Electron Nuclear Double Resonance on Heme Compounds. ENDOR from the Iron Ligands in Protohemin Chloride and Protohemin Bromide

H. L. Van Camp, C. P. Scholes,* and C. F. Mulks

Contribution from the Department of Physics, State University of New York at Albany, Albany, New York 12222. *Received October 20, 1975*

Abstract: Electron nuclear double resonance (ENDOR) and electron paramagnetic resonance (EPR) signals have been obtained from high-spin ferric protohemin chloride and protohemin bromide dimethyl esters frozen in organic solvent. With the magnetic field along the symmetry axis of the heme, magnetic hyperfine and quadrupolar interactions with the heme nitrogens from both compounds have been detected by ENDOR. A small difference in nitrogen hyperfine couplings between the chloride and bromide compounds is seen. Magnetic hyperfine and quadrupolar interactions with the chloride ligand have been detected by ENDOR and magnetic hyperfine structure from the bromide ligand has been observed directly by EPR.

ENDOR can determine with high precision the hyperfine interaction of paramagnetic electrons, centered at the iron (Fe^{3+}) of heme, with surrounding nuclei. From the value of these interactions one may obtain fine details of the electronic distribution at the surrounding nuclei and thus insight into the intimate electronic details of the iron and its environment. A substantial body of ENDOR data on myoglobin and hemoglobin now exists,[1] and changes of biological significance, particularly those reported on hemoglobin $M_{Milwaukee}$,[1b] have been seen by ENDOR. We believe that ENDOR data on heme's electronic structure should, however, be understood in more than the present largely empirical fashion and that systematic model studies are called for on factors which affect heme's electronic structure. The intention of this initial study was to observe nitrogen magnetic hyperfine and quadrupolar interactions from heme frozen in organic solvent and to observe the effect on these nitrogen interactions upon changing heme's axial ligand from chloride to bromide. An additional outcome of our study is the detection of bromine ligand hyperfine interaction directly by EPR and of chloride hyperfine and quadrupolar interactions by ENDOR. We intend in the future to study the effect of other heme axial ligands, particularly those in the bond strength series from methoxide through iodide; this series of axially liganded heme compounds has been studied by far infrared,[2] Mössbauer,[3] NMR,[4] and optical[5] spectroscopies.

Heme esters were chosen for the first study because the work in ref 2-5 was carried out on esters, because they are soluble in many organic solvents,[6] and because there is detailed literature for making them,[7,8] for interconverting them,[8] and for analyzing them.[8] Protohemin esters were used for this initial work because of the relative simplicity of their preparation.

A problem with many porphyrin systems is that they aggregate.[9,10] This aggregation causes spin-spin interactions between paramagnetic heme centers. Spin-spin interactions substantially reduce and broaden EPR signals and by opening competing relaxation pathways may altogether eliminate ENDOR signals. To ensure that we could obtain an ENDOR signal from heme dissolved in organic solvent, it was important to choose a solvent system where heme would not aggregate. A further consideration in the choice of a solvent system for this work is that we must use frozen samples. Since solutes may segregate upon freezing of the solvent,[11] a solvent system which forms a glassy matrix on freezing is more likely to accommodate the heme in nonaggregated fashion in the solid phase. Heme may stay monomeric in solvents like pyridine or *N,N*-dimethylformamide[12] (DMF), but the solvent may replace the desired axial ligand on the ferric heme. With the three aims of providing a nonaggregating solvent, of providing a glassy matrix, and of not replacing the chloride or bromide ligand, we developed the solvent system of tetrahydrofuran (THF)-chloroform plus tetrabutylammonium halide, whose details are given below.

Experimental Section

Apparatus. The ENDOR apparatus was patterned after that successfully used in Feher's lab for ENDOR on heme proteins.[1] However, we used a Varian V-4500 Homodyne system which we modified by adding a circulator and a by-pass reference arm. We use a silvered plastic EPR-ENDOR cavity.[13] For the majority of this work a cavity was used which had three coats of silver, and resonated near 9.0 GHz. Some later work was done with a cavity which had only one coat of silver and resonated near 9.4 GHz. The latter cavity had less EPR sensitivity; however, it enabled us to operate at a higher resonance field strength (for a given electronic *g* value), and because of its thinner silver, it allowed more ENDOR radiofrequency radiation to reach the sample. We measured our magnetic field strength with a Harvey-Wells G-502 NMR Precision Gaussmeter connected to a Hewlett-Packard 5245L Electronic Counter. We measured our microwave frequency with a Hewlett-Packard X-532B Frequency Meter, which was calibrated against a Hewlett-Packard Model 5240A 12.4 GHz Digital Frequency Meter.

Reagents. All solvents were Fisher A.C.S. Certified reagents. THF was distilled under an inert atmosphere over potassium immediately before use. Chloroform (with about 0.75% ethanol as a stabilizer) was stored over CaO for at least 24 h prior to use. NaCl and NaBr were Fisher A.C.S. Certified reagents. Tetra(*n*-butyl)ammonium chloride ($[Bu_4N]^+Cl^-$) and tetra(*n*-butyl)ammonium bromide ($[Bu_4N]^+Br^-$) were purchased from Eastman Organic Chemical and used without further purification. Protohemin starting material was Hemin Equine Type III from Sigma Chemical.

Protoporphyrin IX Dimethyl Ester Iron(III) Chloride. Following the method of Clezy and Morell,[9,14] the propionic acid side groups of protohemin were esterified by reacting overnight at −17 °C in 5% (v/v) sulfuric acid/methanol. With successive washings in 2 M Na_2CO_3, distilled water, and 2 M NaCl, the esterified protohemin was separated into toluene. The resultant solution was evaporated to dryness, taken up in benzene, and chromatographed on alumina.[8] The hematin ester elutant was dried, taken up in chloroform, and reconverted to the chloride by shaking with a slightly acidified (with HCl) 2 M NaCl solution and by passing through a column containing dry NaCl.[8b] The solution of protohemin ester in chloroform was then heated, hot isooctane was added, and the mixture was allowed to cool and form crystals. The crystals were washed with isooctane and dried at room temperature under vacuum. Optical spectra taken on a Cary 14 in chloroform compared favorably with those reported for protohemin ester in ref 8a. Analyses were performed by Galbraith Labs, Inc., Knoxville, Tenn.

Anal. Calcd for $C_{36}H_{36}ClFeN_4O_4$: C, 63.58; H, 5.34; N, 8.24; Cl, 5.21; Fe, 8.21. Found: C, 63.80; H, 5.60; N, 8.14; Cl, 5.48; Fe, 8.26.

Protoporphyrin IX Dimethyl Ester Iron(III) Bromide. The sample

Table I. EPR and ^{14}N ENDOR Results in Protohemin Chloride and Protohemin Bromide Dimethyl Esters[a]

Compd	$g_e\perp$ $g_e\parallel$	EPR measured hyperfine splittings resolved near $g = 2$	^{14}N ENDOR frequency, MHz	$\lvert A_{zz}\rvert$, MHz $\lvert P_{zz}\rvert$, MHz
Protohemin chloride dimethyl ester	5.88 ± 0.02 2.004 ± 0.002	None	2.44 ± 0.03 3.17 ± 0.03 4.40 ± 0.02 5.08 ± 0.02	7.55 ± 0.05 0.35 ± 0.03
Protohemin bromide dimethyl ester	5.84 ± 0.02 2.005 ± 0.002	From bromide 28 ± 1 G or 78 ± 3 MHz	2.29 ± 0.04 3.06 ± 0.03 4.27 ± 0.03 4.94 ± 0.03	7.28 ± 0.05 0.36 ± 0.03

[a] Both protohemin chloride and protohemin bromide dimethyl esters were 3 mM in a (1:1, v/v) THF–chloroform solution which was also 30 mM in $[Bu_4N]^+Cl^-$ or $[Bu_4N]^+Br^-$, respectively. The EPR measurements were taken at $T = 4.2$ K under nonsaturating conditions and with 100-kHz field modulation $\simeq 5$ G peak-to-peak. The value of g_e was experimentally measured at the $d\chi''/dH$ zero crossing near $g \simeq 6$. The ENDOR data were taken at $\nu_e = 9.02$ GHz, $H = 3.22$ kG for the chloride and $\nu_e = 9.02$ GHz, $H = 3.26$ kG for the bromide. An ENDOR radiofrequency field of about 0.5 G peak to peak, as measured at 1 MHz, was used. The rms error in ENDOR frequencies and the coupling constants was obtained from the results of several separate runs on each compound in which alternately increasing and decreasing frequency sweeps were used.

preparation was the same as for the chloride except that NaBr and HBr were used in place of NaCl and HCl.

Anal. Calcd for $C_{36}H_{36}BrFeN_4O_4$: C, 59.69; H, 5.01; N, 7.74; Br, 11.03; Fe, 7.71. Found: C, 59.90; H, 5.03; N, 7.59; Br, 11.26; Fe, 7.90.

Frozen Sample Preparation. Protohemin esters were dissolved in chloroform, an equal volume of THF was added, and a tenfold molar excess (with respect to protohemin) of appropriate tetrabutylammonium halide was then added. Samples with overall volumes of 1.5 ml were placed in large diameter (7.5 mm i.d.) quartz tubes and immediately frozen by plunging into liquid nitrogen.

Results

EPR Results. In our preliminary search for a nonaggregating solvent system heme esters were dissolved in mixtures of several organic solvents (toluene, methylene chloride, chloroform, acetone). For a given heme concentration, EPR signals were found which were two orders of magnitude smaller than would be expected for the same heme concentration in metmyoglobin. We next tried DMF solutions which do give large EPR signals. However, axial ligand replacement in DMF was evident. With the protohemin bromide dimethyl ester dissolved in DMF the EPR spectrum showed a large nonbromide peak in the midst of the bromine hyperfine pattern, even with a tenfold excess of $[Bu_4N]^+Br^-$. ENDOR of the protohemin chloride dimethyl ester in DMF showed more nitrogen peaks than can be accounted for by one heme species.

The Mössbauer work in ref 15 showed a partially nonaggregated spectrum from hemin (non-ester) in THF, and this Mössbauer spectrum gave quadrupole splittings, isomer shifts, and zero-field splittings that were the same as previously seen with crystalline hemin. The implication was that the hemin chloride ligand is not replaced by solvent in THF. We found that hemin ester was quite soluble in a 1:1 (v/v) mixture of THF-chloroform and that this mixture formed a glass. To assure that the halide ligand stayed on the heme,[16] we added a tenfold excess of appropriate tetrabutylammonium halide[17] to our solutions. An unexpected advantage of adding the tetrabutylammonium halide was an approximate twofold increase in magnitude of the heme EPR signal[18] when the tetrabutylammonium salt was added; the heme EPR signals were now comparable in size to EPR signals from equal concentrations of heme in metmyoglobin.

The frozen solution EPR spectra from both protohemin bromide and chloride dimethyl esters are typical high-spin ferric heme spectra[19] with axial symmetry. Values of the electronic g tensor ($g_e\perp$ and $g_e\parallel$) for the two compounds are given in Table I. The EPR line width between derivative extrema near $g_e\perp$ was 52 ± 4 G for the chloride compound and 59 ± 3 G for the bromide compound. The chloride compound had a single symmetric peak at $g_e\parallel$ whose width at half-height is 23 ± 1 G; this peak contained both unresolved nitrogen and unresolved chloride hyperfine structure. At the $g_e\parallel$ extremum the bromide compound gave the four-line hyperfine pattern shown in Figure 1, which is due to magnetic hyperfine interaction with the $I = 3/2$ nuclei of ^{79}Br and ^{81}Br.

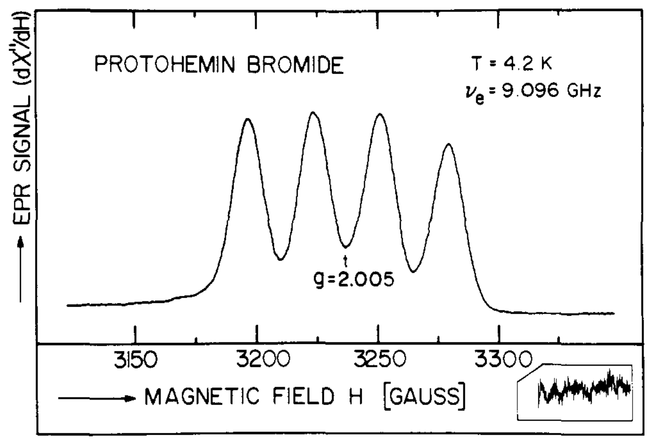

Figure 1. EPR spectrum from a frozen solution of protohemin bromide dimethyl ester, showing bromine hyperfine structure near $g = 2$. The protohemin bromide was in 3-mM concentration with 30-mM $[Bu_4N]^+Br^-$ in 1:1 (v/v) THF-chloroform. $T = 4.2$ K, microwave power $\simeq 20\ \mu W$, 100-KHz field modulation $\simeq$ 5 G peak to peak, 250 G field sweep in 2.5 min with time constant = 0.03 s. Gain on Varian 100 KHz unit is 200. Inset shows noise level with gain = 2000.

ENDOR Results. Information-rich single-crystal-like ENDOR spectra can be obtained from polycrystalline or frozen solution materials merely by doing ENDOR at the g-value extrema.[20] In our case the EPR signal at the $g_e\parallel$ extremum corresponds to an oriented subset of molecules with the magnetic field pointing along the normal to the heme plane.[21] Thus, we observed EPR and ENDOR from molecules where the magnetic field points perpendicular to the heme plane.[22] Because of the fourfold symmetry about the heme iron, the nitrogens are equivalent to one another (from the ENDOR standpoint) when the magnetic field points perpendicular to the heme plane. The four-line ^{14}N nitrogen hyperfine pattern reflects the interaction of the nitrogen nuclei with internal and external magnetic fields and with the local electric field gradient. As can be seen from Figure 2 the entire nitrogen pattern from the bromide compound is shifted to a lower frequency than the pattern for the chloride.[23] We do not attempt to explain the difference in relative intensity of the nitrogen ENDOR lines from one heme compound to another, for the detailed mechanisms to account for ENDOR intensity are

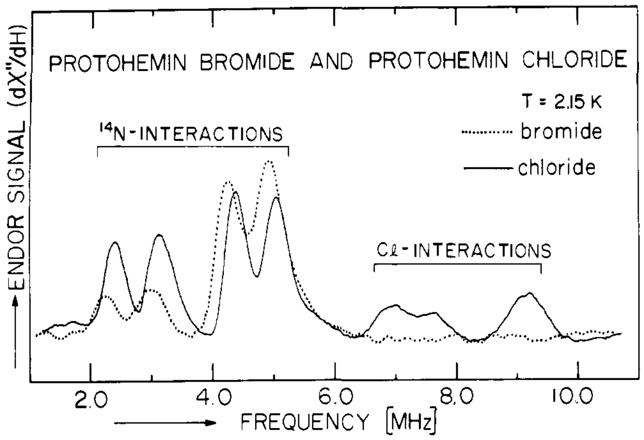

Figure 2. ENDOR spectra from protohemin chloride and protohemin bromide dimethyl esters, reflecting the interaction of the electron spin with ligand nitrogen nuclei. Conditions are as described in Table I. Purpose of this figure is to show the difference in nitrogen hyperfine couplings between the two compounds.

complex[24] and incompletely understood. (Electron spin relaxation rates which can differ from one heme compound to another[25] certainly may affect ENDOR intensities.) Fortunately ENDOR is a spectroscopic technique whose main information comes from the frequency of ENDOR transitions.

For the protohemin chloride we saw additional nonnitrogen lines in the region from 0.5 to 20 MHz, as shown in Figure 3a. We identify the four best resolved and most intense of these as coming from interaction with the ^{35}Cl nucleus. Protohemin *chloride* esters are the only heme compounds which have given such lines and these lines have been seen in the presence and the absence of $[Bu_4N]^+Cl^-$. Protons with weak hyperfine couplings of less than 1 MHz have been previously observed from metmyoglobin[1b] and are centered at the free proton NMR frequency; such protons may be present here too. However, the lines that we see are not centered about the free proton frequency nor does the overall pattern of these lines shift to a higher ENDOR frequency (as would be expected for protons) when we operate with the higher frequency (9.4 GHz) EPR-ENDOR cavity at higher magnetic fields. Several less intense lines seen in the 5.5 to 20 MHz region were identified as coming from ^{37}Cl, whose natural abundance is ⅓ the abundance of ^{35}Cl. After a preliminary analysis of our data, we concluded that there ought to be ^{35}Cl ENDOR in the 0.5 to 2 MHz region, and we made a special effort to resolve ENDOR transitions from this region. Figure 3b shows a low-intensity shoulder from protohemin chloride in the 1- to 2-MHz region, and also shows that there was no such shoulder from protohemin bromide. With our thinly silvered cavity (designed to allow more ENDOR radiofrequency field into the cavity) we sought but did not find any ENDOR in the 20- to 40-MHz region. The frequencies of the ^{35}Cl and ^{37}Cl ENDOR transitions are given in Table II.

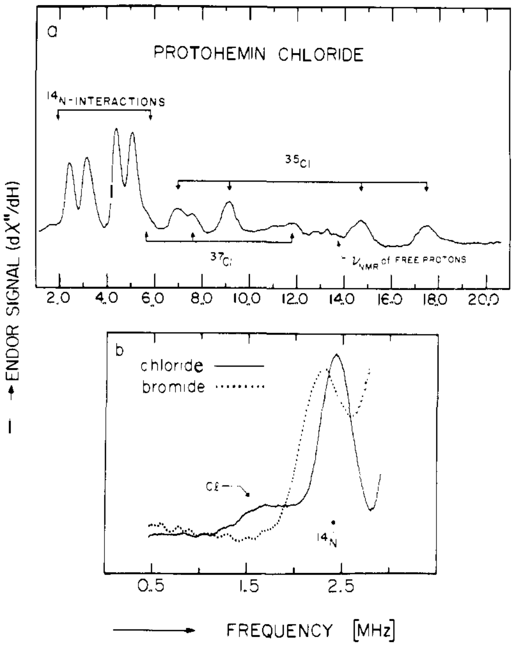

Figure 3. (a) ENDOR spectrum from protohemin chloride dimethyl ester, reflecting the interaction of the electron spin with both chloride and nitrogen ligands. Conditions are as described in Table II. (b) ENDOR spectrum from protohemin chloride dimethyl ester, compared with the ENDOR spectrum from protohemin bromide dimethyl ester in the region from 0.5 to 2.5 MHz. The purpose of this figure is to demonstrate in the 1.5 MHz region the existence of the shoulder which we attribute to a low-lying ^{35}Cl ENDOR transition. The more intense peak lying above 2 MHz is from ^{14}N. These spectra were taken with the thinly silvered cavity to allow more ENDOR radiofrequency field into the sample. This cavity resonated at 9.41 GHz for the chloride sample and 9.40 GHz for the bromide sample. The ENDOR radiofrequency as measured at 1 MHz was about 1 G peak to peak.

Table II. ^{35}Cl and ^{37}Cl ENDOR Transitions in Protohemin Chloride Dimethyl Ester[d]

^{35}Cl ENDOR frequency, MHz	^{37}Cl ENDOR frequency, MHz
1.6 (shoulder)[a]	
6.9 ± 0.1	5.7 ± 0.2[b]
9.18 ± 0.04	7.6 ± 0.1
14.7 ± 0.1	11.7 ± 0.2
17.4 ± 0.1	c

[a] This shoulder shown in Figure 3b was resolved using the cavity which resonated near 9.4 GHz while the rest of the peaks here were determined with a cavity which resonated near 9.0 GHz. [b] Better resolution of this peak was obtained with decreasing ENDOR sweeps from 20 to 1 MHz. Figure 3a was taken with increasing ENDOR sweeps from 1 to 20 MHz. [c] See ref 32. [d] T = 2.15 K, ν_e = 9.03 GHz, H = 3.22 kG, ENDOR radiofrequency field about 0.5 G peak to peak, as measured at 1 MHz. The protohemin chloride dimethyl ester was 3 mM in a (1:1 v/v) THF–chloroform solution which was also 30 mM in $[Bu_4N]^+Cl^-$. As discussed in the text, the most likely parameters for the ^{35}Cl are $|A_{zz}|$ = 16.1 ± 0.1 MHz and $|P_{zz}|$ = 4.0 ± 0.4 MHz.

Theory and Discussion

To explain the interactions of ligand nuclei we use as a first approximation a first-order, axial spin Hamiltonian. Such a spin Hamiltonian has been found effective in explaining previous ^{14}N ENDOR in ref 1. For a system in which the internal hyperfine field and the external magnetic field H point along the axis of symmetry (z), this Hamiltonian can be written for an individual nucleus as:

$$\mathcal{H} = A_{zz}I_zS_z + P_{zz}[I_z^2 - \tfrac{1}{3}I(I+1)] - g_n\beta_nHI_z \qquad (1)$$

where I and S are the nuclear and electronic spin operators, g_n is the nuclear g value, β_n is the nuclear Bohr magneton. P_{zz} is the z component of the quadrupole tensor and it is proportional to the electric field gradient at the ligand nucleus. The third term in the spin Hamiltonian represents the direct nuclear Zeeman interaction of the particular nucleus with the applied magnetic field.

Nitrogen Interactions. For equivalent nitrogens, the above Hamiltonian predicts[1a] two pairs of ENDOR lines denoted by arrows in Table I. The lines in each pair are separated to first order[26] by twice the ^{14}N nuclear Zeeman energy. This amount corresponds, for the respective magnetic fields used with the 9.0 GHz cavity, to 1.98 MHz for the chloride compound and 2.00 MHz for the bromide compound. The average of the four

ENDOR transitions will occur at $\frac{1}{2}|A_{zz}|$. The principal difference in nitrogen hyperfine parameters between the two compounds is the larger hyperfine coupling constant for the chloride compound, which may reflect a slightly larger unpaired electron spin density at the heme nitrogens.

Chloride ENDOR. The axial spin Hamiltonian of eq 1 should be appropriate for interpretation of the chloride ENDOR. X-ray work on hemin[27] has shown the chloride ion to lie essentially on the heme fourfold axis, and our EPR spectra show no evidence for strong nonaxial perturbations to the electron spins. By diagonalizing the spin Hamiltonian for the $I = \frac{3}{2}$ ^{35}Cl or ^{37}Cl nuclei, one predicts the following three pairs of ENDOR transition frequencies:

$$|\tfrac{1}{2}|A_{zz}| \pm g_n\beta_nH| \quad (2a)$$

$$h\nu_{\mathrm{ENDOR}} = |\tfrac{1}{2}|A_{zz}| + 2|P_{zz}| \pm g_n\beta_nH| \quad (2b)$$

$$|\tfrac{1}{2}|A_{zz}| - 2|P_{zz}| \pm g_n\beta_nH| \quad (2c)$$

Transition 2a is within the $\pm\frac{1}{2}$ nuclear spin doublet and involves only magnetic terms. Transitions 2b and 2c are between the $\pm\frac{1}{2}$ and the $\pm\frac{3}{2}$ doublets and involve both magnetic and quadrupole terms.

One would find it difficult to arrive at a unique fit of the ^{35}Cl lines, denoted in Figure 3a to the parameters in eq 2. This is because it is not known which of eq 2a, 2b, or 2c to correlate with the two pairs of intense ^{35}Cl lines, and the relative magnitudes of $|A_{zz}|$ and $|P_{zz}|$ are not known. However, the following additional facts are useful in helping to find the proper values of $|A_{zz}|$ and $|P_{zz}|$:

1. The experimentally obtained frequencies of the two ^{35}Cl lines at 6.9 and 9.18 MHz can be used to compute quite accurately the frequencies of the two less intense lines at 5.7 and 7.6 MHz. The computation is done merely by multiplying the frequencies of the ^{35}Cl lines by the ratio of the ^{37}Cl magnetic moment to the ^{35}Cl magnetic moment.[28] Besides giving us assurance that the ENDOR observed was really from ^{35}Cl and ^{37}Cl, this computation gives us evidence that the transitions are described essentially by the purely magnetic transitions 2a. Thus, for ^{35}Cl we find $|A_{zz}| = 16.1$ MHz.

2. No ENDOR lines are seen above 20 MHz. Lack of lines above 20 MHz ruled out solutions to eq 2 such as $|A_{zz}| = 16.1$ MHz, $|P_{zz}| = 12$ MHz. With the information from the previous paragraph and the frequencies of the two ^{35}Cl lines[29] at 14.7 and 17.4 MHz, we calculated for ^{35}Cl the values $|A_{zz}| = 16.1$ MHz and $|P_{zz}| = 4.0$ MHz to fit eq 2a and 2b.

3. If we substitute into eq 2c the values $|A_{zz}| = 16.1$ MHz and $|P_{zz}| = 4.0$ MHz, which satisfy eq 2a and 2b for ^{35}Cl, we find that the magnetic hyperfine and quadrupole terms almost exactly cancel one another in eq 2c. That predicts two low-energy transitions in the 0.5- to 2.0-MHz range, for both of which the transition energy is approximately $g_n\beta_nH$.[30] Figure 3a shows perhaps a low-intensity hump in the 1- to 2-MHz region. To convince ourselves that this hump is real, we did a thorough study of the 0.5- to 2.5-MHz region. We were satisfied to find that there was indeed the low-intensity ENDOR transition shown in Figure 3b, which occurred only for the protohemin chloride and *not* for the protohemin bromide.

4. The low-energy (eq 2c) ENDOR transitions just discussed must arise from nuclear spin energy levels that lie close to each other. Close-lying levels are particularly susceptible to otherwise negligible, probably off-axial, second-order perturbations, which may arise here simply because in frozen solution not all hemes have their axes exactly aligned with the magnetic field. Second-order perturbations will slightly increase the energy of the 2c transitions and perhaps also broaden the 2c transitions. We did find that the ENDOR seen in Figure 3b is at a slightly higher frequency than the expected value of $g_n\beta_nH$ for ^{35}Cl, and the transition is certainly a broad one. Two of the nuclear spin levels which participate in the 2c ENDOR transitions also participate in the 2a transitions. A second-order perturbation to the nuclear spin levels, which are commonly shared between transitions 2a and 2c, will cause a slight decrease in the Zeeman splitting of the lines from eq 2a. As indicated by the data of Table II, the splitting between the ^{35}Cl lines at 6.9 and 9.18 MHz is about 2.3 MHz, which is less than the expected Zeeman splitting of $2g_n\beta_nH = 2.7$ MHz.

From all of this evidence we were led to believe that our interpretation of the ^{35}Cl ENDOR data with $|A_{zz}| = 16.1 \pm 0.1$ MHz and $|P_{zz}| = 4.0 \pm 0.1$ MHz is essentially the correct one.

Acknowledgments. We would like to acknowledge the considerable technical advice given us in building the ENDOR spectrometer by Mr. R. A. Isaacson of the Feher group. We are grateful to Professor Y. P. Myer of the Chemistry Department at SUNY/Albany for the use of lab space for our chemical preparations and for valuable discussions on heme chemistry. C.P.S. acknowledges support for this work from the following grants: N.I.H. Grant No. AM-17884, N.I.H. Institutional Grant No. H401C, and a SUNY Research Foundation grant.

References and Notes

(1) (a) C. P. Scholes, R. A. Isaacson, and G. Feher, *Biochim. Biophys. Acta*, **263**, 448–452 (1972); (b) G. Feher, R. A. Isaacson, C. P. Scholes, and R. L. Nagel, *Ann. N.Y. Acad. Sci.*, **222**, 86–101 (1973); (c) C. P. Scholes, R. A. Isaacson, T. Yonetani, and G. Feher, *Biochim. Biophys. Acta*, **322**, 457–462 (1973).

(2) G. C. Brackett, P. L. Richards, and W. S. Caughey, *J. Chem. Phys.*, **54**, 4383–4401 (1971).

(3) T. H. Moss, A. J. Bearden, and W. S. Caughey, *J. Chem. Phys.*, **51**, 2624–2631 (1969).

(4) W. S. Caughey and L. F. Johnson, *J. Chem. Soc. D*, 1362–1363 (1969).

(5) W. S. Caughey, H. Eberspaecher, W. H. Fuchsman, and S. McCoy, *Ann. N.Y. Acad. Sci.*, **153**, 722–737 (1968).

(6) J. E. Falk, "Porphyrins and Metalloporphyrins", Elsevier, Amsterdam, 1964, pp 118 and 119.

(7) Reference 6, p 125.

(8) (a) J. O. Alben, W. H. Fuchsman, C. A. Beaudreau, and W. S. Caughey, *Biochemistry*, **7**, 624–635 (1968); (b) N. Sadasivan, H. I. Eberspaecher, W. H. Fuchsman, and W. S. Caughey, *ibid.*, **8**, 534–541 (1969).

(9) W. S. Caughey, J. L. York, and P. K. Iber, "Magnetic Resonance in Biological Systems", A. Ehrenberg et al., Ed., Pergamon Press, Oxford, 1967, pp 25–34.

(10) D. A. Doughty and C. W. Dwiggins, *J. Phys. Chem.*, **73**, 423–426 (1969).

(11) R. T. Ross, *J. Chem. Phys.*, **42**, 3919–3922 (1965).

(12) ENDOR from the nitrogens of protohemin chloride (non-ester) dissolved in 1:1 DMF–methanol has previously been reported in ref 1a. In that work it was useful to have information on ENDOR from a simple heme with no histidine so that nitrogen resonances in metmyoglobin could properly be assigned to heme or to histidine. The exact identity of the heme axial ligand, so long as it was not nitrogen, was not critical. From the absence of chloride hyperfine interactions in the ENDOR spectrum of hemin in ref 1a, it appears that the chloride may have been replaced. Subsequent EPR and ENDOR work in our lab has given evidence for replacement of halide ligand in frozen DMF solutions.

(13) H. L. Van Camp, C. P. Scholes, and R. A. Isaacson, *Rev. Sci. Instrum.*, **47**, 516–517 (1976).

(14) P. S. Clezy and D. B. Morell, *Biochim. Biophys. Acta*, **71**, 165–171 (1963).

(15) G. Lang, T. Asakura, and T. Yonetani, *Phys. Rev. Lett.*, **24**, 981–983 (1970).

(16) Recent studies reported in the following references have indicated that THF, as well as DMF, can act as a very weak ligand for *ferrous* heme. D. Brault and M. Rougee, *Biochemistry*, **13**, 4591–4597 (1974); 4598–4602 (1974).

(17) Tetrabutylammonium halide was used in NMR studies of axial ligand exchange of hemins to provide an excess of exchangeable halide in organic solvent; G. N. La Mar, *J. Am. Chem. Soc.*, **95**, 1662–1663 (1973).

(18) Since ENDOR signals are typically only 1% of the EPR signals from which they are derived, it is important for reasons of good ENDOR signal to noise to have as large an EPR signal as possible.

(19) J. Peisach, W. E. Blumberg, S. Ogawa, E. A. Rachmilewitz, and R. Oltzik, *J. Biol. Chem.*, **246**, 3342–3355 (1971).

(20) (a) J. S. Hyde, "Magnetic Resonance in Biological Systems", A. Ehrenberg et al., Ed., Pergamon Press, Oxford, 1967, pp 63–84; (b) G. H. Rist and J. S. Hyde, *J. Chem. Phys.*, **49**, 2449–2451 (1968); **52**, 4633–4643 (1970).

(21) (a) J. E. Bennett, J. F. Gibson, and D. J. E. Ingram, *Proc. R. Soc. London, Ser. A*, **240**, 67–82 (1957); (b) J. E. Bennett, J. F. Gibson, D. J. E. Ingram, T. M. Haughton, G. A. Kerkut, and K. A. Munday, *ibid.*, **262**, 395–408 (1961); (c) G. A. Helcke, D. J. E. Ingram, and E. F. Slade, *Proc. R. Soc. London, Ser. B*, **169**, 275–288 (1968).

(22) For optimum orientation and best resolution of ENDOR lines we normally set the magnetic field at about 5 G to the high-field side of the $g_e^{\perp}$ peak maximum. For the bromide complex this meant sitting 5 G to the high-field

side of the highest field peak.

(23) (a) We determined that a similar shift of the nitrogen pattern occurred when we used our protohemin compounds dissolved in THF–chloroform solvent but with *no* tetrabutylammonium halide present. In the absence of $[Bu_4N]^+Br^-$ a magnetic hyperfine constant $|A_{zz}|$ of 7.34 MHz was obtained for the protohemin bromide. This value is slightly larger than the average value quoted in Table I for samples with $[Bu_4N]^+Br^-$ present, but the value is almost within experimental error of that average value. In the absence of $[Bu_4N]^+Cl^-$ a magnetic hyperfine coupling of 7.51 MHz was measured for the protohemin chloride; this value is slightly smaller than the average value quoted in Table I but is again within experimental error of it. Quadrupole couplings showed no change from the numbers in Table I. (b) Control experiments were done using the THF–chloroform solvent system with tetrabutylammonium halide but with washed, freshly distilled, *ethanol-free* chloroform. These controls showed no change from the tabulated ENDOR and EPR parameters.

(24) A. Abragam and B. Bleaney, "Electron Paramagnetic Resonance of Transition Metal Ions", Clarendon Press, Oxford, 1970, Chapter 4.

(25) C. P. Scholes, R. A. Isaacson, and G. Feher, *Biochim. Biophys. Acta*, **244**, 206–210 (1971).

(26) We have omitted terms such as $\eta(I_x^2 - I_y^2)$ which could contribute to second-order ENDOR frequency shifts. The upshot of such terms is that ENDOR lines would no longer be separated by twice the known Zeeman energy.

(27) D. F. Koenig, *Acta. Crystallogr.*, **18**, 663–673 (1965).

(28) For ^{35}Cl and ^{37}Cl the respective values of the nuclear magnetic moments in nuclear magnetons are: 0.820 91 and 0.668 33. The corresponding values for the nuclear quadrupole moments in barns are -7.89×10^{-2} and -6.21×10^{-2}. "Handbook of Chemistry and Physics", 55th ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1974, p E-69.

(29) The Zeeman splitting of these two lines agrees well within experimental error with the expected Zeeman splitting for ^{35}Cl of 2.7 MHz at the field used.

(30) For ^{35}Cl $g_n\beta_nH$ was 1.35 MHz for the magnetic field used in conjunction with the cavity which resonated near 9.0 GHz; while $g_n\beta_nH$ was 1.40 MHz for the magnetic field which was used in conjunction with the cavity which resonated near 9.4 GHz.

(31) A nonaxial term such as $P_{zz}(I_xI_z + I_zI_x)\cos\psi\sin\psi$ (see formula 3.61, Chapter 3, ref 24) would contribute to these second order ENDOR frequency shifts.

(32) There should be a ^{37}Cl peak at about 14 MHz; such a peak is predicted to occur at twice the ^{37}Cl nuclear Zeeman energy above the 11.7-MHz peak. The expected peak may lie close enough to the bulk free proton frequency (13.7 MHz in Figure 3a) to suffer interference from protons and to undergo spin cross relaxation with the bulk protons. Such an ENDOR-diminishing cross relaxation with the bulk protons has been seen with the ^{57}Fe ENDOR in metmyoglobin.[1c] When we were using the higher frequency (9.4 GHz) cavity we also saw that the ^{35}Cl line at 14.7 MHz was reduced. With this cavity the proton frequency lies at 14.3 MHz and is close enough to the ^{35}Cl line at 14.7 MHz to cause interference and loss of ENDOR intensity.

Structure of Lithium Dimethylcuprate and Mechanism of the Corey–Posner Reaction

Ralph G. Pearson* and Carl D. Gregory

Contribution from the Chemistry Department, Northwestern University, Evanston, Illinois 60201. *Received September 11, 1975*

Abstract: The structure of lithium dimethylcuprate has been investigated using vapor pressure depression, ^{1}H NMR, and solution x-ray scattering methods. It is found to exist as a dimer in ether solution $[LiCu(CH_3)_2]_2$ with a cyclic structure having approximate D_{2h} symmetry. The kinetics of reaction of this reagent with methyl iodide have been investigated. The reaction appears to involve slow oxidative addition to copper, followed by rapid reductive elimination. From the results of other workers, the oxidative addition step is a nucleophilic substitution and the reductive elimination is concerted. The role of the dimeric structure in facilitating these steps is discussed.

The reaction of lithium diorganocuprates with alkyl halides

$$LiCuR_2 + R'X \rightarrow R\text{-}R' + LiX + CuR \qquad (1)$$

to form carbon-carbon bonds (the Corey–Posner reaction[1]) has been extensively investigated. These studies have been largely concerned with the products of the reaction, from a synthetic point of view.[2-4] Thus, the mechanism of the reaction is still relatively unknown. This is due in large part to the extreme instability of the diorganocuprates, and the consequent difficulty in determining their structures. Without knowledge of the structure of the reagent in reaction 1 it is difficult to postulate any convincing mechanism for the reaction.

This paper considers the structural and mechanistic problem for the case of lithium dimethylcuprate. The most likely structure for this complex has now been determined. Some new kinetic evidence is also presented, and a mechanism is suggested which is consistent with structural, kinetic, and synthetic results.

Experimental Section

Since organocopper reagents are extremely air and water sensitive, all procedures involving them were carried out in an atmosphere of dry, prepurified nitrogen. Standard benchtop techniques[5] employing Schlenk tubes, fritted funnels, side-arm flasks, and syringes with valves were used for synthesis, storage, and kinetics. Nmr tubes with rubber septum caps (Wilmad Glass Co.) were also used. Most of the other copper(I) compounds were also air sensitive, to a lesser degree, and these were handled similarly.

Organic reagents were obtained from Aldrich Chemical Co. (reagent grade) or Eastman Organic Chemicals (White Label Grade) and were used as obtained unless otherwise indicated. Inorganic reagents were from Mallinckrodt or Baker and Adamson, and were analytical reagent grade throughout.

Except as noted, ^{1}H NMR spectra were obtained on a Varian T-60, ir spectra on a Perkin-Elmer 337, and uv-visible spectra on a Cary 14. Low temperature pmr spectra were obtained using a Perkin-Elmer R20B.

Diethyl ether was refluxed over CaH_2 in a nitrogen atmosphere, then distilled and stored under nitrogen.

Methyl iodide for kinetic studies was washed with aqueous sodium bisulfite and dried over P_2O_5 prior to distillation, then stored under nitrogen in the dark.

(2-Toluenesulfonyloxy)propionic acid ethyl ester was prepared according to Kenyon et al.[6] and distilled under vacuum.

Methyllithium was obtained as an ethereal solution from several suppliers: Foote Mineral Co., Apache Chemical Co., and Matheson Coleman and Bell. Concentrations were determined by hydrolysis, followed by titration, and were typically 1.5–1.6 M. Various samples contained different amounts of halide ion contamination of the order of 5% or less. Some samples were slightly discolored. However, all samples gave the same ^{1}H NMR spectrum and the reproducibility of both kinetic runs and syntheses did not depend on the sample used, or the supplier. Solutions appearing to be most free of contaminants were used for vapor pressure and x-ray studies. The reagent was stored under dry nitrogen.