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Assessment of the ESR Spectra of CuKTSM$_2$[1]

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The nitrogen superhyperfine structure in the ESR spectrum for CuKTSM$_2$ in light paraffin oil is particularly well resolved in the rigid-limit and fast-tumbling regions. This oil is an excellent solvent because its viscosity, to a first approximation, is similar to the viscosity in cell membranes, the dielectric properties of paraffin oil are suitable for the solvation of CuKTSM$_2$, and the acyl chains help maintain CuKTSM$_2$ monomers throughout a wide range of concentrations and temperatures. Computer curve fitting, including Monte Carlo and damped-least-squares methods, is used to obtain ESR parameters. The refinement of the ESR parameters based on the determination of the goodness of fit is a new approach to ESR analysis. Near-rigid-limit spectra obtained by increasing the temperature from −40 to about +10 °C have fewer resolved lines in the perpendicular region and no resolved superhyperfine lines in the low-field portion of the parallel region. Use is made of only these lines in the perpendicular region to determine the number of nitrogen donor atoms from the ESR spectrum.

Introduction

In the early 1960s French and Freelander, who screened hundreds of compounds, found that 3-ethoxy-2-oxo-butyraldehyde bis(thiosemicarbazone), H_2KTS, is one of four thiosemicarbazones that have significant activity against sarcoma 180 in mice.[2] Then Petering and co-workers[3,4] proved that H_2KTS is active as the copper complex, CuKTS (Figure 1). High toxicity for H_2KTS was reported following a phase I clinical study,[5] but many of the toxic symptoms can be attributed to interference with copper metabolism.[4] Subequently, Petering and co-workers studied the chemical properties of CuKTS and the interactions that occur upon addition to Ehrlich ascites tumor cells.[6] It was concluded that ligand substitution or addition will be thermodynamically or kinetically unfavorable in vivo, but CuKTS is slowly reduced and dissociated by thiols, primarily glutathione, in cells. For derivatives of CuKTS, a linear free energy correlation between the relative pseudo-first-order rate constants of reaction of these complexes with cells as a function of their reduction potentials indicated that complexes with sufficient reactivity toward sulfhydryl groups are also active against cells.[6] Recent studies show that some copper is transferred to metallothionein.[7]

Previous ESR studies on the interaction of CuKTS derivatives with Ehrlich cells established that CuKTS is rapidly reduced whereas CuKTSM$_2$ (Figure 1) is stable in cells over several hours.[8,9] ESR studies show that the mobility of CuKTSM$_2$ in cells at room temperature is so slow that its spectrum indicates immobilization.[9] As the concentration of CuKTSM$_2$ in Ehrlich cells increases, an additional spectrum characteristic of fast motion is superimposed upon the slow-motion spectrum.[9,10] CuKTSM$_2$ is cytotoxic toward Ehrlich cells at a concentration for which both immobile and mobile CuKTSM$_2$ spectra are recorded. Whether either form or both of these forms are related to cytotoxicity has not yet been determined. It is known that the immobile form dominates in spectra from artificial lipid bilayer preparations above and below the main phase transition temperature.[11] ESR spectra in oriented artificial membranes are consistent with a hypothesis in which CuKTSM$_2$ is well oriented with the plane of the complex perpendicular to the bilayer surface and parallel to the acyl chains.[11] Translational diffusion of CuKTSM$_2$ in a bilayer depends on phospholipid alkyl chainlengths, unsaturation, and the presence of cholesterol.[11,12]

In this article we present ESR spectra of CuKTSM$_2$ in paraffin oil at several different temperatures. Light paraffin oil is a particularly suitable solvent because the motion of CuKTSM$_2$ in paraffin oil may resemble, to a rough first approximation, the motion of CuKTSM$_2$ in artificial bilayers and cell membranes.[9] The oil is a mixture of saturated hydrocarbon chains of various lengths. Translational diffusion of CuKTSM$_2$ in paraffin oil is slow, and collisions between CuKTSM$_2$ molecules (Heisenberg exchange) are infrequent.[13] Rotational diffusion is probably faster because the average size of the solvent molecule is similar to the size of CuKTSM$_2$ and because the possibility of microscopic

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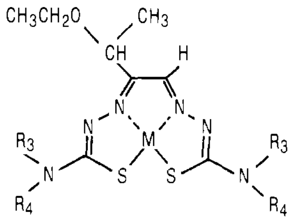

	R_3	R_4
H_2KTS	H	H
H_2KTSM	H	CH_3
H_2KTSM2	CH_3	CH_3

Figure 1. Structures of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) copper(II) complexes.

alignment of the hydrocarbon chains about $CuKTSM_2$ exists. The study of the motion of $CuKTSM_2$ over a wide range of rotational correlation times without changing the solvent is possible due to the wide range of viscosity changes from 156 cP at 4 °C to 13 cP at 13 °C.[13] To perform a detailed analysis of the motional behavior of $CuKTSM_2$ in isotropic (paraffin oil) and anisotropic (membrane) environments, it is crucial to obtain the exact ESR spectral parameters for $CuKTSM_2$. Good simulations of the rigid limit spectra have enabled us to evaluate spectra in the near-rigid, slow-motion, and fast-motion domains. From this evaluation it became evident that the resolution of the nitrogen superhyperfine structure in the $g_\perp$ region may often be used to determine the number of nitrogen donor atoms under conditions where the structure in the $g_\parallel$ region is not resolved. These methods are expected to be extended to the two-nitrogen–two-sulfur (2N1S) configuration of type I complexes after taking into account a reduction in ESR parameters and an increase in rhombicity (work in progress).

Experimental Section

Materials. H_2KTSM_2, 3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazone), was generously supplied by Eugene Conts, and $CuKTSM_2$ was generously supplied by H. G. Petering. Isotopic ^{65}CuO or ^{63}CuO was purchased from Oak Ridge National Laboratory, Oak Ridge, TN. Stock solutions of cupric ion were made up in concentrated HCl. All solvents were reagent grade. The pH was adjusted with NaOH. Paraffin oil (light) was purchased from MCB, Manufacturing Chemists, Inc.

Spectrometers. The ESR spectrometers are located at the National Biomedical ESR Center at the Medical College of Wisconsin and at the University of Wisconsin—Milwaukee. The S-band spectrometer operates at about 3 GHz. The S-band cavity is a loop–gap resonator.[14] Samples (2 mM solutions of $CuKTSM_2$ in light paraffin oil) are deoxygenated and contained in either a flat cell or a disposable pipette sealed with clay. The temperature is controlled with a Varian variable-temperature controller. The temperature is recorded with a Fluke 2100A digital thermometer. A copper–constantan thermocouple is placed into the top portion of the sample.

Simulations. The simulation program is a modified version of the program obtained from John R. Pilbrow, Monash University, Clayton, Victoria, Australia.[15] This program takes into account g- and A-strain contributions to the line width and generates the spectrum in the frequency-swept domain. The equation for the Gaussian half-width is

$$\sigma_\nu = \left(\sigma_R{}^2 + \left[\frac{\Delta g}{g}\nu_0(H) + \Delta A(M_I)\right]^2\right)^{1/2} \qquad (1)$$

where σ_R is the residual line width, $\Delta g/g$ and ΔA represent the g- and A-strain contributions, and ν_0 is the microwave frequency. Six principal strain values are included in the set of ESR parameters. For spectral fitting, either a Monte Carlo[16] or a damped-least-squares[17] method is employed. Initial values of $g_\parallel$ and $A_\parallel$ are determined from the experimental spectra, and $g_\perp$ and $A_\perp$ are obtained after a few simulations. When the line positions are properly adjusted, these parameters are fixed and the strain parameters are set free to minimize the least squares.

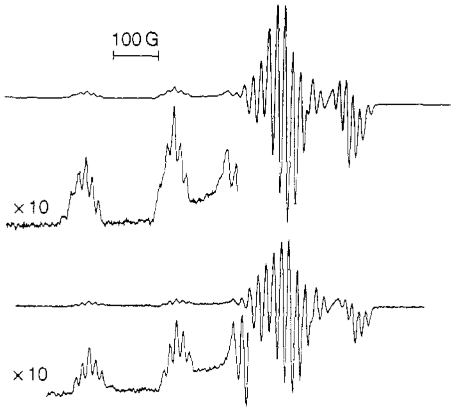

Figure 2. Rigid-limit X-band ESR spectra of $CuKTSM_2$ (2 mM) in light paraffin oil. The copper complex contains naturally abundant isotopes of copper, i.e. 69% ^{63}Cu and 31% ^{65}Cu (top spectra) and only ^{63}Cu (bottom spectra). Spectrometer conditions: microwave frequency, 9.1 GHz; modulation amplitude, 5 G; modulation frequency, 100 kHz; microwave power, 50 (top spectra) and 5 mW (bottom spectra); temperature, −40 (top spectra) and −196 °C (bottom spectra).

The damped-least-squares method, which proved to be successful for fitting overlapping exponential decay curves,[18] in many cases works well for ESR spectra. It utilizes the Gauss–Newton minimization procedure to which a scalar factor "p" is included. This helps to "damp" the process toward the minimum and assures the convergence in the iterative steps. The damped-least-squares method is outlined as follows: N is the number of spectral points; n is the number of spectral adjustable parameters; $\mathbf{X}$ is a column vector of parameters, $\mathbf{X} = x_1, ..., x_n$; $\mathbf{F}$ is a column vector of residuals $f_j = E_j - S_j$, (for $j = 1, ..., N$), where E_j and S_j are the jth experimental and simulated points, respectively. Typically N may be 1000, corresponding to digitization of the spectrum at 1000 field positions. $\mathbf{A}$ is a ($N \times n$) matrix of partial derivatives of a residual with respect to each ESR parameter

$$a_{ij} = \partial f_i/\partial x_j$$

$\mathbf{B}$ is a matrix, and $\mathbf{G}$ is a column vector defined by

$$\mathbf{B} = \mathbf{A}^T\mathbf{A} \qquad \mathbf{G} = \mathbf{A}^T\mathbf{F}$$

The iterative step for the parameter vector $\mathbf{X}$ is

$$X_m + 1 = X_m - (B_m + pC_m)^{-1}G_m \qquad (2)$$

where $\mathbf{C}$ is a diagonal matrix formed from $\mathbf{B}$ by including only the elements on the main diagonal of $\mathbf{B}$, and $\mathbf{X}_0$ is a vector comprised of an initial best guess for the ESR parameters. The standard error of the adjusted parameter, ∂_{x_i}, is then

$$\sigma_{x_i} = [b_{ii}L/(N - n)]^{1/2} \qquad (3)$$

where b_{ii} are diagonal elements of $\mathbf{B}^{-1}$ and L is defined as

$$L = \sum_{i=1}^{N}(E_i - S_i)^2 \qquad (4)$$

The goodness of fit parameter χ^2 is defined as

$$\chi^2 = L/(\mathrm{var}(N - n)) \qquad (5)$$

where var is a variance of noise in the base line of the ESR spectrum and other parameters are as defined above. χ^2 is used to quantitatively express the matching between simulated and experimental spectra.

Results

The nitrogen superhyperfine structure of the ESR spectrum for $CuKTSM_2$ in paraffin oil at low temperatures is particularly well resolved (Figure 2). The five lines in the $g_\parallel$ region of the ESR spectrum for $CuKTSM_2$ (Figure 2, bottom spectrum), formed from the single ^{63}Cu isotope, originate from the coordi-

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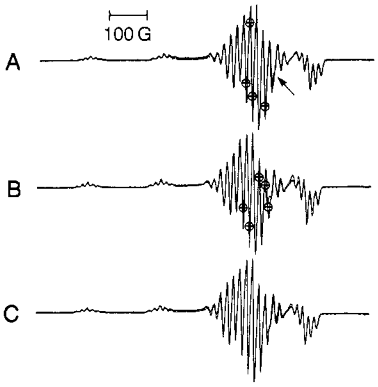

Figure 3. Experimental spectra (solid line) described in Figure 2, for which the copper complex contains ^{63}Cu, and simulated spectra (dotted line) following iterative methods (see Experimental section): (A) spectrum for the square-planar configuration; (B) spectrum showing separate hyperfine tensors for nitrogen donor atoms N(1) and N(2); (C) best-fit spectrum following minimization technique. The simulations can be reproduced by using 3061 G for the field set, 9.100 GHz for the microwave frequency, and the parameters in Table I. Calculation of the spectral positions in Figures 5–7 can be obtained by comparison with the rigid-limit spectrum in Figures 2 and 3 and by the use of the parameters in Table I. An arrow indicates the position for an additional inflection not obtained in spectrum A. A line through the circles indicates positions for which the simulated spectrum does not fit the experimental spectrum.

nation of Cu to two equivalent nitrogen donor atoms. When $CuKTSM_2$ is formed with ^{63}Cu and ^{65}Cu (the natural abundance is 69% and 31%, respectively), the superposition of two spectra is evident in the $g_\parallel$ region where six lines are present (Figure 2, top spectrum). In the region around $g_\perp$ a maximum of 13 out of a possible 20 or more lines can be resolved for both unenriched and isotopically enriched samples.

A good fit between computer simulations of the rigid-limit spectrum of $CuKTSM_2$ in paraffin oil and the experimental spectrum suggests that the assumptions of colinear and axial **g**, $\mathbf{A}^{Cu}$, and $\mathbf{A}^{N}$ tensors are reasonable first approximations (Figure 3a and Table I). Applying a minimization method, either Monte Carlo or damped least squares, gives a value for χ^2 (eq 5) (Figure 3A). The simulated spectrum overlaps with the experimental spectrum except for one missing line in the $g_\perp$ region (arrow, Figure 3A). If the simulation program is modified to account for magnetic nonequivalency of the two nitrogen superhyperfine tensors, as described by Guzy et al.[19] for copper phthalocyanin, χ^2 drops (Figure 3B and Table I). Inclusion of in-plane **g**-tensor anisotropy in the simulations results in better matching of the simulated and experimental spectrum, and χ^2 drops further (Figure 3C and Table I). Noncolinearity of the **g** and **A** tensors does not change χ^2 significantly (data not shown). Previously Moores and Belford[20] found the principal in-plane axes of the **g** tensor oriented along the metal ligand bonds for copper doped into single crystals of orthorombic bis(*N*-methylsalicylaldiminato)nickel(II), and Hitchman and Belford[21] found in-plane **g** axes lying between the copper–oxygen bonds for copper diluted into crystals of bis(benzoylacetonato)palladium. Our data is not sensitive to the direction of the in-plane axes of the **g** tensor relative to the **A**-tensor principal axes, and the set of the spectral parameters for $CuKTSM_2$ (Table I) does not include noncolinearity of the **g** and **A** tensors.

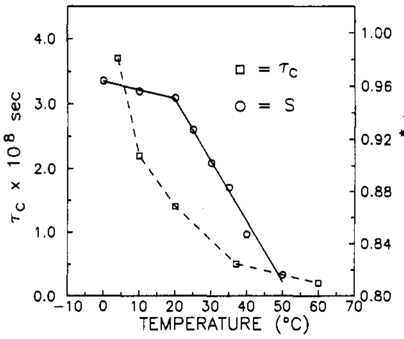

Figure 4. Plot of the *S* parameter (○) and τ_c (□) versus temperature for $CuKTSM_2$ in paraffin oil.

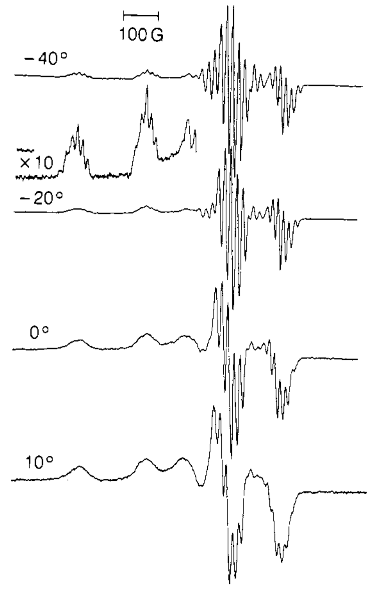

Figure 5. Rigid-limit and near-rigid-limit X-band ESR spectra of $CuKTSM_2$ (2mM with 69% ^{63}Cu and 31% ^{65}Cu) in light paraffin oil as a function of temperature. Spectrometer conditions: microwave frequency, 9.1 GHz; microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G.

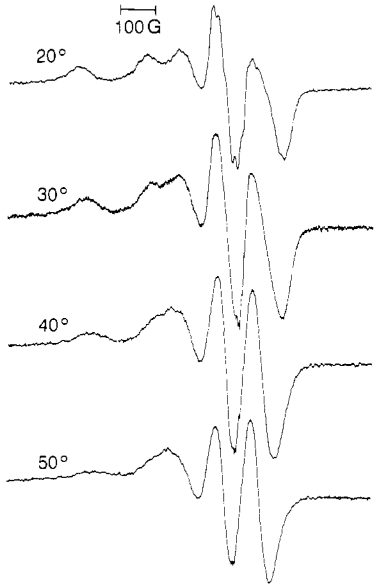

Figure 6. X-Band ESR spectra of $CuKTSM_2$ in light paraffin oil under slow-motion conditions (parameters given in the caption of Figure 5).

Motion of $CuKTSM_2$ in paraffin oil is identified by adopting the nomenclature used by Campbell and Freed[22] for vanadyl complexes and utilizing the *S* parameter defined as

$$S = A_z^*/A_z \qquad (6)$$

where A_z is the rigid-limit value for the copper hyperfine splitting in the *z* direction and A_z^* is the splitting at a given temperature. Freed[23] has shown that the parameter *S* is proportional to the

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Table I. ESR Parameters[a,b] for $CuKTSM_2$

g Values and Hyperfine Constants

assumptions	g_x	g_y	g_z	A_x^{Cu}	A_y^{Cu}	A_z^{Cu}
square planar	2.0515	2.0515	2.1399	112	112	570
$A_x^N \neq A_y^N$	2.0515	2.0515	2.1399	115	115	570
$g_x \neq g_y$	2.0520	2.0510	2.1399	112	112	570

Superhyperfine Constants

assumptions	$A_x^N(1)$	$A_y^N(1)$	$A_z^N(1)$	$A_x^N(2)$	$A_y^N(2)$	$A_z^N(2)$
square planar	48.7	48.7	39.8	48.7	48.7	39.8
$A_x^N \neq A_y^N$	49.4	40.1	41.8	40.1	49.4	41.8
$g_x \neq g_y$	50.8	41.1	42.8	41.1	50.8	42.8

Line Width and Strain Parameters

assumptions	W_x	W_y	W_z	$C(1)_x$	$C(1)_y$	$C(1)_z$	$C(2)_z$	χ^2
square planar	9.8	9.8	11.2	0.0007	0.0007	0.0005	2.5	82
$A_x^N \neq A_y^N$	9.8	10.2	11.2	0.0007	0.0007	0.0005	3.0	71
$g_x \neq g_y$	8.6	9.5	11.2	0.0007	0.0007	0.0005	3.0	28

[a] Expressed in MHz to facilitate comparison with ENDOR data (to convert from MHz to cm^{-1} divide the value given in the table by 2.9979×10^4. [b] The Gaussian half-width, $\sigma_v = \{\sigma_R^2 + [\Delta g/g\gamma_0(H) + \Delta A(M_I)]^2\}^{1/2}$ where σ_R is the residual line width, w_i; $C(1)_i = \Delta g/g$; and $C(2)_i = \Delta A$ where $i = x, y$, and z. $\Delta g/g$ and ΔA represent the g and A "strain" contributions from ref 15. The units for w_i and ΔA are MHz. $\Delta g/g$ is dimensionless. Only an A-strain contribution in the z direction was included in the parameter file.

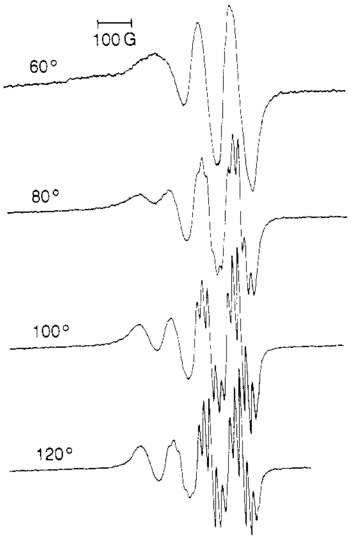

Figure 7. X-Band ESR spectra of $CuKTSM_2$ in light paraffin oil under fast-motion conditions (parameters given in the caption of Figure 5).

correlation time, τ_c, characterizing the molecular motion. The temperature dependence of S for $CuKTSM_2$ is shown in Figure 4. The near-rigid-limit spectra (Figure 5) are those for which $S > 0.95$. These spectra retain most of the features of the rigid-limit spectrum. The anisotropy in the magnetic interactions in the slow-motion spectra (Figure 6) is evident, and S is greater than 0.81 and less than 0.95 (Figure 4). For the fast-motion spectra all anisotropies are averaged out (Figure 7), and S does not change with temperature (data not shown). The hyperfine anisotropy for copper in $CuKTSM_2$, $|A_z - A_x|$, is about 4.5×10^8 Hz. To average out this anisotropy the molecule has to reorient with a rotational correlation time, τ_c, on the order of 2.2×10^{-9} s. The rotational correlation time (Figure 4) for a molecule in an isotropic solvent can be estimated by means of the Stokes–Einstein equation (eq 7). The hydrodynamic radius, r, is assumed

$$\tau_c = 4\pi r^3\eta/3kT \qquad (7)$$

to be 6×10^{-8} cm, and the viscosity in paraffin oil is taken from Subczynski et al.[13] A semiempirical formula (eq 8) relates S with τ_c.[23] Curve fitting gives a and b equal to 2.34×10^{-10} s/rad and −1.47, respectively.

$$\tau_c = a(1 - S)^b \qquad (8)$$

In the near-rigid limit (τ_c longer than 3.7×10^{-8} s), the resolution of the nitrogen superhyperfine structure diminishes as the temperature increases (Figure 5). The predominant feature at 10 °C is the $M_I = +^1/_2$ line. The spectral anisotropy for the $+^1/_2$ line is smaller than that for the $M_I = -^3/_2$ and $-^1/_2$ lines. As molecular motion increases, the lines for which spectral anisotropy is large are broadened more than the lines for which spectral anisotropy is small (i.e. the $M_I = +^1/_2$ lines). In the absence of nitrogen superhyperfine structure, this effect is noticed for $VO(acac_2(pm))$ in toluene at −114 °C for which the $-^1/_2$ line line dominates the spectrum.[22]

As the motion increases from the near-rigid limit to the slow-motion region (τ_c between 3.7×10^{-8} and about 10^{-9} s) the nitrogen superhyperfine structure is lost (Figure 6). We have previously used the reduction of the resolution of the nitrogen lines to describe rotational motion of $CuKTSM_2$ in phospholipid bilayers through a parameter defined as the Cu-motion parameter.[11] This motion parameter has been found to be sensitive to the properties of artificial bilayers such as the fluid/gel phase transition, but spectral simulations based on the stochastic Liouville equation[22] seem to be the only approach to quantitatively characterize the reorientation of $CuKTSM_2$ in either an isotropic solvent or a membrane (work in progress).

As the motion increases from slow to fast ($\tau_c < 10^{-9}$ s) the nitrogen superhyperfine structure of the ESR spectrum for $CuKTSM_2$ reappears (Figure 7). The averaging of the **g** and **A** tensors is apparent. The spectra begin to approach the motionally narrowed limit.

At a lower microwave frequency of 3.4 GHz (S-band), the spectra in the near-rigid limit are dominated by two intense spectral features comprised of the $M_I = -^1/_2$ and the $M_I = +^1/_2$ lines (Figure 8). As motion increases, the lines in the $g_\parallel$ and $g_\perp$ region are broadened until only the $M_I = -^1/_2$ and $+^1/_2$ features are clearly resolved. The five lines for the $M_I = +^1/_2$ line at −10 °C appear to be diagnostic for two nitrogen donor atoms, but simulations of the $M_I = +^1/_2$ line (data not shown) suggest a more complicated pattern consisting of overlap between perpendicular and parallel components. The perpendicular component is actually on the low-field side of the more intense parallel component. This nitrogen hyperfine structure becomes poorly resolved in the slow-motion and fast-motion domains (Figure 9).

Discussion

It seemed important to us to be able to simulate the rigid-limit ESR spectrum of $CuKTSM_2$ before attempting to simulate less

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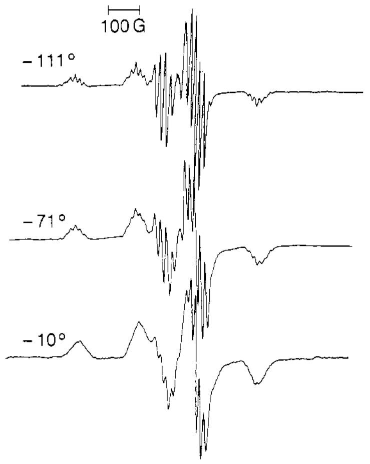

Figure 8. Rigid-limit and near-rigid-limit S-band ESR spectra of $CuKTSM_2$ (1 mM with 69% ^{63}Cu and 31% ^{65}Cu) in light paraffin oil as a function of temperature. Spectrometer conditions: microwave frequency, 3.359 GHz; microwave power, 16 dB; modulation frequency, 100 kHz; modulation amplitude, 2.5 G.

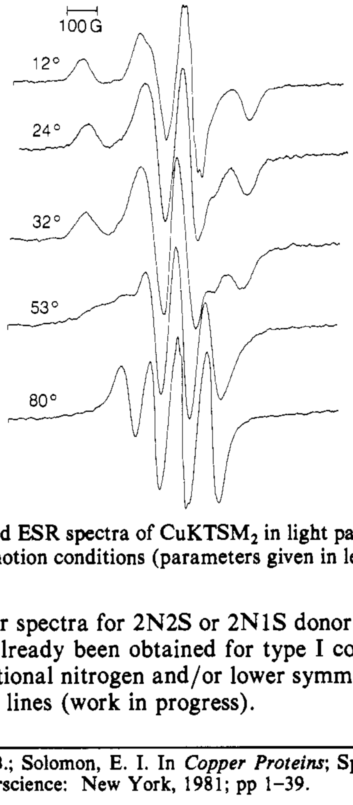

Figure 9. S-Band ESR spectra of $CuKTSM_2$ in light paraffin oil under slow- and fast-motion conditions (parameters given in legend of Figure 8).

resolved copper spectra for 2N2S or 2N1S donor atoms. Such spectra have already been obtained for type I complexes[24] for which an additional nitrogen and/or lower symmetry increases the number of lines (work in progress).

In the absence of nitrogen superhyperfine structure in the $g_\parallel$ region, investigators have often attempted to count the number of resolved lines in the $g_\perp$ region to determine the number of nitrogen donor atoms. To our knowledge no one has offered a firm rationale to explain why this method sometimes accounts for the right number of nitrogen donor atoms. One method to obtain nitrogen hyperfine on a single copper line in the $g_\perp$ region is as follows: Minimize the spectral anisotropy for a single M_I line (i.e. $M_I = +^1/_2$ line for $CuKTSM_2$) by adjusting the microwave frequency. Increase the temperature until the M_I lines are broadened more than the line that has minimal spectral anisotropy. Check spectral features to verify that the peak-to-peak signal height of this line is an order of magnitude greater than the height of the other M_I lines. The number of resolved superhyperfine lines is often attributed to a single M_I line. The five-line pattern for the most intense line of the $CuKTSM_2$ spectrum at 10 °C (Figure 5) is attributed to the $M_I = +^1/_2$ line in the $g_\perp$ region. The inference is that under the right conditions of minimal spectral overlap and appropriate broadening, the resolution in the perpendicular region can be used to account for the number of nitrogen donor atoms.

It is desirable to obtain well-resolved spectra for copper complexes bound to nitrogen donor atoms like those obtained for copper phthalocyanine[19] or CuKTS doped in NiKTS.[25] Analysis of the resolved nitrogen hyperfine structure in the $g_\parallel$ region is sufficient to determine the number of nitrogen donor atoms. But, especially for copper complexes in biological media, for which numerous axial ligands and strains are present, the $g_\parallel$ lines are often broadened enough to obliterate the nitrogen hyperfine structure in the $g_\parallel$ but not the $g_\perp$ region. For example, ESR spectra for the 2N2S copper complexes formed from analogues of tetradentate salicylaldimine[26] have lines resolved in the $g_\perp$ but not the $g_\parallel$ region. The pattern of these lines is due to both copper and nitrogen hyperfine coupling. If the solvent is changed from frozen chloroform/toluene or dimethylformamide/chloroform mixtures to frozen pyridine, a five-line pattern dominates the $g_\perp$ region. No other lines are well resolved. If the temperature was increased and the same five-line pattern remains in the $g_\perp$ region, an assignment of two almost equivalent nitrogen donor atoms could be justified for the complex.

In summary, if the spectral anisotropy of a single M_I line of a copper ESR spectrum is minimized, the intense feature in the $g_\perp$ region may be used to identify the number of nitrogen donor atoms in a copper complex. The spectral anisotropy can be minimized by changing the microwave frequency. Raising the temperature helps to isolate a well-resolved single M_I line.

Registry No. $CuKTSM_2$, 53109-51-6.

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