

Comparison of Rotational-Echo Double-Resonance and Double-Cross-Polarization NMR for Detection of Weak Heteronuclear Dipolar Coupling in Solids

Allyson M. Christensen and Jacob Schaefer*

Department of Chemistry, Washington University, St. Louis, Missouri 63130, USA

Karl J. Kramer

US Grain Marketing Research Laboratory, Agricultural Research Service, US Department of Agriculture, Manhattan, Kansas 66502, USA

Insect exoskeleton, multiply labeled with ^{13}C and ^{15}N , was examined by rotational-echo double-resonance (REDOR) and double-cross-polarization (DCP) magic-angle spinning ^{13}C NMR. Low levels of incorporation of label make the analysis of these samples a practical test of the relative advantages of REDOR and DCP for the detection of weak, heteronuclear dipolar coupling between rare spins in solids. The sensitivity of REDOR for the detection of directly bonded ^{13}C - ^{15}N pairs is an order of magnitude greater than that of DCP when neither label is involved in homonuclear dipolar coupling of strength comparable to the spinning frequency. However, if either of the ^{13}C or ^{15}N labels undergoes homonuclear spin flips, DCP gains in relative sensitivity and is easier to use for spin counting than REDOR.

KEY WORDS REDOR Double-cross-polarization Solids NMR ^{13}C - ^{15}N dipolar coupling Magic-angle spinning Insect cuticle

INTRODUCTION

Rotational-echo double-resonance¹ (REDOR) and double-cross-polarization² (DCP) magic-angle spinning NMR are both capable of detecting weak heteronuclear dipolar coupling between rare-spin labels in biological solids. A typical REDOR experiment involves the dephasing of transverse carbon magnetization by ^{15}N π pulses synchronized with the magic-angle spinning. All ^1H - ^{13}C and ^1H - ^{15}N interactions are suppressed by decoupling. The dephasing arises from local field gradients and depends directly on the strength of the ^{13}C - ^{15}N dipolar coupling. If there are no other spin couplings, analysis of REDOR dephasing is simple.^{3,4}

Analysis of a DCP polarization transfer, on the other hand, is complicated.⁵ A typical DCP experiment involves two polarization transfers: the first from protons to, say, carbons for sensitivity enhancement, and the second from carbons to nitrogens to characterize ^{13}C - ^{15}N dipolar coupling. The second transfer is done in the presence of couplings with protons to relieve otherwise stringent Hartmann-Hahn matching requirements.⁵ The transfer rate therefore has an involved dependence⁶ on the rare-spin dipolar coupling, the amplitude modulation of this coupling by magic-angle spinning and the amplitude and frequency modulation of the ^1H - ^{13}C and ^1H - ^{15}N dipolar couplings by spinning.

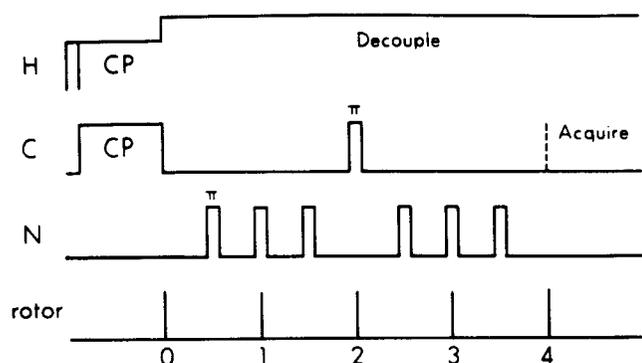
Despite these complications, DCP ^{13}C and ^{15}N NMR have been used successfully to examine metabolism,⁷ protein structure⁸ and cross-linking.⁹ Recently both REDOR and DCP have been used in structural studies of two samples of ^{13}C - ^{15}N labeled tobacco hornworm pupal exuviae (exoskeleton). One sample was obtained by injection of wandering fifth-instar hornworm larvae with L-[ring- $^{15}\text{N}_2$]histidine and [β - $^{13}\text{C}_1$]dopamine, and the other sample by injection with L-[ring- $^{15}\text{N}_2$]histidine and uniformly labeled [ring- $^{13}\text{C}_6$]dopamine. After several days, covalently bonded ^{13}C - ^{15}N labels appeared in newly synthesized exoskeleton from the formation of low concentrations of cross-links between histidine-ring nitrogens of proteins and β and ring carbons of catecholamines derived from dopamine. A comparison of the results of these experiments provides a useful evaluation of the merits of the two techniques under the conditions of a demanding application.

EXPERIMENTAL

Pulse sequences for REDOR and DCP with ^{13}C detection are illustrated in Fig. 1. REDOR requires control of the spinning speed to about $\pm 0.1\%$ because the dephasing pulses and start of data acquisition are rotor synchronized.³ DCP requires the same level of accuracy in spinning-frequency control because the rare-spin transfer rate has a strong dependence on the spinning speed through the amplitude modulation of the rare-spin dipolar coupling.⁶ REDOR relies on high-quality,

* Author to whom correspondence should be addressed.

REDOR



DCPMAS

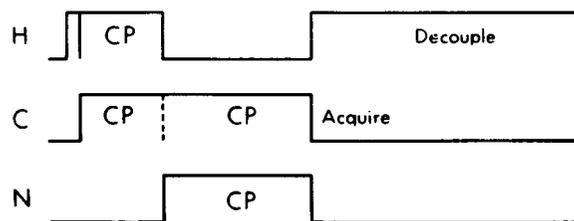


Figure 1. Pulse sequences for REDOR and DCP magic-angle spinning (MAS) ^{13}C NMR. Although REDOR spectra necessarily have spinning sidebands, DCP spectra can be obtained either with or without total suppression of spinning sidebands.¹³

phase-shifted ^{15}N π pulses to dephase carbon magnetization with no dependence on ^{15}N frequency offsets.^{3,10} DCP depends on long, stable, droop-free spin-lock pulses for maximum polarization transfer between heteronuclear rare spins.⁵ Both experiments are performed as differences. The REDOR difference is between spectra obtained with and without the dephasing π pulses. The DCP difference is between spectra obtained with and without polarization transfer between rare spins. Both difference spectra contain information about only those carbons that are dipolar coupled to nitrogen.

Carbon-13 NMR spectra in these experiments were obtained at room temperature at 50.3 MHz with magic-angle spinning at 3.205 kHz. Proton-carbon cross-polarization transfers were performed at 38 kHz and proton dipolar decoupling at 80 kHz. The single, 13-mm diameter, radiofrequency coil was connected by a low-loss transmission line to a triple-resonance tuning circuit.¹¹ REDOR spectra were obtained using four rotor cycles of dephasing with alternating 0° and 90° ^{15}N π pulses every half rotor cycle. A four rotor-cycle dephasing period is optimum for the detection of directly bonded ^{13}C - ^{15}N pairs, for which the REDOR dephasing is *ca* 80% of the full-echo signal.³ More distant ^{13}C - ^{15}N pairs do not interfere because little dephasing accumulates from weak coupling in just four rotor periods. For example, if ^{13}C and ^{15}N labels are

separated by 4 Å, the four-rotor cycle dephasing drops to 0.4% of the full-echo signal.^{3,12} DCP spectra were obtained using a 3-ms carbon-nitrogen spin-lock transfer with radiofrequency field amplitudes mismatched by one spinning frequency.^{5,6} Rotors with 1-g sample capacities were made from ceramic (zirconia) barrels fitted with plastic (Kel-F) end-caps and were supported at both ends by air-pumped journal bearings. Only 100 mg of one of the labeled insect exoskeletons and 150 mg of the other were available, so the samples were positioned in the center of the rotor by Kel-F sample holders.

RESULTS AND DISCUSSION

The natural-abundance ^{13}C NMR spectrum of pupal exuviae of tobacco hornworm shows resolved contributions from the methyl carbons of chitin (δ_c 23, Fig. 2, bottom right), the methylene carbons of lipids (δ_c 30), the oxygenated carbons of chitin (that at δ_c 105 is the best resolved), various aromatic carbons of protein side-chains (δ_c 120), the diphenolic carbons of catechols (δ_c 145) and the carbonyl carbons of proteins (δ_c 175). The relative intensities of these peaks can be used for a semi-quantitative compositional analysis of the organic content of intact tissue.⁹ Hornworm pupal exuviae contain *ca* 10% water (determined by gravimetric analysis), 30% protein, 35% chitin, 20% catechols and 5% lipid.^{9,12} Most of the ^{13}C label from [β - $^{13}\text{C}_1$]dopamine appears in carbons with resonances at δ_c 30, 60 and 80, which are characteristic of methylene, nitrogen-substituted and oxygen-substituted sp^3 carbons, respectively.¹²

Slow ^{13}C - ^{15}N polarization transfer and fast spin-lock relaxation are the main causes of the poor sensitivity of double-cross polarization.⁵ Because a spin-locked polarization transfer is avoided, the inherent sensitivity of REDOR is greater than that of DCP, as illustrated in Fig. 2. The REDOR difference spectrum (Fig. 2, top left) is more than five times as intense as the double-cross difference spectrum (Fig. 2, top right), even though the latter was observed after almost twice as many scans. The 60 ppm REDOR difference signal in Fig. 2 arises from only 0.4 μmol of ^{13}C label.¹² The observed, directly bonded ^{13}C - ^{15}N pairs result from covalent cross-links between histidine ring nitrogens of proteins and β carbons of catecholamines derived from dopamine^{9,12} (Fig. 3, top).

Two-bond ^{15}N - ^{15}N coupling in double-labeled histidine is sufficiently weak that homonuclear ^{15}N spin flips are suppressed by the 3-kHz spinning.^{5,8} This, together with the fact that the concentration of cross-links is low, means that the ^{13}C - ^{15}N pairs in the insect exoskeleton labeled by [ring- $^{15}\text{N}_2$]histidine and [β - $^{13}\text{C}_1$]dopamine can be considered to be completely isolated. On the other hand, the six ^{13}C nuclei of the ring-labeled dopamine have multiple dipolar couplings that are comparable to the magic-angle spinning frequency. These ^{13}C nuclei are not isolated. The strong homonuclear ^{13}C coupling results in poorly resolved ^{13}C NMR spectra of [ring- $^{13}\text{C}_6$]dopamine itself (data not shown). Hence the ^{13}C - ^{15}N pairs in insect exoskeleton labeled with

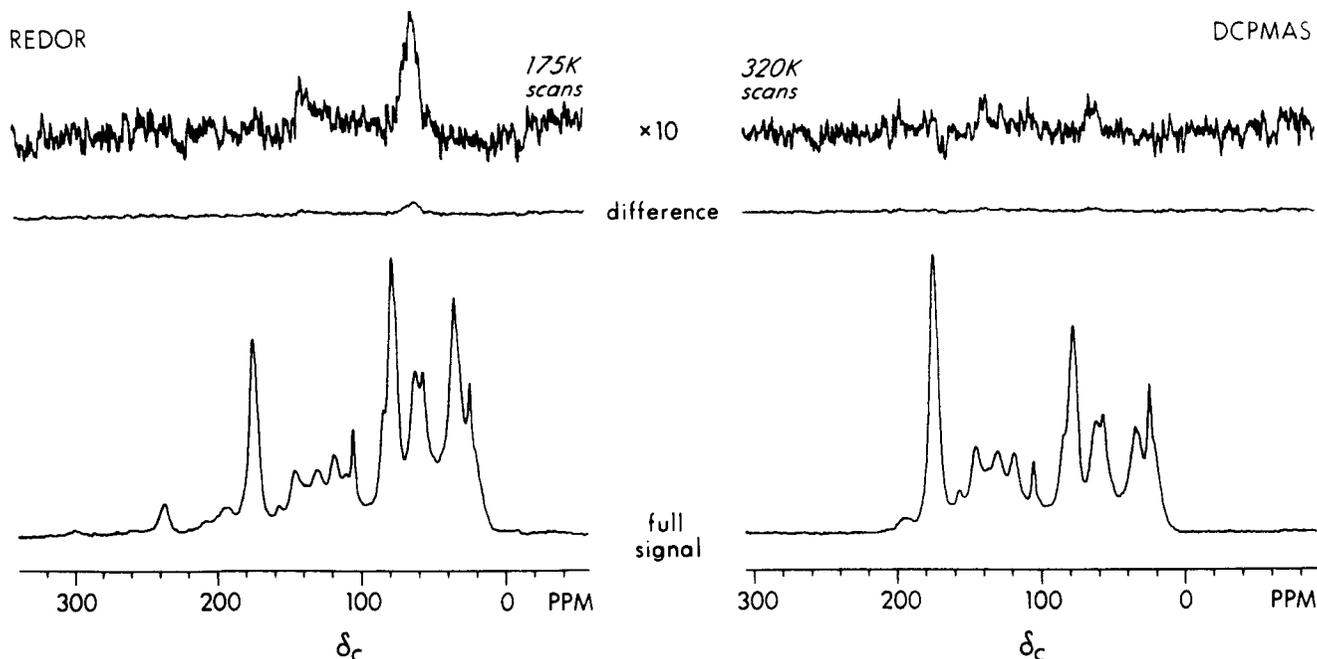
[^{15}N]Histidine and [β - $^{13}\text{C}_1$]Dopamine

Figure 2. REDOR (left) and DCP (right) full-signal (bottom) and difference (middle, top) magic-angle spinning ^{13}C NMR spectra of tobacco hornworm pupal exuviae labeled with L-[ring- $^{15}\text{N}_2$]histidine and [β - $^{13}\text{C}_1$]dopamine. Spectra were obtained at 50.3 MHz with magic-angle spinning at 3.205 kHz. REDOR dephasing was summed over four rotor cycles with ^{15}N π pulses every half rotor period. DCP spectra were obtained with total suppression of spinning sidebands following a 3-ms carbon-nitrogen spin lock. More than half of the methylene-carbon intensity at 30 ppm is lost during the DCP spinlock. The natural-abundance methyl-carbon peaks at 23 ppm are equal in intensity in the two spectra. The difference peak at 140 ppm is due to natural-abundance ^{13}C in ^{15}N -labeled rings of histidine residues in hornworm protein.

[ring- $^{15}\text{N}_2$]histidine and [ring- $^{13}\text{C}_6$]dopamine are not isolated.

The sensitivity advantage of REDOR is lost when multiple labels of the same type are not isolated but are

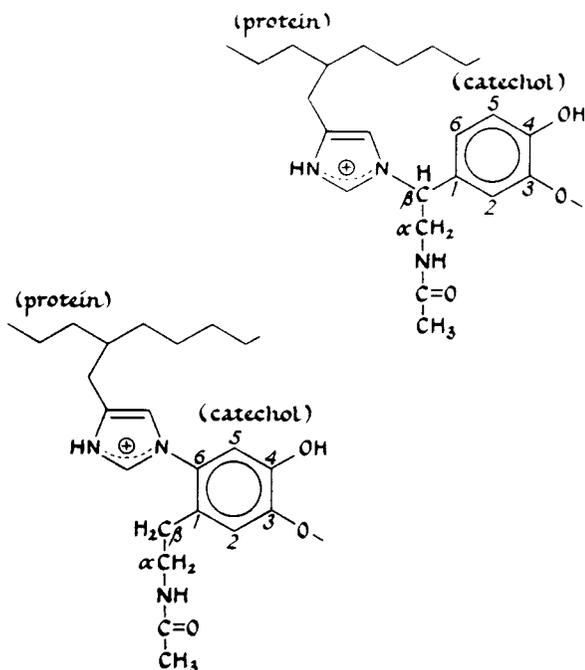


Figure 3. Cross-links formed in hornworm exoskeleton.^{9,12} Proteins are covalently attached to catecholamines derived from dopamine.

strongly coupled to one another. A comparison of spectra for tobacco hornworm pupal exuviae labeled with L-[ring- $^{15}\text{N}_2$]histidine and [ring- $^{13}\text{C}_6$]dopamine (Fig. 4) shows a REDOR difference (top left) that is no larger than the DCP difference (top right). The observed ^{13}C - ^{15}N couplings arise from cross-links between histidine nitrogens and ring carbons of catechols⁹ (Fig. 3, bottom). After contributions to the difference signals from natural-abundance ^{13}C in the histidine rings have been removed, the DCP difference signal is larger than the REDOR difference signal. The loss in sensitivity for REDOR occurs because the dipolar couplings between ^{13}C nuclei in this exoskeleton sample cause dephasing of transverse carbon magnetization in both halves (with and without dephasing ^{15}N π pulses) of REDOR experiments. Furthermore, quantitative analysis of the resultant diminished echo amplitudes is generally not possible because of the correlations between ^{13}C - ^{13}C and ^{13}C - ^{15}N couplings.

When quantitative measurements of dipolar couplings are required in samples containing isolated pairs of unlike rare spins, we believe that REDOR is a better choice than DCP. However, for samples containing strongly coupled multiple homonuclear labels, DCP is quantitatively more reliable than REDOR. Mutual spin flips among carbons actually simplify the ^{13}C - ^{15}N DCP experiment by inhibiting oscillatory ^{13}C - ^{15}N polarization transfers.⁵ Even though analysis of DCP results to obtain internuclear distances is difficult, requiring comparisons to model compounds⁸, the use of DCP for spin counting of ^{13}C - ^{15}N spin pairs is straightforward.⁷⁻⁹ For example, it is possible to make

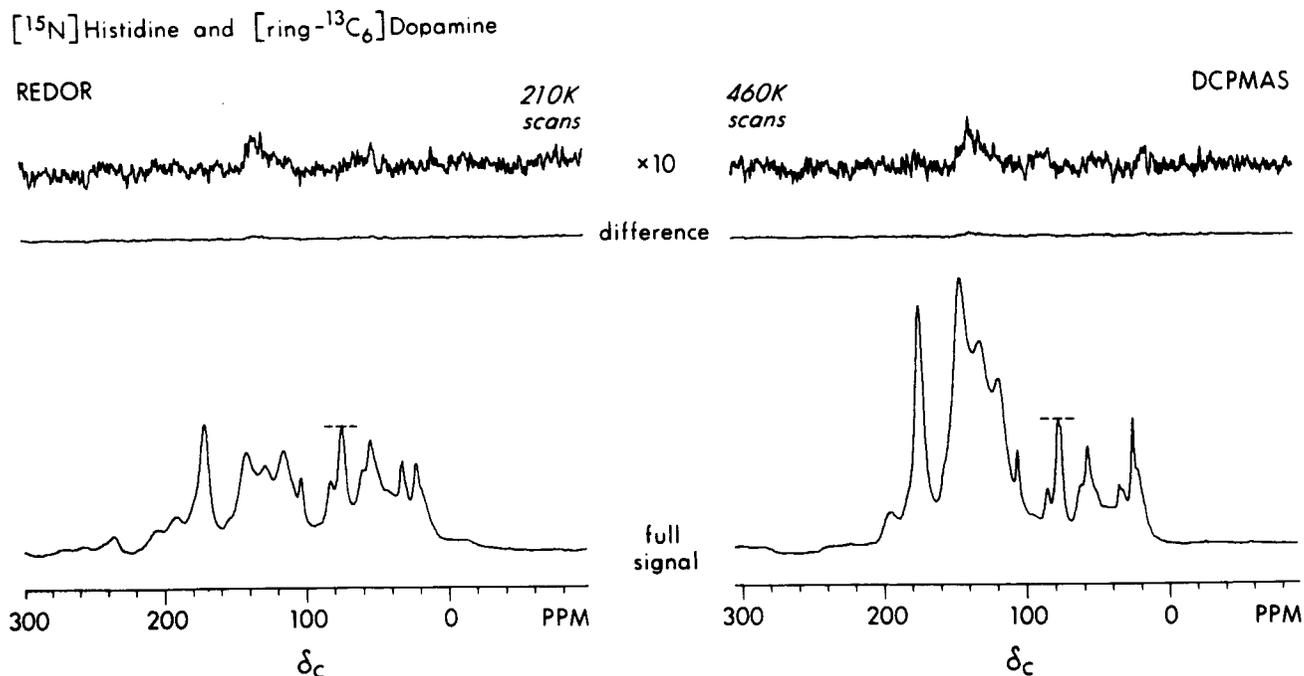


Figure 4. REDOR (left) and DCP (right) full-signal (bottom) and difference (middle, top) magic-angle spinning ¹³C NMR spectra of tobacco hornworm pupal exuviae labeled with L-[ring-¹⁵N₂]histidine and [ring-¹³C₆]dopamine. Experimental conditions as in Fig. 2. The natural-abundance chitin peaks at 80 ppm indicated by dashed lines are equal in intensity. About half of the 140 ppm signal in the REDOR difference spectrum and one quarter of the 140 ppm DCP difference signal are due to natural-abundance ¹³C in the histidine ring.

a direct comparison of the two DCP spectra shown in Figs 2 and 4. Carbon–nitrogen cross-polarization transfer rates and proton relaxation rates are the same for the two samples.^{8,9} The spectra can be scaled relative to one another by the intensity of the methyl-carbon peak at 23 ppm which arises just from natural-abundance carbons. Direct comparison of the difference spectra shows that one third of the histidine nitrogens involved in cross-linking to compounds derived from dopamine

are bound to β -carbons (Fig. 2, top right, 60 ppm) and two thirds to ring carbons (Fig. 4, top right, 140 ppm).

Acknowledgements

This work was supported by NSF grant DIR-8720089 and USDA grant 88-CRCR-3684.

REFERENCES

1. T. Gullion and J. Schaefer, *J. Magn. Reson.* **81**, 196 (1989).
2. J. Schaefer, R. A. McKay and E. O. Stejskal, *J. Magn. Reson.* **34**, 443 (1979).
3. T. Gullion and J. Schaefer, *Adv. Magn. Reson.* **13**, 55 (1989).
4. G. R. Marshall, D. D. Beusen, K. Kocielek, A. S. Redlinski, M. T. Leplawy, Y. Pan and J. Schaefer, *J. Am. Chem. Soc.* **112**, 963 (1990).
5. J. Schaefer, E. O. Stejskal, J. R. Garbow and R. A. McKay, *J. Magn. Reson.* **59**, 150 (1984).
6. E. O. Stejskal, J. Schaefer and J. S. Waugh, *J. Magn. Reson.* **28**, 105 (1977).
7. G. S. Jacob, J. Schaefer, E. O. Stejskal and R. A. McKay, *J. Biol. Chem.* **260**, 5899 (1985); **262**, 254 (1987).
8. E. O. Stejskal, J. Schaefer and R. A. McKay, *J. Magn. Reson.* **57**, 471 (1984).
9. J. Schaefer, K. J. Kramer, J. R. Garbow, G. S. Jacob, E. O. Stejskal, T. L. Hopkins and R. D. Speirs, *Science* **235**, 1200 (1987).
10. T. Gullion and J. Schaefer, *J. Magn. Reson.* in press.
11. R. A. McKay, US Pat. 4446 431 (1984).
12. A. M. Christensen, J. Schaefer, K. J. Kramer, T. D. Morgan and T. L. Hopkins, *J. Am. Chem. Soc.* submitted for publication.
13. W. T. Dixon, *J. Chem. Phys.* **77**, 1800 (1982).