Estimation of Protein Turnover in Soybean Leaves Using Magic Angle Double Cross-polarization Nitrogen 15 Nuclear Magnetic Resonance*

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Protein turnover plays a central role in the overall growth and development of plants. Although measurements of the rates of protein turnover are obviously valuable in studies of the regulation of general plant metabolism, none of the methods currently in widespread use are both inherently accurate and free from species-specific technical difficulties (1, 2). The development of new methods for measuring protein turnover in plants remains an important goal. In this paper, we report the use of 13C and 15N labeling and solid state magic angle double cross-polarization 15N nuclear magnetic resonance to estimate the rates of total protein turnover in attached leaves of whole soybean plants.

The combination of high power resonant decoupling and high speed mechanical sample spinning has been demonstrated to produce liquid-like high resolution 13C nmr spectra of a wide variety of organic solids (3). Individual resonances from chemically different carbon atoms can be observed. The high resolution technique becomes a high sensitivity one when the rare-spin magnetization is obtained from a cross-polarization transfer from the abundant protons. These techniques work as well for 15N as for 13C.

Cross-polarization 13C and 15N nmr of intact (lyophilized) biological material provides a direct and convenient nondestructive measure of the total uptake and metabolic fate of the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The entire 7-day period after which they were returned to the growth chamber. Each subsequent 14-h light period was followed by a 10-h dark period. After exposure to $^{13}$CO$_2$, trifoliate leaves were removed from the plants over the next 6 weeks and metabolism stopped by immersion in liquid N$_2$. After lyophilization, the leaf samples were ready for NMR analysis. About 300 mg of powdered leaf tissue was pressed into a pellet and inserted into a magic-angle rotor.

**Spin-lattice**

The synthesis of benzyl(oxycarbonyl)glucylamide derivative (Fig. 1) was performed in two steps. Benzyl(oxycarbonyl)glucylamide (Merek) in 4 N NaOH with benzyl(oxycarbonyl)chloride (Aldrich Chemical Co., Milwaukee, WI) for 30 min at 0°C, after which the solution was extracted with ether, acetylated to pH 2.5 with concentrated HCl, and extracted with ethyl acetate. The ethyl acetate extract was dried over MgSO$_4$. The resulting amide nitrogens were predominantly in the peptide spin system. The carbon lock transfer from the carbon-nitrogen Hartmann-Hahn condition (12), a matched spin-lock cross-polarization condition, with 13C-ammonium formate (Sigma Chemical Co., Rochestor, NY) at -5°C. After 25 min, a solution of [15N]glucine (95 atom % $^{15}$N; Merek) in 1 N NaOH was added and stirred for 3 h at room temperature (8). The aqueous layer was isolated, extracted with ether, and precipitated with cold 1 N HCl. The precipitate was washed with cold 1 N HCl, cold water, and dried. The purity of the [15N]glucine (12) was assessed by melting point, thin-layer chromatography (TLC), and nmr. Magic Angle 15N 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 26-kHz radio frequency field (13). The pulse sequence employed in these experiments is shown in Fig. 1. The first pulse is the same as used for $^1$H-15N cross-polarization described above. (The 'H rf field is adiabatically reduced from its maximum value of about 50 kHz used for spin locking and decoupling to the 26-kHz value needed to match the 15N rf field, the latter the most difficult to generate in a single, triply-tuned coil.) The proton rf field is turned off and the 15N magnetization held spin locked for a variable time, $\tau$, during which a 13C rf field is turned on. If the 13C rf field is on resonance and its amplitude satisfies a carbon-nitrogen Hartmann-Hahn condition (12), a matched spin-lock transfer from 15N to 13C drains polarization from the nitrogen spin system. The carbon 13C rf field is phase-modulated to prevent an accumulation of carbon polarization, thereby helping to ensure simple kinetics describing the 15N to 13C transfer (5). A direct difference experiment between single and double cross-polarization procedures (Fig. 1) results in the accumulation of a 15N signal arising exclusively from those nitrogens directly bonded to 13C. Data collection for difference spectra typically required 12 to 24 h.

### RESULTS

15N NMR and Double Cross-Polarization—In the absence of a 15N-13C double cross-polarization drain (Fig. 1, 13C rf field off-resonance), the 15N magnetization, $S$, is a function of the rotating frame hold time, $\tau$, given by

$$S = e^{-\tau/T_\rho(N)} = S_0(\tau) = S_0$$

where we have assumed the decay of $S$ is described by a single relaxation time, $T_\rho(N)$, which may be related to either spin-spin or spin-lattice processes in the solid (13).

An example of the decay of $S$ in the absence of a drain with increasing $\tau$ for a double-labeled tyrosine leaf is shown in Fig. 2. For short $\tau$, the spectrum is dominated by a signal (about 100 ppm downfield from solid ammonium sulfate) due to amide nitrogens predominantly in peptide linkages of proteins. Minor signals are due mostly to lysine, arginine, and histidine side chain nitrogens (4). The peaks at the extremes of the spectrum are spinning side bands arising from the 1.4-kHz mechanical sample spinning. In this paper, we will be concerned only with the main chain peptide-amide nitrogen resonance. After a 7-ms hold time, this resonance has decayed to 60% of its original intensity (Fig. 2), indicating $T_\rho(N)$ is about 10 ms.

In the presence of a single 15N-13C drain (Fig. 1, 13C rf field on resonance), an additional decay process for $S$ is introduced so that

$$S = S_0 e^{-\tau/T_\rho(N)}$$

where we have assumed that the drain process itself is adequately described by a single exponential time constant (5). For a massively labeled protein main chain, two types of 15N-13C couplings can occur in fragments such as 15N-13C-15N-13C, where the subscript a denotes an amide carbon, and b an aliphatic carbon. Different couplings arise because the carbon-nitrogen bond lengths in this fragment are different, and hence, 15N-13C cross-polarization transfer (drain) rates are also different.

If the probability that $C_a = 13C$ is $p_a$ and the probability that $C_b = 15N$ is $p_b$, then the fraction of 15N atoms drained by both $C_a$ and $C_b$ is $p_ap_b = p_{ab}$ (with rate $T_{ab}^{-1}$), by $C_b$ only is $p_b(1-p_a) = f_a$ (with rate $T_a^{-1}$), by $C_a$ only is $(1-p_a)p_b = f_b$ (with rate $T_b^{-1}$), and by neither is $(1-p_a)(1-p_b) = f_0$. Since $f_0 + f_a + f_b + f_0 = 1$, we have made the implicit assumption that all protein is labeled with both 13C and 15N (i.e., the protein pool is homogeneous). Thus, in the presence of multiple drains on $S$,

$$S = S_0(f_{ab} e^{-\tau/T_{ab}} + f_a e^{-\tau/T_a} + f_b e^{-\tau/T_b} + f_0)$$

The relative difference signal arising from the direct difference experiment of Fig. 1 is then

$$S(S - S_0) = 4 f_a R_{ab} + 4 f_b R_a + 4 f_0 R_0$$

where

$$R_{ab} = 1 - e^{-\tau/T_{ab}}$$

1 The abbreviation used is rf, radiofrequency.

2 E. O. Stejskal, J. Schaefer, and R. A. McKay, manuscript submitted for publication.
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The pulse sequence of Fig. 1 was used with the $^{13}$C rf field off-resonance and $\tau$ varying from 0.5 to 7.0 ms. The decay of each line in the spectrum determines its $T_2(T_{1N})$. The major line (which occurs about $100$ ppm downfield from external solid ammonium sulfate) arises predominantly from peptide amide nitrogens. The two lines at the extremes of each spectrum are spinning side bands (SSB) and arise from the 1.4-kHz mechanical sample spinning.

$$R_a = 1 - e^{-\tau/T_a}$$

(6)

$$R_b = 1 - e^{-\tau/T_b}$$

(7)

Values for $T_a$ and $T_b$ can be measured on known, specifically labeled amino acids. We find, for example, that for $[2-^{13}$C-$^{15}$N]$^{15}$N-labeled glycine, $T_a = 10.9$ ms and for $[4-^{13}$C-$^{15}$N-(amide)]asparagine, $T_a = 3.6$ ms.

We make the reasonable assumptions that

$$T_{ad} = T_a^{-1} + T_b^{-1}$$

(8)

(many body spin interactions are absent and spin-spin cross-relaxation rates are additive, cf. below) and that

$$p_a = p_b = p$$

(9)

(uniform labeling). Equation 4 now becomes

$$\Delta S/S_0 = p(R_a + R_b) - p^2 R_a R_b$$

(10)

and

$$p(R_a + R_b) = B (\Delta S/S_0)$$

(11)

where

$$B = 2/[1 + (1 - 4Q)^{1/2}]$$

(12)

$$Q = R_a R_b (\Delta S/S_0)/(R_a + R_b)^2$$

(13)

This means a plot of the product of $B$ and the experimentally determined $\Delta S/S_0$ against the sum of $R_a$ and $R_b$ is a straight line with a slope equal to $p$, the main chain $^{13}$C concentration determined in the determination of $p$ (±10%). Values for $p$ for all leaves examined in the 1- and 7-day labeling experiments are presented in Tables I and II, respectively. The numbering system used to identify the trifoliolates is shown in Fig. 5. The

Examples of the determination of $\Delta S/S_0$ for two levels of $^{13}$C in soybean leaves are shown in Fig. 3. Because the experimental direct difference signal is accumulated in real time (i.e., it is not the difference of two spectra collected sequentially), the effects of long term systematic drifts in the performance of the spectrometer are automatically cancelled. Reliable values for $\Delta S/S_0$ can be determined from the integrals of these main chain $^{15}$N nmr signals even for low levels of $^{13}$C.

Typical plots of $B(\Delta S/S_0)$ versus ($R_a + R_b$) are shown in Fig. 4. Reasonably straight lines are observed for about a 5-fold variation in $p$, supporting the assumptions leading to Equation 10. The scatter in these plots gives some idea of the precision in the determination of $p$ (±10%). Values for $p$ for all leaves examined in the 1- and 7-day labeling experiments are presented in Tables I and II, respectively. The numbering system used to identify the trifoliolates is shown in Fig. 5. The

FIG. 2. Single cross-polarization magic-angle $^{15}$N nmr spectra of a soybean leaf (Plant 9, trifoliolate no. 9, Table II). The pulse sequence of Fig. 1 was used with the $^{13}$C rf field off-resonance and $\tau$ varying from 0.5 to 7.0 ms. The decay of each line in the spectrum determines its $T_2(T_{1N})$. The major line (which occurs about $100$ ppm downfield from external solid ammonium sulfate) arises predominantly from peptide amide nitrogens. The two lines at the extremes of each spectrum are spinning side bands (SSB) and arise from the 1.4-kHz mechanical sample spinning.

FIG. 3. Magic angle cross-polarization $^{15}$N nmr spectra of $^{15}$N-enriched lyophilized soybean leaves exposed to $^{13}$CO$_2$ for 7 days (left) and exposed only to normal CO$_2$ (right) during active photosynthesis. The spectra at the top of the figure were obtained using the pulse sequences of Fig. 1 with a nitrogen spin lock of 7 ms and the carbon rf field off-resonance by 60 kHz. The spectra at the bottom of the figure are the result of double cross-polarization direct difference experiments in which the carbon rf field is first off-resonance and then on-resonance. This procedure was repeated 30,000 times. The positive signal which accumulates is a measure of the concentration of $^{15}$N-$^{13}$C pairs in the labeled leaf protein.

FIG. 4. Reasonably straight lines are observed for about a 5-fold variation in $p$, supporting the assumptions leading to Equation 10. The scatter in these plots gives some idea of the precision in the determination of $p$ (±10%). Values for $p$ for all leaves examined in the 1- and 7-day labeling experiments are presented in Tables I and II, respectively. The numbering system used to identify the trifoliolates is shown in Fig. 5.
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0.155. Naturally, if we make $T_c \gg T_1$ (which, however, can be demanding on the spectrometer), then a steady state $\Delta_S/S_0$ can be measured and the determination of $p$ is independent of the exact values of the drain rates. In any event, we conclude that possible complicating effects of multiple labels observed in these experiments are minor. This would be true even if the level of $^{13}$C incorporation were much higher than observed for both growing and mature leaves.

**Determination of Turnover Rates for Protein Main Chains**—In the 7-day labeling experiment, four trifoliolates from plant 1 were harvested 1 day after the $^{13}$CO$_2$ exposure. The two trifoliolates taken from the main branch at this time had $^{15}$N-$^{13}$C concentrations which were 8.5% and 6.9%, respectively, of the total $^{15}$N present. Since all nitrogens are assumed

\[ (R_a + R_b) \rightarrow \frac{\Delta S}{S_0} = f_a R_a \]  

leading to

\[ 1 - \frac{(\Delta S/S_0)}{P_a} = e^{-\tau/T_c} \]  

Thus, a semilog plot of $1 - (\Delta S/S_0)/p_a$ against $\tau$ is a straight line with slope $T_c$. For the triple-labeled dipeptide derivative, we find a drain rate time constant (denoted $T_{13}$) of 4.4 ms (Fig. 6), about 25% greater than that observed for double label asparagine. We attribute this relatively small but measurable increase not to a slightly longer N–C bond length for the peptide, but rather to complicated $^{13}$C–$^{13}$C spin dynamics. As mentioned above, a similar effect of triple labels on $T_{13}$ is not present since the aliphatic $^{13}$C is already tightly coupled to protons and the presence of an additional $^{13}$C carbonyl dipolar interaction is negligible.

A variation in $T_a$ from 3.6 to 4.4 ms in the plots of Fig. 4 has only a minor effect on $p$, changing the value derived from the straight line, second from the left, for example, from 0.143 to

**Table I**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Stem or branch</th>
<th>Node</th>
<th>Trifoliolate no.</th>
<th>Harvest</th>
<th>$^{13}$C (main chain)</th>
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</table>

$^a$ Numbering system illustrated in Fig. 5.

**Table II**

<table>
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<th>Stem or branch</th>
<th>Node</th>
<th>Trifoliolate no.</th>
<th>Harvest</th>
<th>$^{13}$C (main chain)</th>
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</thead>
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<td>41</td>
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<td>2</td>
<td>8</td>
<td>6</td>
<td>10.6</td>
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</table>

$^a$ Numbering system illustrated in Fig. 5.
to be \(^{15}\text{N}\), these values represent the main chain \(^{13}\text{C}\) concentrations. The former trifoliolate was fully expanded (that is, had reached its mature size) at the time of \(^{13}\text{CO}_2\) exposure and had a dry weight of 729 mg at the time of harvest. The latter was fully expanded but was probably in the initial state of senescence (decay) at the time of exposure; this trifoliolate had a dry weight of 492 mg at the time of harvest.

Two trifoliolates taken from a lateral branch after 1 day were both fully expanded, similar in weight (516 and 558 mg dry weight), and had similar \(^{15}\text{N},^{13}\text{C}\) concentrations (9.1 and 8.8\%, respectively, Table II). Younger trifoliolates (for example, trifoliolates I-8, I-9, and I-12, Table II), which were not fully expanded, were harvested from lateral branches at 7 and 13 days. These trifoliolates had higher concentrations of \(^{15}\text{N},^{13}\text{C}\). On average, mature leaves exposed to \(^{13}\text{CO}_2\) for 7 days (14-h daylight \(^{13}\text{CO}_2\) exposure for each day) accumulated about 9\% \(^{13}\text{C}\) in their protein main chains. By comparison, fully expanded leaves exposed to \(^{13}\text{CO}_2\) for 1 day (14-h daylight \(^{13}\text{CO}_2\) exposure period) accumulated an average \(^{15}\text{N},^{13}\text{C}\) concentration which was about 3\% of the total \(^{15}\text{N}\) in the leaf (Table I). If we assume the kinetics of \(^{13}\text{C}\) incorporation into protein main chain is described by a simple first order process, then

\[
\frac{dc}{dt} = -kc + kc_1
\]  

where \(c\) is the isotopic concentration of the protein carbon pool of interest, \(c_1\) is the isotopic labeling concentration, and \(k\) is the rate constant describing the incorporation. The time constant of the process is given by

\[
T_t = \frac{\Delta t}{\ln(|c_1 - c_1|)}
\]

In Equation 17, \(c_0\) is the \(^{13}\text{C}\) natural abundance (1.1\%) and \(c_1\) is the main chain \(^{13}\text{C}\) concentration after incorporation for a time, \(\Delta t\). For the 1- and 7-day experiments, \(c_1\) is 3 and 9\%, respectively (cf. above). Based on this analysis, we find incorporation time constants of 44 and 74 days, respectively, for the 1- and 7-day \(^{13}\text{CO}_2\)-labeling experiments. Since we have assumed the protein carbon pool is constant, the incorporation time constant and turnover half-life are one and the same.

The loss of \(^{15}\text{N}-^{13}\text{C}\) in trifoliolates harvested as a function of time can be used to estimate independently the rate of turnover of protein in these leaves. The first order kinetic turnover lifetime is calculated using the formula,

\[
T_t = \frac{\Delta t}{\ln(|c_1 - c_1|)}
\]

where \(c_1\) is now the \(^{15}\text{N}-^{13}\text{C}\) concentration of a trifoliolate harvested at an earlier time, \(c_0\) is the concentration in a second trifoliolate measured at a later time, and \(\Delta t\) is the difference in days between the two harvesting times. We compare only trifoliolates which were at similar developmental stages on different plants. Thus, the \(^{13}\text{C}\) concentrations immediately after \(^{13}\text{CO}_2\) labeling may reasonably be assumed to be the same for the two trifoliolates. Comparisons were made with trifoliolates from both the main and lateral branches, but only for older leaves. Thus, possible problems associated with \(^{13}\text{C}\) incorporation during growth were avoided. The resulting turnover lifetimes (calculated using Equation 18) are presented in Table III. The average lifetime for these interplant comparisons is 28 days.

**TABLE III**

<table>
<thead>
<tr>
<th>Trifoliolates compared*</th>
<th>Main chain (^{13}\text{C}) concentration</th>
<th>(\Delta t)</th>
<th>(c_1)</th>
<th>(c_0)</th>
<th>Lifetime</th>
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<tr>
<td></td>
<td></td>
<td>days</td>
<td>%</td>
<td>%</td>
<td>days</td>
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<td>I-7, I-10</td>
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<td>Average</td>
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* Plant and trifoliolate designation from Table II.

**DISCUSSION**

The incorporation time constant of \(^{15}\text{N}-^{13}\text{C}\) pairs into leaf protein for the 7-day labeling is about 50% greater than that observed for the 1-day labeling experiment. There are two factors associated with the \(^{13}\text{CO}_2\) labeling of the 7-day experiment which could together account for the longer time constant. First, in the 7-day experiment, the plants were left in the labeling chamber during the 10-h dark period, and the \(^{13}\text{CO}_2\) which accumulated during the night as a result of respiration of older, unlabeled leaves was not flushed out before the beginning of the daylight period. As a result, on the 2nd and subsequent days of \(^{13}\text{CO}_2\) exposure, the plants first refixed all of the \(^{13}\text{CO}_2\) present in the chamber. This refixation took approximately 1 h, and so resulted in about a 10% dilution of \(^{13}\text{CO}_2\) with \(^{12}\text{CO}_2\).

Second, the plants fixed \(^{12}\text{CO}_2\) at a slower rate during the latter part of the 7-day experiment. About 20% less \(^{12}\text{CO}_2\) was fixed in the last 4 days as was fixed in the first 3 days. This was probably a reaction of the plants to the confined conditions of the labeling chamber. Because of these two factors, we suspect that the incorporation time constant determined from the 1-day experiment is the more reliable.

A modest variation was observed in the values obtained for the turnover lifetime of \(^{15}\text{N}-^{13}\text{C}\) pairs (Table III). We believe these variations result from the formation and metabolism of double label by each trifoliolate at a rate determined by its individual developmental stage. Obviously, the most accurate turnover lifetimes would be those determined from comparisons of trifoliolates which were at closely similar developmental stages, or, even better, by determinations based on the removal at different times of small fractions of the same trifoliolate. Unfortunately, the sensitivity of our present apparatus precludes the latter possibility. Because soybean plants I and II were not necessarily identical, only the average turnover lifetime presented in Table III should be considered significant. Some of the individual comparisons may indeed reflect developmental differences between trifoliolates.
Although the double cross-polarization method described here for measuring the rate of turnover of leaf protein is as yet not an exact method, we believe there are sufficient advantages of the technique to encourage further refinement. For example, the use of high levels of radioactive labels are not involved, so that destructive effects of the labeled compounds on the plant tissue are avoided, as are health hazards to laboratory workers. There are, in principle, no perturbations to the system being studied. That is, labels are introduced naturally. Leaves are not detached until the end of the experiment, and no destructive tissue extraction procedures are involved. Reutilization of label is less of a problem than with the use of $^{14}\text{CO}_2$ or $^{14}\text{C}$-amino-acid labelings because only $^{13}\text{C}$ atoms associated with main chain peptide amide nitrogens are measured. Thus, gross underestimates of the turnover rates, due to the reutilization of amino acids from slowly metabolized labeled side chains, do not occur.

Of course, some of the $^{15}\text{N}-^{13}\text{C}$ bonds detected with our double cross-polarization method could have appeared as the result of reincorporation of the $^{13}\text{C}$ in carboxyl carbon atoms of amino acids into protein amide carbon atoms. We do not believe this is a serious problem since the measured incorporation time constant for the 1-day labeling experiment (44 days) is in reasonable agreement with the average turnover half-life determined by comparisons of leaves of different plants (28 days).

The incorporation and turnover rates reported here indicate that the protein of a mature, fully expanded leaf of soybean is fairly long lived with a lifetime of about 30 days. This suggests that the synthesis of protein does not put a significant demand on the energy budget of expanded leaves.

REFERENCES