Published in final edited form as:

Appl Magn Reson. 2010 January 1; 37(1-4): 763–779. doi:10.1007/s00723-009-0083-6.

Simulating suppression effects in Pulsed ENDOR, and the ‘hole in the middle’ of Mims and Davies ENDOR Spectra

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Abstract

All pulsed ENDOR techniques, and in particular the Mims and Davies sequences, suffer from detectability biases (‘blindspots’) that are directly correlated to the size of the hyperfine interactions of coupled nuclei. Our efforts at ENDOR ‘crystallography’ and ‘mechanism determination’ with these techniques has led our group to refine our simulations of pulsed ENDOR spectra to take into account these biases, and we here describe the process and illustrate it with several examples. We first focus on an issue whose major significance is not widely appreciated, the ‘hole in the middle’ of pulsed ENDOR spectra caused by the $n = 0$ suppression hole in Mims ENDOR and by the analogous $A \rightarrow 0$ suppression in Davies ENDOR (**Section I**). This section discusses the issue for nuclei with $I = \frac{1}{2}$ and also for ^2H ($I = 1$), using the treatment of **Section II**. In **Section II** we discuss the general treatment of suppression effects for $I = 1$, illustrating it with a treatment of Mims suppression for ^{14}N ($I = 1$) (**Section II**).

Pulsed Mims ENDOR[1,2] affords exquisite sensitivity and resolution in studies of weakly hyperfine-coupled ($|A| < 1$ MHz) nuclei. When combined with new biochemical labeling and intermediate-trapping techniques,[3] Mims ENDOR studies on a variety of enzymatic systems have provided structural information on many systems that cannot be obtained by other means. The Davies ENDOR sequence complements the Mims sequence in that it is preferentially used to interrogate larger couplings.[2,4] The prospect of performing ENDOR ‘crystallography’ and ‘mechanism determination’ with these techniques[5,6] has forced our group to devise methods of dealing with the fact that all pulsed ENDOR techniques, and in particular the Mims and Davies sequences, suffer from detectability biases (‘blindspots’) that are directly correlated to the size of the hyperfine interactions of coupled nuclei, and to refine our simulations of pulsed ENDOR spectra to take into account these biases.

Mims three-pulse and refocused Mims (Re-Mims) four-pulse[7] ENDOR spectra are subject to hyperfine suppression, according to the detectability function,

$$\text{ENDOR } (A \text{ (MHz)}, \tau \text{ (}\mu\text{s)}) \propto \sin^2(\pi A \tau) \quad (1)$$

where ϑ is the interval between the first and second pulses in the three-pulse Mims or four-pulse Re-Mims ESE sequence. This generates ‘holes’[8] (zeroes) in the intensity for contributions with $A\tau = 0, 1, 2, \dots$ and intensity maxima when $A\tau = \frac{1}{2}, \frac{3}{2}, \frac{5}{2}, \dots$. A particularly dramatic demonstration of such a series of detectability maxima and minima was presented and exploited in a study that employed $^{19}\text{F}/^1\text{H}$ Mims ENDOR to examine the binding of trifluoroethanol to a mixed-valence, FeII/FeIII diiron center, Figure 1.[9] Davies pulsed ENDOR suppresses contributions with $A \rightarrow 0$, according to the detectability function,[10,11]

$$\text{ENDOR}(A(\text{MHz}), t_p(\mu\text{s})) \propto \frac{1.4(A t_p)}{0.7^2 + (A t_p)^2} \quad (2)$$

where t_p is the duration of the first pulse in the Davies three-pulse ESE sequence. This function produces an effect analogous to the $n = 0$ Mims hole. When resonances from strongly-coupled nuclei overlap with resonances from weakly-coupled nuclei, increasing the microwave pulse strength and decreasing pulse time will bias the detection towards the more strongly-coupled peaks that underlie the weakly-coupled resonances.

In general, these suppression effects are treated like the weather, as something whose effects can be minimized by optimizing pulse parameters, but can't be avoided and should be endured without unseemly attention. In this report we describe our longstanding approach to 'weather management': by combining simulations that incorporate suppression effects with pulse-parameter manipulation guided by these simulations, we can overcome – and sometimes even favorably exploit – suppression effects.

We first focus on an issue whose major significance is not widely appreciated, the 'hole in the middle' of pulsed ENDOR spectra caused by the $n = 0$ suppression hole in Mims ENDOR and by the analogous $A \rightarrow 0$ suppression in Davies ENDOR (**Section I**). This section discusses the issue for nuclei with $I = 1/2$ and also for ^2H ($I = 1$), using the treatment of **Section II**. In **Section II** we discuss the general treatment of suppression effects for $I = 1$, illustrating it with a treatment of Mims suppression for ^{14}N ($I = 1$).

I. The 'hole in the middle' of Mims and Davies ENDOR Spectra

IA. Analysis and Example

The one common problem associated with both the Davies and Mims ENDOR protocols is that their ENDOR detection functions go to 0 in the limit of small hyperfine interactions. This 'central, ' $n = 0$ ', suppression hole' as $A \rightarrow 0$ is well understood to create a serious problem in Mims ENDOR, which is the preferred protocol for small hyperfine couplings. It is likewise a problem for $A \rightarrow 0$ in Davies ENDOR, although this is not routinely thought of as an important limitation because this sequence is preferentially used to interrogate larger couplings. In fact, this central hole is *always* a problem in both protocols when the hyperfine tensor/matrix principal values do not all have the same sign, namely when the anisotropic (traceless) component of the hyperfine interaction dominates the isotropic component, for in such cases there necessarily occur orientation for which $A \rightarrow 0$.

Such is the case when the through-space dipolar interaction is being used to determine the location of, say, a ^2H , ^{15}N , or even ^{13}C atom/nucleus of a substrate/inhibitor more than 3-4Å away from a paramagnetic center. In such cases, the couplings are small and Mims ENDOR is called for.[12,13] This problem is often exacerbated by relatively short phase memories in metalloproteins (even at 2K) which can limit the τ values to less than 1μs and often as short at 0.500μs.

Hyperfine interactions for $[^2\text{H}_x\text{O}]$ moieties bonded to a metal center can have couplings large enough to call for the use of Davies ENDOR and can have hyperfine interactions dominated by the anisotropic interaction.[14,15] and in such cases the central hole in Davies ENDOR is no less a problem.

In this section we first present simple, idealized examples that illustrate the difficulties introduced by the $n = 0$ Mims hole and demonstrate how these difficulties can be treated in the

case of a 'free radical' center, namely one where the g anisotropy is too small to introduce orientation selection, and one where orientation selection is present. We can use this technique to illustrate how the 'hole' at A=0 in Mims ENDOR can produce serious difficulties in the interpretation of measured spectra. We compare the results from two different powder pattern ENDOR spectra for ^{13}C simulated using the standard statistical weighting factors for simple spherical averaging (powder pattern).

Hyperfine Interaction Model—Consider an $I = \frac{1}{2}$ nucleus where $|v_N| \gg |A|$. There is a simple 1:1 correspondence between the observed ENDOR frequencies and the hyperfine coupling $|A|$

$$\nu_{\pm}(\theta, \varphi) = \left| \nu_N \pm \frac{1}{2}A(\theta, \varphi) \right| = \left| \nu_N \right| \pm \left| \frac{1}{2}A(\theta, \varphi) \right| \quad (3)$$

Under these circumstances, we can simulate Mims ENDOR spectra by simply multiplying the output of a simulation without the suppression effects ('statistical weighting') by the suppression function centered at the Larmor frequency. An analogous approach can be taken for Davies suppression as $A \rightarrow 0$. This approach works not only for $I = \frac{1}{2}$, but also for ^2H ($I = 1$) ENDOR when the quadrupole splitting is much smaller than the hyperfine coupling, as is common, or even is not resolved.

As an example, the hyperfine tensor is modeled as an isotropic component plus a point-dipole component

$$A(\theta, \phi) = a_{iso} + \frac{g_e \beta_e g_N \beta_N}{r^3} (3 \cos^2 \theta - 1) = a_{iso} + \frac{19.9 \text{ MHz}}{(r/\text{\AA})^3} (3 \cos^2 \theta - 1) \quad (4)$$

where θ is the angle between the applied magnetic field and the electron-nuclear vector; for concreteness, in this example we take the nucleus to be a ^{13}C . In our simulation program the contributions of g anisotropy in determining the orientation hyperfine interaction is properly incorporated; in this example it is omitted.

Mims n = 0 hole for a paramagnet with unresolved g anisotropy—In the absence of orientation selection, as with a free radical center, the ENDOR spectrum is a 'spherical average' over all orientations of the external field relative to the hyperfine coordinate frame. Consider two model hyperfine interactions with the same turning points in $|A(2, \nu)|$. In the first, the hyperfine coupling is a purely point-dipole interaction ($a_{iso} = 0$; Dipolar Model 1) with $r = 4 \text{\AA}$, the second is dominated by the isotropic interaction ('Isotropic' Model 2) $a_{iso} = 0.4 \text{ MHz}$ and point-dipole distance of $5.75 \text{\AA}$. These two hyperfine interactions would have very different physical interpretations. The first model would represent the coupling to, say, a ^{13}C probe nucleus of a substrate with no direct bond to the paramagnetic center. In the second case, the isotropic coupling reflects covalent bonding to the probe atom to the paramagnetic center that leads to delocalization/polarization of spin density onto the ^{13}C probe nucleus, while the anisotropic component would likely be interpreted as a combination of a through-space interaction and a contribution from local p-electron spin density.[16]

Statistical Powder ENDOR patterns for these two models, calculated without including suppression effects (Figure 2 top) are quite distinct even though the turning points in the spectrum occur at the same frequencies, since both hyperfine tensors have the same absolute values of their principal components $|A_{\perp}| = 0.31 \text{ MHz}$, $|A_{\parallel}| = 0.62 \text{ MHz}$. Because model 1 is purely dipolar, the principal tensor components have opposite sign which leads to the classic

'Pake' pattern centered at the ^{13}C Larmor frequency, with a substantial amount of the intensity appearing between the two perpendicular turning points. Model 2 is dominated by the isotropic component, and no intensity would be observed in the region near the Larmor frequency. Therefore the most diagnostic portion of the patterns occurs in a frequency region most affected by the Mims suppression effects near $A=0$. Any attempt to measure these ENDOR spectra using a Mims ENDOR sequence must take into account that the intensities at various frequencies are modulated by the suppression function given in Eq. [1]. Multiplying these two patterns by a Mims ENDOR intensity function assuming a $\tau=0.75\mu\text{s}$ (Figure 2, bottom) demonstrates that the predicted experimental spectra would actually be extremely similar in overall appearance, where the differences between the models become exceedingly subtle intensity and lineshape effects.

In a spherical/powder-averaged spectrum, the best solution to this quandary is to vary τ over as wide a range as is experimentally accessible and compare the *trends* in the lineshapes and intensities of the Mims ENDOR spectra. In Figure 3, the left panel shows the Mims ENDOR spectral simulations obtained for model 1 for τ values of 0.5, 0.75, 1.00, 1.25, and $1.5\mu\text{s}$ and the right-hand panel shows the same τ values for the second model. Each spectrum is normalized to its maximum intensity. Even with as long a τ value as $1.50\mu\text{s}$, the differences in the lineshapes between the models are still fairly subtle if we focus solely on this individual pair of simulations. Yet, the trend from the shortest to the longest τ values in model 1 demonstrate that the $A_{\perp}$ peaks show an increasing prominent tailing edge towards $\nu(^{13}\text{C})$ that are absent in the model 2 simulations. This small difference in simulations is often the determining factor in assigning experimental spectra to one or the other models when orientation selection is not possible.

Mims $n = 0$ hole for a paramagnet with orientation selection—In systems with orientation-selection, the mapping of the ENDOR powder pattern onto the EPR envelope provides a simple method for distinguishing between these two patterns using a single τ value. Figure 4 shows side-by-side comparisons of orientation-selected Mims ENDOR simulations for model 1 (left) and model 2 (right) assuming a g tensor of (2.2, 2.0, 1.8) and $\tau=0.5\mu\text{s}$. The $A_{\parallel}$ direction is coaxial with g_3 in both simulations. The two patterns are easily distinguished by the shapes of the peaks and the trends across the EPR envelope. Both patterns have identical spectra at the EPR extrema, $g=1.8$ (top) and $g=2.2$ (bottom). However, the lineshapes in the field-dependent ENDOR spectra in model 1 show shoulders towards the Larmor frequency (smaller $|A|$) between g_1 and g_2 and a single peak at g_2 , whereas in model 2, the shoulders appear in the direction of larger $|A|$ values, with no intensity towards smaller $|A|$. In addition, there is a huge loss in simulation intensity between g_2 and g_3 in the dipolar model as a significant percentage of the ENDOR transitions in this field region have vanishingly small $|A|$ values. No similar decrease in intensity is observed in the model that is dominated by the isotropic component. Though the arguments concerning the lineshape analysis have been used commonly, the fact that the intensity of the Mims ENDOR peaks will also vary wildly across the EPR envelope when the hyperfine is predominantly dipolar in nature has not.

Davies $A \rightarrow 0$ Suppression—Analogous to the Mims $n = 0$ suppression.

IB. Mims, $n = 0$ suppression hole, Experiment

Efforts are usually made to collect Mims data with $\tau \gg 1/|A_{\text{max}}|$, where A_{max} corresponds to the outer edge of the Larmor-centered ENDOR pattern, so as to place the $n = 1$ Mims holes at $A = 1/\tau \gg |A_{\text{max}}|$, well outside the data region, with the implicit expectation that lineshapes will largely be preserved, although perhaps with an overall reduction of signal intensity. However, our results show that unless the spread in hyperfine values that contribute to the spectrum is much less than $1/\tau$, then it is likely that the $n = 0$ hole will have a strong effect on

lineshapes, in some cases even generating false maxima that may be misinterpreted as structured features created by orientation selection, or as independent signals from different nuclei.

The effects of suppression are particularly well demonstrated for a spherically-averaged (powder) spectrum of a radical by the ^{31}P ENDOR response collected from a radical signal that arises in lysine amino mutase (LAM) reacting with a substrate analog, dehydrolysine (DHLys). The DHLys is coupled to PLP, Inset 1, and as a result Mims ENDOR spectra from the radical show a ^{31}P signal from the phosphate.[17]

A recent study of a possible model for nitrogenase intermediates, compound **1** shown in Inset 2, illustrates the effects of the $n = 0$ Mims hole in analyzing data collected for a center with resolved g values and resulting orientation selection. We used this approach to determine the ^2H ($I = 1$) hyperfine tensors for the hydrazido [=ND₂] portion of the molecule.[18]

Spherical/Powder Average Spectra; ^{31}P ENDOR from the DHLys Radical: [17]—

The DHLys radical (Inset 1) has a narrow enough g spread even at 35 GHz that there is no orientation selection, and so an ENDOR spectrum is a superposition of responses from all orientations relative to the external field ('powder average'). The radical's ^{31}P spectrum shows two pairs of peaks centered about the ^{31}P Larmor frequency: a broad outer pair with a splitting of about 0.8 MHz, and a narrow inner pair with a splitting of 0.13 MHz, Figure 5, **upper**. The 'obvious' interpretation of this spectrum would be to assign these two doublets to two distinct ^{31}P . The broader doublet rather looks like it might be associated with a hyperfine tensor dominated by the isotropic interaction, with the breadth of each branch determined by a lesser dipolar interaction.

However, detailed analysis through simulations that incorporated Mims suppression instead show that the spectrum arises from a single ^{31}P whose hyperfine tensor is dominated by an axial dipolar interaction. Figure 5, **lower** displays the 'statistical' simulation, generated by summing the contributions from all orientations without modifying those contributions by suppression. The simulation has features that correspond to the experiment, but their relative intensities are wildly different. It displays a Pake pattern with high-intensity perpendicular peaks, which correspond in frequency to the sharp inner pair of ENDOR lines, and an edge which corresponds to the outer edge of the data. Inclusion in the simulation of the Mims suppression function with the experimental $\vartheta = 0.452\mu\text{s}$ not only suppresses the perpendicular peaks relative to the outer part of the pattern, but generates a maximum in the previously gently-sloping intensity profile close to the point of maximum Mims sensitivity ($A = 1/2\tau(\mu\text{s})$). The resultant lineshape closely matches the experimental data, Figure 5, **middle**. What is not obvious from the scaled spectra is just how much a penalty in intensity is paid because of the suppression effect. Plotted to scale, the upper (experimental) and middle (simulated) spectra would have a maximum intensity of no more than roughly the intensity of lower spectrum at $|v - v(^{31}\text{P})| \sim 0.5$ MHz.

Orientation Selection and the ^2H ENDOR of **1 (Inset 2)[18]**—Figure 6 shows experimental ^2H ENDOR spectra from the [-ND₂] of **1**, as collected at $g_1 = 2.00$ for $\vartheta = 0.200$ and $0.800\mu\text{s}$ (spectra a,d) and $g_2 = 1.94$ for $\vartheta = 0.200$ and $1.400\mu\text{s}$ (i,l); these are extracted from a 3-D pattern of spectra at multiple values of ϑ collected at fields across the EPR envelope of the $S = 1/2$ EPR signal. At $\vartheta = 0.200\mu\text{s}$ the spectra consist of v_+v_- doublets (quadrupole splitting not resolved) that could be easily thought of as representing isotropically coupled ^2H , and the full patterns collected with $\tau = 0.200\mu\text{s}$ could be readily interpreted with an isotropic dominated tensor, $\mathbf{A}^{\text{is}} = [0.5, 0.8, 2.7]$ MHz. However, a detailed analysis of the hyperfine interaction showed that a dipolar dominated tensor, $\mathbf{A}^{\text{di}} = [-1.0, 0.7, 2.7]$ MHz, also could be used to simulate the $0.200\mu\text{s}$ data.

Simulations that incorporated Mims suppression carried out with higher and higher values of ϑ predicted that spectra taken near g_1 would show little difference at all accessible values of ϑ for either tensor, reflecting the fact of a minimal difference between the statistical spectra predicted for this field by the two models (g,h). This expectation is confirmed by comparing the calculated and experimental at this field collected for $\vartheta = 0.200$ (a-c) and $0.800\mu\text{s}$ (d-f). However the statistical spectra at $g_2 = 1.94$ show qualitative differences (o,p), and spectra at g_2 should change significantly as ϑ increases if the dipolar model were correct, but should change little if the isotropic model is. As can be seen in Fig 6, the $\vartheta = 1.400\mu\text{s}(!)$ spectrum at g_2 clearly does differ from that with $\vartheta = 0.200\mu\text{s}$. The simulation with suppression for $\mathbf{A}^{\text{is}}$ clearly fails at $\vartheta = 1.400\mu\text{s}$ (n vs l), while that for $\mathbf{A}^{\text{di}}$ just as clearly does an excellent job of reproducing the spectra (m vs l).

IC. Davies A $\rightarrow$ 0 Suppression, Experiment

A case in point is our recent reinvestigation of the types of protonated oxygen (OH_x) species bound to the $\text{Fe}^{\text{III}}/\text{Fe}^{\text{IV}}$ diiron cluster of Intermediate **X** of *Escherichia coli* ribonucleotide reductase (RNR).[3] The question to be resolved was the possible presence, either individually or simultaneously, of an hydroxo bridge, which we denoted **B**, and a terminal aqua ligand (OH_x) bound to Fe^{III} , denoted $\mathbf{T}_x$ (Figure 7). In this case the two types of ^1H both have dipolar hyperfine tensors, but they exhibit the two limiting types of dipolar interaction: a $\mathbf{T}_x$ proton exhibits the classical axial form expected for the interaction of two point dipoles, $\mathbf{T} \cdot [T, -T/2, -T/2]$; **B** exhibits a rhombic interaction tensor, $\mathbf{T} \cdot [T, 0, -T]$. The best tool for this study turned out to be the ^2H Davies ENDOR protocol. Surprisingly, the suppression hole had a major positive influence on our ability to distinguish among models for the center, Figure 7.

Figure 8A shows the 2-D field-frequency pattern of Davies ^2H ENDOR spectra collected across the EPR envelope of **X** in D_2O . As shown, model-free simulation of this pattern yielded spectra that reproduce experiment with superb fidelity. These simulations employed a single (type of) contributing deuteron whose hyperfine tensor, $\mathbf{A}_T^{\text{ex}}$, has the axial character predicted for a terminal ($\mathbf{T}_x$) aqua ligand, namely a terminal hydroxo ($\mathbf{T}_1$) or a terminal water ($\mathbf{T}_2$ in Figure 7) that has two magnetically equivalent deuterons, $\mathbf{A}_T^{\text{ex}} = [-1.55, -1.15, 3.2]$, $\cdot [-T/2, -T/2, T]$. In contrast, a bridging (**B**) hydroxyl ^1H would exhibit a rhombic interaction tensor, $\mathbf{T} \cdot [T, 0, -T]$, [14, 15] and such a model does *not* fit the 2D pattern. Note, the ^2H simulations employed the strategy for simulating suppression effects for an $I = 1$ nucleus, as described below.

The Davies hyperfine suppression makes an important contribution to the shapes of the $\mathbf{T}_x$ simulations of Figure 8A, but we here discuss its even greater role in answering the more difficult question: could **X**, contain *both* **T** and **B** protons, the $[\mathbf{T}_x+\mathbf{B}]$ model of Figure 7? To predict the ENDOR response for a hypothetical **X** with both **T** and **B** deuterons ($[\mathbf{T}_x+\mathbf{B}]$, Figure 7) we assumed the presence of either one or two **T** deuterons, described by $\mathbf{A}_T^{\text{ex}}$ derived above, plus an hydroxo bridge **B** deuteron represented by an optimized ^2H hyperfine tensor, $\mathbf{A}_B^0 = [A_1 = -3.031, A_2 = -0.948, A_3 = 3.179]$ calculated through use of previously published equations [19] and summed the spectra from each type of deuteron to generate the 2-D field-frequency patterns expected if both **T** and **B** deuterons were present. We minimized the predicted detectability of **B** by employing for the **T** deuteron(s) the field-dependent linewidth used in Figure 8A, while using for **B** a linewidth at all fields that equaled the largest linewidth found for the **T** deuteron(s) at any field (the value near g_1).

Figure 8B displays the composite 2-D ^2H ENDOR pattern calculated for models that contain either $x = 1$ and $x = 2$ protons on the terminal OH_x , along with the **B** deuteron, $[\mathbf{T}_1+\mathbf{B}]$ and $[\mathbf{T}_2+\mathbf{B}]$ models respectively, each overlaid onto the experimental ^2H ENDOR pattern for **X** (WT). One 2-D pattern [Figure 8B; statistical] was calculated simply as a ‘statistical/geometric’ sum of the contributions from the subset of orientations that contributes to the EPR spectrum at the g -value of observation,[20] without inclusion of suppression. In such a simulation, the

integrated ENDOR intensity of a **T** and **B** deuteron would be equal. As the **B** spectrum is broader and is calculated with a greater component ENDOR linewidth, the relative statistical intensities of features associated exclusively with **B** would be much less than those of the **T** proton(s), making it difficult to determine whether there is or is not intensity from a **B** proton, relative to the experimental signal/noise ratio.

The second 2-D pattern [Figure 8B; suppression] of summed simulations in Figure 8B properly incorporates the Davies ENDOR suppression effect. As can be seen, in the high and low-field regions of the pattern, the presence of **B** again does not alter the spectra appreciably. However, for fields near the middle of the pattern ($g = 1.996-1.999$), **B** has such a large relative intensity that its presence could not have been missed with the signal/noise of the experimental traces. In short, Davies suppression qualitatively alters our ability to distinguish among alternate models for **X**. The reason for this is that at the intermediate g values the sharp **T** doublet has a smaller hyperfine coupling and is suppressed to a greater degree in Davies ENDOR. As a result the *relative* intensities of the **T_x** and **B** signals are so greatly altered that one can have no doubt that *if* a **B** proton were present, its signal would be observed along with that of the **T_x** proton (s), enabling us to state with confidence that **X** does not contain a **B** proton either alone or in addition to the **T** proton(s), in agreement with our proposal of over a decade ago.[19]

II. Mims ENDOR Simulations for I=1 nuclei

There are two commonly encountered $I = 1$ nuclei for which the simplistic Mims hole treatment described above fails, ^{14}N – always – and ^2H – when quadrupole couplings are well resolved and it loses its honorary $I = \frac{1}{2}$ status. For $I=1$ centers, the four first-order ENDOR transition frequencies are given by

$$\nu_{\pm}(m) = |\nu_N \pm \frac{1}{2}A(\theta, \phi) + 3P(\theta, \phi)(m - \frac{1}{2})| \quad (5)$$

where $P(\theta, \phi)$ is the orientation-dependent nuclear quadrupole interaction (NQI) and $m=0,1$. The addition of the quadrupole term in Eq. [5] breaks down the 1:1 mapping between the ENDOR transition frequency and the hyperfine coupling, so that it is incorrect to simply multiply the ‘statistical’ simulation by a hyperfine-hole pattern. This problem is especially acute for ^{14}N simulations as $|3P|$ can be as large as 4MHz for a coordinated imidazole ligand, which is similar in magnitude both to ν_N in a Q-band ENDOR spectrum and to the A values. This prevents the use of any simple sequential-perturbation method as all of the terms in Eq. [5] are essentially equal in energy splitting. Extracting the ENDOR frequencies by exact diagonalization of the matrix is a straightforward approach, but does not provide access to a hyperfine term that can be used in the Mims suppression formula.

We have adapted the method described in Bowman and Massoth[21] which treats the calculation of ^{14}N ENDOR/ESEEM frequencies in a two-step process. The first step calculates the effective orientation-dependent hyperfine coupling for an $S = \frac{1}{2}$ center with anisotropic and (possibly) non-coaxial $\mathbf{g}$ and $\mathbf{A}$ and uses the equations of Thuomas and Lund[22] to calculate an effective field (K) generated by the nuclear-Larmor and hyperfine terms in the spin Hamiltonian treated on an equal footing, as described earlier.[23] At this point we extract the effective A value that is to be used in the Mims suppression function for that specific orientation; the same procedure is used for all nuclear spins, I , even for $I = \frac{1}{2}$, rather than post-multiplying by the Mims suppression function in that case. The second step involves the use of the analytical formula described by Muha for the eigenvalues of an $I=1$ nucleus in an arbitrary magnetic field,[24] in this case the combined Larmor/hyperfine field, K . This procedure has been incorporated into a simulation package, EndorSim (modeled after the pioneering program GENDOR, which calculates statistical spectra),[20,23,25][26] that is capable of calculating

orientation-selective ENDOR spectra with Mims suppression for $I = \frac{1}{2}, 1$. This two-step procedure for treating suppression effects can be generalized to systems with $I = 3/2$ using a parallel treatment by Muha,[27] and to $I > 3/2$ by use of perturbation methods or a standard matrix diagonalization routine to obtain the ENDOR frequencies in the second step.

In many systems there are multiple ^{14}N ligands with $|A| < 6\text{MHz}$ that have similar spin-Hamiltonian parameters. The ability to hyperfine-edit ENDOR spectra from these systems using the ϑ dependence of Mims ENDOR allows us to correlate peaks associated with an individual ^{14}N and to extract the hyperfine and quadrupole splitting for each ^{14}N . As an example of such a system, we have recorded Mims ENDOR spectra at a series of different τ values of the six nitrogen donor ligands in a frozen aqueous solution of *trans*-[(Imidazole)₂(NH₃)₄Ru(III)]Cl₃ at $g_2 = 2.2$. The four NH₃ ligands are symmetry-equivalent as are the two imidazole ligands.[28]. The g_2 axis in this ion corresponds to the Im-Ru-Im direction, commonly labeled as the molecular Z axis. This direction is parallel to principal axes of both A and P for the two imidazole ligands and for the four ammine ligands. When the external field lies along a principal-axis direction of both A and P it represents a 'double turning point' in the observed ENDOR frequencies. In orientation-selective ENDOR spectra collected at fields that include orientations with such double-turning points, those orientations dominate the ENDOR response, and this tends to create a pseudo-single-crystal-like spectrum even if the field is not at an extreme of the EPR envelope (g_1 or g_3).

In Figure 9 (left), Mims ^{14}N ENDOR spectra recorded at 2K across the frequency region from 2-8 MHz are shown at 5 different τ values: 0.360 μs , 0.420 μs , 0.500 μs , 0.560 μs , and 0.720 μs . The experimental spectra are dominated by five peaks whose intensities respond differently with each τ value. From these hyperfine-edited spectra it is relatively straightforward to assign the resonances to the two different classes of ^{14}N ; the coordinated imidazole ^{14}N with $|A_{\text{Im}}| = 3.6\text{MHz}$ and $|P_{\text{Im}}| = 0.73\text{MHz}$ and the ammine ^{14}N with $|A_{\text{Am}}| = 2.8\text{MHz}$ and $|P_{\text{Am}}| = 0.32\text{MHz}$. At $\tau = 0.320\mu\text{s}$ and $0.720\mu\text{s}$, $|A_{\text{Am}}|\tau = 1, 2$ respectively so that Mims holes suppress the ENDOR contribution from the ^{14}N of the ammine ligands and these spectra are dominated by the ^{14}N of the imidazole ligands as shown by the 'goalposts' on the 0.360 μs spectrum. The situation is reversed at $\tau = 0.560\mu\text{s}$, as $|A_{\text{Im}}|\tau = 2$ and the imidazole ENDOR contribution is suppressed leaving the spectrum of the ammine ligand.

Using the EndorSim program we simulated the orientation-selective ENDOR spectra as a function of τ both for the imidazole and ammine ligands, the resulting simulations shown in Fig. 9 (right) are sums of these individual simulations. Spectra taken between $g_1 = 3.04$ and $g_2 = 2.20$ (data not shown) provided the additional information for determinations of the full hyperfine and quadrupole tensors. The correspondence between the experimental and the simulated spectra are excellent. Of particular interest in the simulations is the fact that the low-frequency peak, which corresponds to transitions that arise from the v_- branch of both ^{14}N classes, is matched with some precision for all τ values. These spectra demonstrate that the Mims ENDOR simulation protocol for $I=1$ nuclei described above is valid in systems that do not obey simple first-order quadrupole splitting patterns.

Summary

The inevitable presence of 'holes' in pulsed-endor spectra both presents obstacles to analysis and provides opportunities to manipulate and edit spectra. We show here how to overcome the obstacle of the 'hole in the middle' of Mims and Davies spectra through simulation and variation of ϑ , and how to manipulate and edit spectra of $I = \frac{1}{2}$ nuclei in the same fashion.

Acknowledgments

We thank Prof. Michael J. Clarke (Boston College) for the preparation of *trans*-[(Imidazole)₂(NH₃)₄Ru(III)]Cl₃. This work has been supported by the NIH (HL 13531, BMH) and NSF (MCB0723330, BMH). It has benefitted from the superb technical support of Mr. Clark Davoust.

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Figure 1.

τ -dependence of the $^1\text{H}/^{19}\text{F}$ ENDOR spectra of mixed-valence (FeII/FeIII) diiron center (denoted Hmv) with bound $\text{CF}_3\text{CD}_2\text{OH}$ (trifluoroethanol, TFE). Arrows indicate Mims suppression holes; normal and feathered arrows refer the proton and fluorine signals, respectively. All spectra were taken on a sample NOT containing DMSO, which replaces TFE, except as indicated for one of the spectra with $\tau = 0.400 \mu\text{s}$. ($0.148 \mu\text{s}$) Re-Mims (4-pulse) sequence with a $\pi/2$ microwave pulse = 32 ns, with 20 μs RF pulse, $\tau = 0.148 \mu\text{s}$; 34.638 GHz MW frequency, $g = 1.840$; pulse sequence repetition time - 25 ms; 40 averaged data shots per point; 20 scans. ($0.400 \mu\text{s}$) Mims sequence with a $\pi/2$ microwave pulse = 50-52 ns, with 20 μs Rf pulse, $\tau = 0.400 \mu\text{s}$; 34.596 GHz MW frequency, $g = 1.840$; pulse sequence repetition

time - 25 ms; 40 averaged data shots per point; 3 scans. (0.400 μ s + DMSO) As in (0.400 μ s) but spectrum of Hmv + TFE +DMSO, τ =0.412 μ s; 34.741 GHz MW frequency, pulse sequence repetition time - 30 ms; 30 averaged data shots per point; 2 scans; (overlaid with (0.400 μ s) spectrum). (0.480 μ s) As in (0.400 μ s) but τ = 0.480 μ s; 1 scan; (0.600 μ s) As in (0.400 μ s) but τ = 0.600 μ s; 2 scans; (1.000 μ s) As in (0.400 μ s) but τ = 1.000 μ s; 5 scans. Figure adapted from reference [9].

Figure 2.

(Top) Powder-averaged simulation of axial ^{13}C ENDOR spectra assuming two different models, a purely dipolar model (Dipolar; solid line) and a model with dominant isotropic interaction ('Isotropic'; dashed line). Both models have $2|A_{\perp}| = |A_{\parallel}| = 0.61\text{MHz}$, but in the dipolar model $A_{\parallel} = -2A_{\perp}$. (Bottom) Simulated powder-averaged Mims ENDOR spectra for both models using $\tau = 0.750\mu\text{s}$. The simulations were obtained by multiplying the traces in the top of the figure by the Mims ENDOR suppression function for a τ value of $0.750\mu\text{s}$ $\sin^2(\pi(2(\nu - \nu(^{13}\text{C}))(0.750)) = \sin^2(\pi A \tau)$. The major differences between the two spectra in the top of the figure are lost due to the Mims hole function at $A=0$.

Figure 3. Simulated dependence of the powder pattern of Dipolar and 'Isotropic' models as a function of τ . In the dipolar model (right panel) the frequency region between the two perpendicular turning points shows increasing intensity as τ increases. The ability to distinguish between these two patterns is best obtained by following the trends in the region nearest the Larmor frequency as a function of τ rather than looking at any individual spectrum.

Figure 4.

Orientation-selective Mims ENDOR simulations for both hyperfine models using $\tau = 0.500\mu\text{s}$ assuming a rhombic g tensor of (2.2, 2.0, 1.8). In both models, the unique direction of the hyperfine tensor is coaxial with $g_3 = 1.80$. With the added dimension of the orientation selection, it is relatively easy to distinguish between the two patterns even with a single τ value.

Figure 5.

35 GHz Mims pulsed ENDOR (top) and simulations (bottom) of DHLys radical (Inset 1) at 2 K. Experimental conditions: microwave pulse lengths 52 ns, RF pulse length 60 μs , $\tau = 0.452$ μs , repetition time = 10 ms, microwave frequency 34.483 GHz. Simulations: $A = [0.16, 0.16, 1.6]$ MHz, ENDOR linewidth = 0.05 MHz. Middle simulation has Mims holes included with $\tau = 0.452$ μs , bottom simulation is the statistical distribution only.

Figure 6.

35 GHz Mims and Re-Mims pulsed ENDOR (solid lines) and dipolar (dashed lines) and isotropic (dotted lines) simulations of Compound **1** (Inset 2) at 2 K. Spectra collected at g_1 and g_2 and $\tau = 200$ (Re-Mims), 800 and 1400 ns (Mims), and simulations performed with and without holes included as indicated in the figure.

Terminal aqua ligand: $\mathbf{T_x}$

Hydroxo bridge: $\mathbf{B}$

$\mathbf{T_B \approx [-T, 0, T]}$

Terminal + bridge: $\mathbf{[T_x + B]}$

Figure 7.

Alternate, models for exogenous ligands to $\mathbf{X}$, with the portions relevant to this study in bold. *Top*, oxo bridge plus terminal aqua ligand ($\mathbf{T_x}$); *middle*, hydroxo bridge ($\mathbf{B}$); *bottom*, terminal aqua plus bridge $\mathbf{[T_x + B]}$. Approximate characteristics of the $^{1,2}\text{H}$ cluster dipolar hyperfine tensors are indicated.

Figure 8.

Black solid lines = Experimental 2-D field-frequency plots of Davies ^2H ENDOR spectra of **X**(WT) in D_2O . (---) lines are simulations with the three models considered here (Fig 7): (A) [**T_x**]; (B) [**T_x**+**B**] with and without (statistical) suppression, considered here.

Conditions: π -pulse length = 200 ns, τ = 0.600 μs , repetition time = 50 ms, MW frequency = 34.826 GHz, T = 2 K. All spectra have been centered at the ^2H nuclear Larmor frequency.

(A) Experiment with **T** proton simulations

(---) overlaid. Simulation parameters: $\mathbf{g} = [g_1 = 2.0056, g_2 = 1.9977, g_3 = 1.993]$, $\mathbf{A} = [A_1 = -1.55, A_2 = -1.15, A_3 = 3.2]$ MHz, (Euler angles $\alpha = 10.5^\circ, \beta = 72^\circ, \gamma = 0$), line widths used = 0.09 (minimum) to 0.25 (maximum) MHz.

(B) Optimized spin Hamiltonian parameters for the bridging proton (**B**) by including a_{iso} . (Optimized; $\mathbf{A}_B^0$); $\mathbf{g} = [g_1 = 2.0056, g_2 = 1.9977, g_3 = 1.993]$, $\mathbf{A} = [A_1 = -3.031, A_2 = -0.948, A_3 = 3.179]$ MHz, (Euler angles $\alpha = 0.0^\circ, \beta = 115.0^\circ, \gamma = 75.0^\circ$), line width = 0.25 MHz.

(B) Statistical/Right: Experiment with simulations of [**T_x**+**B**] models: $x = 1$

(---), $x = 2$

(---) without suppression ($\tau = 0$). Spin Hamiltonian parameters for the simulations of terminal and the optimized bridging hydroxides: see Fig 8A and 8B. For $x = 1$, the intensities of the **T** and **B** deuteron signals were added and

the resultant scaled to the maximum of the experiment; for $x = 2$, twice the **T** intensity was added to the **B** intensity before scaling.

Suppression/Left: similar conditions as statistical, but with suppression ($\tau = 150$ ns).

Figure 9.

(Left) Q-band Mims ENDOR spectra of *trans*-[(Im)₂](NH₃)₄Ru(III)³⁺ in a water/glycol glass at 2K as a function of τ . The resonances from the ammine ligands are suppressed by selecting $\tau=0.360\mu\text{s}$, leaving intensity only from the coordinated imidazole ligands, denoted with the goalpost diagram. The spectrum obtained with $\tau=0.560\mu\text{s}$ suppresses the imidazole transitions leaving the first-order assignment of the ammine resonances. (Right) Simulated Mims ENDOR spectra using EndorSim for the sum of the imidazole and ammine ENDOR patterns using equal weight. The assignments from the left panel are repeated to guide the eye. The overall experimental patterns are well matched by the simulations, showing the EndorSim protocol is valid.

Inset 1.

Inset 2.