15N]glyphosate, [2-13C,15N]glyphosate, and [1-13C]glyphosate, respectively. (The numbering of the carbons in glyphosate is shown in Structure 1.) Labeled cells were harvested for NMR analysis at an absorbance of 1.15 (mid-log phase) by centrifugation at 10,000 × g for 10 min. The cells were washed once with 0.025 M potassium phosphate, pH 7.0, centrifuged again, frozen in liquid nitrogen, and then lyophilized. One liter of growth medium harvested 1.15 yielded about 0.6 g of lyophilized cells.

Fast cross-polarization rates for protonated nitrogens, long proton rotating-frame lifetimes, and high concentrations of protons in these biological samples ensure representative relative NMR intensities for all nitrogens. Portions of this paper (including part of “Results,” and additional Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass.

Detection of Glyphosate by HPLC—The glyphosate concentration of culture media was measured by HPLC using a procedure that involved post-column oxidation of glyphosate to phosphate followed by addition of molybdate; the reduced phosphomolybdate complex was detected photometrically at 660 nm. Aminomethylphosphonic acid was also detected by this procedure.

RESULTS

CPMAS 13C NMR of [3-13C,15N]Glyphosate-grown PG2982—The 50-MHz CPMAS 13C NMR spectrum of PG2982 grown in glyphosate medium containing 1 mM [3-13C,15N]glyphosate is shown in Fig. 1 (top), and directly below it the spectrum generated by PG2982 grown in glyphosate medium containing 1 mM glyphosate at natural abundance. Subtraction of the two spectra, normalized for sample weight and number of scans, produces a difference spectrum due only to (label Fig. 1, second from bottom). The difference spectrum shows the metabolism of the phosphonomethyl carbon of the glyphosate molecule. This spectrum contains 6 signals: two...
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Fig. 2. Degradation of glyphosate by PG2982 as determined by CPMAS NMR.

Table I
Carbon-13 chemical shifts of some purine nucleotides and amino acid residues

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-8</th>
<th>Methyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine monophosphate</td>
<td>155</td>
<td>152</td>
<td>117</td>
<td>138</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Adenosine monophosphate</td>
<td>153</td>
<td>149</td>
<td>119</td>
<td>141</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymidine monophosphate</td>
<td>173</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycyl residue</td>
<td>173</td>
<td>57</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seryl residue</td>
<td>174</td>
<td>61</td>
<td>67</td>
<td>20</td>
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<td></td>
</tr>
<tr>
<td>Threonyl residue</td>
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<td></td>
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<td></td>
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<tr>
<td>Methionyl residue</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The observed $^{13}$C labeling pattern is that expected from reactions mediated by tetrahydrofolate, a coenzyme responsible for the transfer of one-carbon fragments in organisms. A $^{13}$C-labeled single-carbon fragment can enter this coenzyme system in a limited number of ways, and is always transferred to specific sites within products (shown in Fig. 2). These sites include the C-2 and C-8 positions of the purine ring (155 and 138 ppm), the methylene-carbon positions of serine and serine-derived residues (57 and 62 ppm), and the methylcarbon positions of methionine (17 ppm) and thymidine (13 ppm) residues (cf. Table I). In addition to routing into single-carbon transfers, a fraction of the C-3 carbon pool is lost (presumably as CO$_2$) since the total incorporation per milligram of cell growth is about half that for C-2 and C-1 (cf. below).

The P-C bond of glyphosate must be cleaved prior to, or at the time of, transfer of the C-3 carbon into the tetrahydrofolate pathway. The product of the first step in the metabolism of glyphosate is presently being investigated using crude extracts of PG2982.

This PG2982 sample was also examined by $^{13}$C NMR at 15.1 MHz. After subtraction of the appropriate 15.1-MHz natural abundance spectrum, the difference spectrum (Fig. 1, bottom) shows that the methyl- and methylene-carbon resonances are only slightly broadened compared to those observed at 50.3 MHz, while the aromatic region of the 15.1-MHz spectrum is severely broadened. We attribute the lack of resolution in the aromatic region at the lower field strength to a contribution to the $^{13}$C line width from $^{15}$N and $^{14}$N coupling (arising from directly bonded nitrogens). This broadening is not removed by magic-angle spinning. An effect of aromatic carbon resonances at 155 and 138 ppm, two methylene-carbon resonances at 62 and 57 ppm, and two methylcarbon resonances at 17 and 13 ppm. None of these signals is due to [3-$^{13}$C,$^{15}$N]glyphosate whose CPMAS spectrum consists (at 50 MHz) of a single 300-Hz wide line at $\delta_0 = 45$. (This line is too broad to reveal scalar coupling; dipolar splitting is removed by spinning.)
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Fig. 3. CPMAS 50.3-MHz $^{13}$C NMR spectra of *Pseudomonas* PG2982 grown on $[1-^{13}C]$glyphosate (left), and $[2-^{13}C,^{15}N]$glyphosate (right). Top left, spectrum of cells grown on $[1-^{13}C]$glyphosate; bottom left, difference spectrum generated by subtracting an appropriately scaled natural abundance spectrum. Top right, spectrum of cells grown on $[2-^{13}C,^{15}N]$glyphosate; bottom right, difference spectrum produced as described above. The spectra at the right are displayed at about twice the vertical gain of those at the left.

Fig. 4. CPMAS 20.3-MHz $^{15}$N NMR spectra of *Pseudomonas* PG2982 grown on $[2-^{13}C,^{15}N]$glyphosate (left) and $[3-^{13}C,^{15}N]$glyphosate (right). The spectra at the bottom of the figure were obtained using the pulse sequence of Fig. 1 (Supplement) with a nitrogen spin lock of 3 ms and the carbon radiofrequency field off resonance. The spectra at the top of the figure are the double cross-polarization difference spectra generated using the pulse sequence of Fig. 1 (Supplement) with the carbon radiofrequency field alternatively off and on resonance.
this type has been reported previously for carbons bonded to nitrogens in amino acids (15, 16).

**CPMAS** $^{13}$C NMR of [2-$^{13}$C,15N]- and [1-$^{13}$C]Glyphosate-grown PG2982—If cleavage of the phosphonomethyl carbon-nitrogen bond of glyphosate were the first step in the breakdown of glyphosate, a product of the reaction would be glycine. Fig. 2 includes the four major paths for metabolism of cellular glycine. They are: 1) direct incorporation of glycine into protein; 2) interconversion of glycine into serine or threonine which can then be incorporated into protein; 3) synthesis of the purine backbones of nucleic acids; and 4) metabolism of glycine to glyoxyxlate. (The glyoxyl nitrogen, C-2, and C-3 carbons shown in the figure are marked so that routing can be followed.)

We attribute the three major signals in the difference spectrum of cells grown on [2-$^{13}$C,15N]glyphosate (Fig. 3, bottom right) to the breakdown of glyphosate to glycine followed by its routing into paths 1, 2, and 3 of Fig. 2. The 119-ppm signal is assigned to the (nonprotonated) C-5 carbon of the purine ring of nucleotides, the 43-ppm signal to the methylene carbon of glycy1 residues of proteins, and the broad 53-ppm signal to the a-carbon of seryl and threonyl residues within protein. In addition, the minor signals which appear in the difference spectrum result from glycine metabolized by path 4 to glyoxylate followed by scrambling via the glyoxylate pathway, or by conversion to serine followed by its metabolism via serine dehydratase. Based on the relative signal intensities, about 35% of the glycine from glyphosate is incorporated directly into protein as glycy1 residues, about 35% is converted to serine (and a minor amount of threony1) followed by incorporation into protein, about 20% is used to make purines, and about 10% is metabolized resulting in scrambling of the label.

The difference spectrum for PG2982 grown on [1-$^{13}$C]glyphosate (Fig. 3, bottom left) contains only two resonances, at 175 and 152 ppm. We attribute the carbonyl-carbon signal at 175 ppm to metabolism of [1-$^{13}$C]glyphosphate to [1-$^{13}$C]glycine followed by its incorporation into protein via paths 1 and 2 of Fig. 2. The 152-ppm signal is due to the incorporation of [1-$^{13}$C]glycine into purines by path 3 (labeling the C-4 carbon of the purine ring) The relative intensities of the two signals shows that the flux along pathway 3 is about one-fourth that along the sum of paths 1 and 2, in agreement with results from the C-2 labeling experiments. The C-1 carbon of glycine metabolized via glyoxylate is lost as CO2. Thus, slightly less label is incorporated from the C-1 than the C-2 position.

**CPMAS and DCPMAS** 15N NMR of [13C,15N]Glyphosate-grown Pseudomonas sp. PG2982—The two 13C CPMAS NMR spectra of double-label glyphosate-grown PG2982 are virtually identical (Fig. 4, bottom), each with an aromatic (purine) nitrogen signal at about 215 ppm (17, 18), an intense amide nitrogen (peptide) signal at 85 ppm, weak broad signals for arginine and lysine side chain nitrogens at 60 and 10 ppm, respectively, and narrow resonances at 0 and 22 ppm for NH2 and an unknown metabolite, respectively. About 8% of the amide-nitrogen signal and most of the NH2 signal is due to natural abundance. The remainder of each spectrum is due to the uptake of label. About 20% of the total signal intensity due to uptake of label is in the 215-ppm purine-nitrogen signal.

The results of the three carbon-labeling experiments described above lead to the expectation that some 70% of the nitrogen from glyphosate incorporated into PG2982 enters protein directly, much of it in the form of glycyl residues, another 20% enters purines, and the remainder is used as a general nitrogen source. This expectation is, in fact, consistent with the observed 15N NMR spectra. The intensity of the peptide resonance is indeed about four times that of the purine resonance. In addition, the peptide peak is asymmetric and has an unusual high-field average chemical shift (85 ppm) characteristic of a significant glycy1 content. Finally, weak lysine- and arginine-residue signals (although well above natural abundance levels) are consistent with a minor routing of labeled nitrogen into PG2982 through a general pool. PG2982 is not under nitrogen stress in these growth experiments, since ammonia is readily available from the medium. Thus, the glycine derived from glyphosate is not used predominately as a general source.

Proof for the cellular breakdown of glyphosate directly to glycine was provided by DCPMAS 15N NMR of PG2982 grown on [15N,13C]glyphosate. This NMR method enables direct observation of a labeled 13C-15N chemical bond (see Supplement). A 15N signal in a DCP 15N NMR spectrum arises exclusively from 15N nuclei directly bonded to 13C. The DCP difference spectrum from the [2-$^{13}$C,15N]glyphosate-labeled PG2982 (Fig. 4, top left) contains two strong signals, an 85-ppm amide resonance, and a 215-ppm purine signal. By contrast, the DCP difference spectrum from the [3-$^{13}$C,15N]glyphosate-labeled PG2982 has no strong signals (Fig. 4, top right). Based on these DCP results, the absence of significant scrambling of the C-2 and C-1 labels (Fig. 3), and the absolute levels of 13C and 15N incorporated into PG2982 (see Supplement), the C-2 carbon-nitrogen bond must be largely preserved during metabolism and the C-3 carbon-nitrogen bond broken.

An examination of the pathways of glycine metabolism (Fig. 2) shows that the 13N-13C-labeled bond in glycine will be retained in pathways 1 and 2, giving rise to the 85-ppm amide DCP signal of Fig. 4, top left, and in pathway 3, producing an N-7 nitrogen, C-5 carbon bond within purines (215-ppm DCP signal of Fig. 4, top left). The chemical shifts of the observed strong DCP difference signals are only consistent with the degradation of [2-$^{13}$C,15N]glyphosate to [2-$^{13}$C,15N]glycine, followed by its incorporation into proteins and purines via the pathways of Fig. 2. Glyphosate degrades to glycine.

The weak signal at 215 ppm from the [3-$^{13}$C,15N]glyphosate-labeled PG2982 (Fig. 4, top right) is due to the double-labeled N-7, C-8 bond of a purine ring; the N-7 label coming from the [15N]glycine intermediate and the C-8 label from the C-3 carbon of glyphosate by way of the tetrahydrofolate pathway. De novo purine synthesis from unlabeled carbon sources is responsible for the isotopic dilution at the C-8 carbon position.

The intensities of the DCP spectra can be used for a quantitative determination of the appearance of labeled chemical bonds in the purines and proteins of PG2982, as well as an independent assay of the relative fluxes along the pathways of Fig. 2. These determinations are presented in the Supplement.

**Discussion**

**Glyphosate Metabolism**—The data presented in this report establish that Pseudomonas sp. PG2982 cleaves glyphosate directly to glycine. The phosphonomethyl carbon of the glyphosate molecule enters into the tetrahydrofolate-directed pathway of single-carbon transfers, and the phosphonate group, the only source of phosphorus for the bacterium, is used for growth. Glycine derived from glyphosate is further metabolized by the standard pathways shown in Fig. 2.

Direct evidence for the breakdown of glyphosate to glycine was supplied by DCPMAS 15N NMR (Fig. 4), which shows...
that metabolism of the glyphosate molecule preserves a C-2 carbon-nitrogen bond within glycine and its metabolites whereas the C-3 carbon-nitrogen bond is broken. Thus, the enzymatic breakdown of glyphosate within PG2982 involves cleavage of the phosphonomethyl carbon-nitrogen bond, releasing glycine. This is in contrast to mixed cultures of soil microbes which break glyphosate down to aminomethylphosphonic acid (2, 3), cleaving the C-2 carbon-nitrogen bond in the process.

Studying Cellular Metabolism by CPMAS NMR—The results of conventional metabolic radiolabeling experiments on organisms like PG2982 often appear complex. For instance, a labeled glycyl unit which is only incorporated into protein still results in significant labeling of all proteins. Interpretation of the resulting radiolabel pattern of the proteins is not straightforward. Has the label remained unchanged within the glycyl unit, has it been transferred to amino acids derived specifically from glycine, or has it been partially scrambled to other carbon positions?

Solid-state CPMAS NMR is well suited to this sort of study of metabolism. The solid-state NMR experiment produces a spectrum arising from all the components of the sample, proteins and nucleic acids included. Yet the labeling patterns are often simple to interpret. For example, a labeled glycyl fragment which has not been scrambled by metabolism will give rise to a single glycyl NMR signal even though the fragment may be present in a thousand different proteins. This ease of analysis is also clearly illustrated by the labeling of nucleic acids in PG2982. The C-3 carbon of glyphosate was found by NMR to end up in only two of the carbon sites of purines, the C-2 and C-8 positions. A comparable experiment with [3-14C]glyphosate would have required a considerable effort to determine the positions of the radiolabels in these nucleic acids.

The application of solid-state NMR to the determination of the metabolism of a compound is also distinctly different from the more familiar use of solution-state in vivo FT NMR on live cells (20). In the FT NMR experiment, only low-molecular weight metabolites in solution are observed. When steady-state concentrations of metabolites of a pathway are low, yet the flux through the pathway into polymeric material is high, FT NMR gives no information about this path. The metabolites are simply not observed.

The solid-state NMR experiment gives signals for everything present in the lyophilized material, the polymeric high-molecular weight material included. Consequently, even in the situation where the steady-state level of intermediates is below detectability, the end products produced by metabolism can still be used to infer the identity of the intermediates. This was precisely the situation with the metabolism of glyphosate in PG2982. Few identifiable intermediates were observed in any of the spectra. No labeled free glycine was detected. Yet, the complete pathway for glyphosate degradation via glycine was established based on the labeling patterns of the end products.

Acknowledgments—We thank Nancee M. Kimack for help with the growth of Pseudomonas PG2982. Thanks are due to Clark Porter and Joel Ream for measuring EPSP synthase inhibition by stable isotope-labeled glyphosate compounds, and to Robert Merz for measuring herbicidal activity of the labeled materials by direct application to plants. Claude A. Ricketts and John A. Long performed HPLC analyses of residual glyphosate within growth media. We also thank Larry Hallas and Terry Balthazor for providing a slant of PG2982 and for helpful suggestions concerning its growth. All of these individuals are a part of Monsanto Co., St. Louis.

REFERENCES
Solid-state NMR determination of glyphosate metabolism in a Pseudomonas sp.

Gary S. Jacob, Jacob Schafer, R. G. Steijakal, and R. A. McKay

Double-Cross Polarization NMR and Chemical Bond Labeling

Double cross-polarization NMR is an extension of cross polariz-ation to systems containing three different spin-1/2 nuclei, in par-ticular systems containing 1H, 13C, and 15N. The experiment involves first a transfer of polarization from abundant protons to either carbon or nitrogen, followed by removal of the proton rf field together with a spin-lock contact of carbon and nitrogen. Either 13C or 15N spins can then be observed, resulting in spectra of high sensitivity which contain information about carbon-nitrogen dipolar coupling. The technique has been used for the detection of labeled 13C-15N chemical bonds in complicated intact biological systems (9).

Double cross-polarization 15N NMR spectra of double-labeled glyphosate in PG2982 were obtained using matched spin-lock transfers first from 1H to 15N and then from 15N to 13C. If the 13C rf field is on resonance and its amplitude satisfies a carbon-nitrogen Hartmann-Hahn condition, a spin-lock transfer from 15N to 13C drains polarization from 15N. A direct difference experiment between single polarization and double cross-polarization procedures then results in the accumulation of a DCPMAS difference 15N-signal arising exclusively from those nitrogen directly bonded to 13C.

The pulse sequence for the experiment is shown in Fig. 1, and the experimental conditions described in the caption. It is possible to show (12) that the ratio of the 15N DCP difference signal, $AS$, to the ordinary CPMAS signal after the 15N spin-lock hold, $S_0$, is given by $f/(1-e^{-7/2T_{2\text{H}}})$, where $f$ is the fraction of 15N spins under observation with a 13C neighbor, $t$ is the contact time between carbon and nitrogen in a matched spin-lock interaction, and $T_{2\text{H}}$ is the spin-lock transfer time constant, often referred to as $T_{\text{D}}$. Typical values for $f$ are 0.5 to 5%, and for $T_{2\text{H}}$, 3 to 20 msec. For a system in which $f$ is known, $T_{2\text{H}}$ can be determined using the pulse sequence of Fig. 1 by varying $t$.

**Double-Cross Polarization Pulse Sequence**

\[ \text{H} \quad r \quad \text{N} \quad \text{C} \]

Figure 1. Double-cross-polarization pulse sequence. A 15N direct difference signal can be accumulated by shifting the 13C radiofrequency field off resonance for one scan and on resonance for the next. The second NMR signal is subtracted from the first. The time $t$, during which the 15N is off, is called the 15N hold time when the 13C rf field is off resonance, and the drain time when the 13C is on resonance. The 13C rf field is phase modulated by 100-Mh Gaussians to prevent an accumulation of carbon magnetization. Data acquisition occurs with the 15N radiofrequency field turned back on for dipolar decoupling. Amplitudes of rf fields are typically 35 kHz for spin-lock contacts, and 50 kHz for decoupling. The first contact between 1H and 15N typically lasts for 2 msec, and the second between 13C and 15N for 3 msec. The sample is contained in a cylindrical double-bearing magic-angle rotor with an internal volume of 700 pl.

The 15N isotopic enrichment of a labeled bond can be determined from observed absolute intensities in the 15N CPMAS experiment (11). The labeled C-N chemical bond concentration is then determined by the fraction of 15N atoms in the bond with a 13C neighbor, which is equal to $f$. Using appropriate model compounds to determine experimental $T_{2\text{H}}$'s for the chemical bonds of interest (9,12), we determine $f$ by measuring the 15N $AS/S_0$ for a single contact time. The ratio $AS/S_0$ and the concentration $f$ are related by the family of straight lines shown in Fig. 2.

---

*Figure 2.* The fraction, $f$, of 15N atoms of a chemical bond with a 13C neighbor as a function of the 15N DCP difference signal, $AS$, observed for a carbon-nitrogen contact time, $t$, of 3 msec, for typical values of the carbon-nitrogen cross-polarization transfer time, $T_{\text{CM}}$. The difference signal is scaled by the CPMAS signal after the 15N spin lock, $S_0$. The calculated lines correspond to $f = (AS/S_0)/(1 - e^{-7/2T_{2\text{H}}})$.

The DCPMAS 15N NMR spectrum from the [2-13C, 15N]glufosinate-grown sample (Fig. 4, top left, full text) shows only two difference signals, one at 215 ppm due to the N-7 nitrogen of purines, and the other at 89 ppm due to amide nitrogen (see full text for line assignments). The ratio of the difference signal, $AS$, to the full signal, $S_0$, is 0.43 for the 215-ppm signal. This indicates that the purine 15N-7 has a 90% (±10%) enriched carbon neighbor, consistent with incorporation of [2-13C, 15N]glufosinate into purines (Fig. 2, pathway 3, full text). The enrichment is determined directly from Fig. 2 using the experimental $AS/S_0$ of 0.43 and a $T_{2\text{H}}$ of 5 msec, the latter typical of aromatic carbon-nitrogen bonds (12). The 15N enrichment of the carbon next to the labeled amide nitrogen is also high, as evidenced by a $S_0/N$, of about 0.18. If we assume the enriched carbon is aliphatic, then $T_{2\text{H}}$ is 10 to 20 msec (9,12), and the enrichment is 90% (f = 0.9) determined from Fig. 2.

The 13C CPMAS spectrum for cells grown on [2-13C, 15N]glufosinate (Fig. 3, top right, full text) gives an integrated intensity about 2.2 times that of cells at natural abundance. Thus, incorporation of 13C label is equal to (1.2)(1.2) = 1.5% of total carbon. Assuming nitrogen and carbon comprise about 15 and 50%, respectively, of cellular dry weight (21), we expect 15N concentrations of about (1.2)(10/50)(15) = 4.5% of total nitrogen for a one-to-one incorporation of 15N and 13C from [2-13C, 15N]glufosinate. The 13C CPMAS spectrum (Fig. 4, bottom left, full text) gives a signal about 22 times greater than natural abundance (not shown), corresponding to a 3.6% 13C incorporation of the sample nitrogen, in close agreement with the expected value. Thus these can be only minor nitrogen from glyphosate released back into the growth medium. In fact, based on CPMAS 13C and 15N NMR, over 90% of the 15N from glyphosate taken up by PG2982 ends up in purines and proteins, and a little amount of 13C from the C-2 site. This is in agreement with our DCIPS experiments (see full text), and is consistent with little scrambling of 15N label in PG2982.