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Proton Magnetic Resonance Study of High- and Low-Spin Hemin Derivatives*

Robert J. Kurland,† Robert G. Little,‡ Donald G. Davis,§ and Chien Ho||

ABSTRACT: Proton magnetic resonance spectra of several high-spin and low-spin hemin derivatives in deuterated dimethyl sulfoxide (Me_2SO-d_6) solution have been measured: (chloroprotoporphyrin IX)iron(III), (hemin); (chloro-2,4-diethyldeuteroporphyrin IX)iron(III), (mesohemin); (chlorodeuteroporphyrin IX)iron(III), (deuterohemin) and corresponding complexes with bromide, iodide, cyanide, and imidazole as axial ligands. Both the proton magnetic resonance and optical spectra show that a mixture of the chloride and Me_2SO complexes exist in hemin–Me_2SO solution at hemin concentrations of 0.01 M or greater, whereas at concentrations of 0.01 M or less in the bromo or iodo derivatives, only the Me_2SO complex is present. For the low-spin complexes (with cyanide and imidazole as axial ligands), the relative signs of the Fermi contact shifts are consistent with a π-electron mechanism for the contact interaction. However, the shifts for the 2,4 protons in the deuterohemin–Me_2SO complex are of the same sign and magnitude (approximately) as those for the α-methylene protons at the 2,4 positions in the mesohemin–Me_2SO complex. This last result suggests that other mechanisms (in addition to a π-electron one) may be involved in the contact interaction for the high-spin Me_2SO complexes. For the low-spin complexes, the proton magnetic resonance shifts yield an approximate estimate of 10^{-2} (positive) for the π-electron spin density at each peripheral carbon of the porphyrin ring.

The use of proton magnetic resonance to give information about metal–ligand bonding in paramagnetic transition metal complexes is well established (Eaton and Phillips, 1965). An analysis of the paramagnetic shifts can, in certain simple cases, provide a measure of the distribution of unpaired electrons in the ligands (McConnell and Chesnut, 1958; McConnell and Robertson, 1958; Kurland and McGarvey, 1970).

The work reported here, which deals with the proton magnetic resonance spectra of both high-spin ($S = {}^5/_2$) and low-spin ($S = {}^1/_2$) forms of hemin derivatives, was undertaken with several objectives: first, the proton magnetic resonance spectra of the hemin derivatives should be useful as models in understanding the proton magnetic resonance spectra of myoglobin, hemoglobin, and cytochromes; second, qualitative information about the relative stabilities of various hemin[1]–ligand complexes can be deduced from the proton magnetic resonance spectra; third, the paramagnetic proton magnetic resonance shifts can be interpreted (in principle) to give information about the electronic structure

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* Contribution from the Department of Chemistry, State University of New York at Buffalo, Buffalo, New York, 14214 (R. J. K.), and from the Department of Biophysics and Microbiology and the Department of Biochemistry, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15213 (C. H.). This paper was presented in part to the 156th National Meeting of the American Chemical Society, Sept 9–13, 1968, Atlantic City, N. J., and to the 3rd International Conference on Magnetic Resonance in Biological Systems and Symposium on Non-Heme Iron Proteins, Oct 14–18, 1968, Airlie House, Warrenton, Va. Supported in part by research grants from the National Science Foundation (GB-25053 and GP-10463), the National Institutes of Health (HE-10383 and FR-00292), and the American Heart Association.

† To whom inquiries should be made.

‡ Present address: Department of Chemistry, University of California at Irvine, Irvine, Calif. 92664.

§ Present address: Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N. C. 27706.

|| John Simon Guggenheim Fellow, 1970–1971.

[1] Abbreviations used that are not listed in Biochemistry 5, 1445 (1966), are: hemin, (chloroprotoporphyrin IX)iron(III); mesohemin, (chloro-2,4-diethyldeuteroporphyrin IX)iron(III); deuterohemin, (chlorodeuteroporphyrin IX)iron(III).

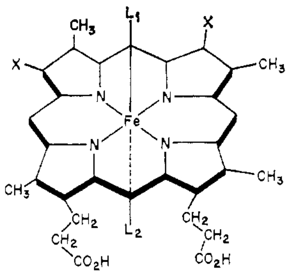

FIGURE 1: Hemin structural diagram, where L_1 and L_2 represent axial ligands and X = CH = CH_2 (hemin), X = CH_2CH_3 (mesohemin), X = H (deuterohemin).

of hemin and, thereby, the nature of the iron–porphyrin bonding.[2]

Preliminary results for several hemin derivatives have been reported previously by us (Kurland *et al.*, 1968) and by others (Kowalsky, 1963; Wüthrich *et al.*, 1969; Caughey and Johnson, 1969; Hill and Morallee, 1970). We present here a more detailed account of our proton magnetic resonance studies of hemin derivatives with some new results bearing on hemin complexes with Me_2SO. Structural diagrams for the hemin derivatives are shown in Figure 1.

Experimental Section

Materials

Chlorohemin was obtained from the Eastman Organic Chemicals as the recrystallized product (97+ %). The commercial hemin samples were used for most of the proton magnetic resonance studies. However, it was found that upon further recrystallization of the commercial sample by the procedure of Fischer (1941), there was no observable difference in the proton magnetic resonance spectra between the commercial sample and the recrystallized product. The elementary analyses for C, H, Cl, Fe, and N of the commercial sample and the recrystallized product were in excellent agreement with the theoretical values.[3]

Chloromesohemin was obtained by catalytic hydrogenation of chlorohemin in alkaline aqueous solution, which converts the vinyl groups in positions 2 and 4 to ethyl groups. This procedure is a modification of that used by Davies (1940) and the controlled hydrogenation procedure is essentially that used by Baker *et al.* (1964) in their preparation of mesoporphyrin IX from protohemin IX. The catalyst used in the hydrogenation reaction was Pt_2O obtained from the Englehard Industries, Inc. (lot no. 50). The hydrogen gas was obtained from the Air Reduction Co. The crude product was recrystallized twice by the procedure used in the recrystallization of the chlorohemin (Fischer, 1941). The elementary analyses gave percentages of C, H, and N for the chloromesohemin which were about 9% less than the theoretical values for this compound; moreover, the iron analysis for the compound was 13.85% as compared to the theoretical value of 8.51%.[3,4]

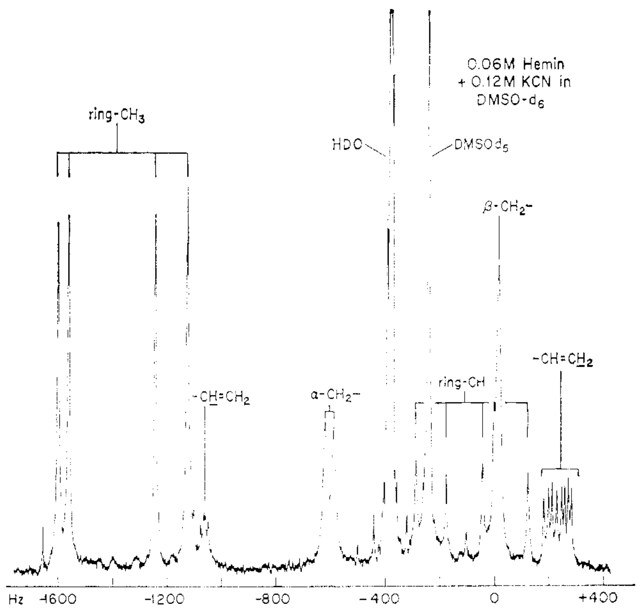

FIGURE 2: 100-MHz nuclear magnetic resonance spectrum of 0.06 M hemin in Me_2SO-d_6 with *ca.* 0.12 M KCN added. Scale is in hertz from Me_4Si.

Nevertheless, the ratio of C to H was in good agreement with the theoretical value for this ratio. An emission spectroscopic analysis of the chloromesohemin was carried out, and it was found that there was about 5% Pt present in the sample.[5] The visible spectrum (over the region 500–600 nm) for the mesoporphyrin iron(II) cyanide was the same as that obtained by Davies (1940).

Chlorodeuterohemin was obtained by the reaction of chlorohemin with resorcinol (Fisher Certified reagent) which converts the vinyl groups in positions 2 and 4 to hydrogens, according to the procedure of Falk (1964). The crude product was recrystallized by the procedure used to recrystallize chlorohemin (Fischer, 1941). The elementary analyses of C, H, Cl, Fe, and N for this compound are in excellent agreement with those of the theoretical values. The visible spectra of deuterohemin in ether and in pyridine are also in excellent agreement with those reported by Falk (1964).

Bromohemin was prepared from chlorohemin by a modification of the recrystallization procedure used for chlorohemin (Fischer, 1941), with bromide compounds being used rather than chloride.

Imidazole was obtained from Sigma as the fluorimetric grade. Me_2SO-d_6 was purchased either from Merck, Sharp and Dohme of Canada or from Nuclear Magnetic Resonance Specialities. Me_4Si was purchased from Nuclear Magnetic Resonance Specialities.

All other chemicals were obtained as the best available grade from the major suppliers and were used without further purification.

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[2] Zerner *et al.* (1966) have given an extensive review of theoretical calculations on porphyrins and underscore, in their work, the lack of experimental information available to test the accuracy of such calculations.

[3] The elementary analyses were carried out by Dr. S. M. Nagy of the Massachusetts Institute of Technology.

[4] It should be mentioned that the elementary analysis of the iron content is obtained from the residue weight at the end. Hence, it could contain any nonvolatile material that is present in the sample.

[5] The emission spectroscopic analysis was carried out by Dr. E. S. Hodge of the Mellon Institute, Pittsburgh, Pa. Although this analytical result might suggest that a platinum–porphyrin complex could be present, evidently this was not the case, at least in the solution samples: inasmuch as the platinum–porphyrin complex would be diamagnetic, its proton magnetic resonance lines would lie in the range of chemical shifts 0–10 ppm and would be easily distinguishable by virtue of line widths and shifts from those of the paramagnetic iron(III) porphyrin; however, lines which would correspond to the platinum porphyrin complex were not observed.

TABLE I: Line Positions and Assignments in Low-Spin Paramagnetic Hemin Derivatives.[a]

Compound	Ring CH$_3$	α-CH$_2$	β-CH$_2$	Ring H	X[b]
0.06 M Hemin + 0.12 M KCN in Me$_2$SO-d_6	−15.92	−6.18	+0.18	−2.88	−11.22, −10.60[c]
	−15.54	−5.86		−1.76	
	−12.38			−0.42	+1.89, +2.17[d]
	−11.22			+1.24	+2.52, +2.78[d]
0.06 M Deuterohemin + 0.24 M KCN in Me$_2$SO-d_6	−17.36	−6.92	+0.58	−1.38	+26.2[e]
	−15.93	−5.78		−1.14	+25.9[e]
	−13.80			−0.56	
	−11.72			+0.12	
0.06 M Mesohemin + 0.12 M KCN in Me$_2$SO-d_6[f]	−14.43 (9)	−5.98 (8)	+0.15 (4)	−1.98 (1)	+0.46 (3)
	−14.09 (3)			−0.82 (1)	+0.54 (3)
				−0.72 (1)	
				−0.51 (1)	
0.015 M Hemin + 0.03 M imidazole in Me$_2$SO-d_6[g]	−19.2	−5.6	−0.3	−1.1	−12.6[c]
	−19.0			−1.8	+2.2, +2.6[d]
	−14.90				
	−12.60			g	+3.6, +4.1[d]

[a] Shifts measured in ppm relative to Me$_4$Si (at 100 and 60 MHz and at an ambient temperature of 27°); (−) and (+) denotes downfield and upfield shifts, respectively; precision, except where noted, ±0.02 ppm. [b] Substituents in 2,4 positions; hemin: X = CH = CH$_2$; mesohemin: X = CH$_2$CH$_3$; deuterohemin: X = H. [c] C*H*=CH$_2$. [d] CH=C*H*$_2$. [e] 2,4 H. [f] Number in parentheses denotes relative intensities. [g] Precision, ±0.1 ppm; two of the ring H lines masked by solvent lines.

Methods

The proton magnetic resonance spectra were taken on a Varian HA-100- or on an HA-60-type nuclear magnetic resonance spectrometer, except where otherwise noted. Because of the limited-sweep range (−20 to +10 ppm) of these instruments (when operated in the field-frequency control mode) only the low-spin spectra could be recorded without instrumental modification.[6]

Optical spectra of the chloro- and bromohemin samples were taken as 10^{-2}–10^{-4} M in Me$_2$SO on a Cary-14 recording spectrophotometer.

Results

In Figures 2–4 are depicted the proton magnetic resonance spectra of the low-spin cyanohemin derivatives taken at 100 MHz; line positions span the range +2600 to −1800 Hz from Me$_4$Si at 27°. Samples were taken as 0.06 M of the free-acid form of the hemin in Me$_2$SO-d_6, with stoichiometric multiples of KCN added. The assignments for the various groups of these three cyanohemin derivatives are given in the figures and Table I. The line positions and assignments have been made on the basis of relative intensity considerations, except as noted below. The vinyl proton peaks may be assigned on the basis of their characteristic proton–proton spin-coupling pattern.[7] Since the lines corresponding to the α and β positions cannot be distinguished from intensity considerations alone, other criteria, which are discussed below, have been used. It may be noted that cyanide concentration in excess of those reported here gave no apparent change in the spectra.

The 100-MHz proton magnetic resonance spectrum of the low-spin complex of hemin with imidazole is shown in Figure 5. The sample was taken as 0.015 M hemin–0.03 M imidazole solution in Me$_2$SO-d_6 at 27°. Several observations, not evident from Figure 5 itself, should be noted. First, lines

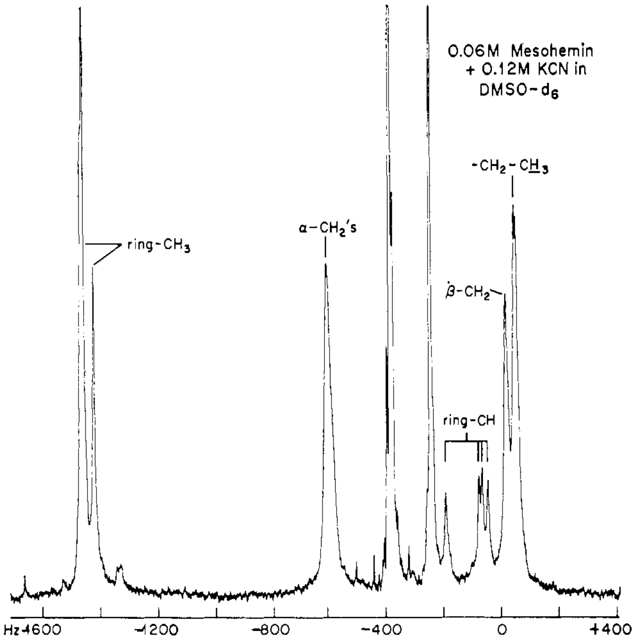

FIGURE 3: 100-MHz nuclear magnetic resonance spectrum of 0.06 M mesohemin in Me$_2$SO-d_6 with *ca.* 0.12 M KCN added. Scale is in hertz from Me$_4$Si.

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[6] R. J. Kurland and D. Wisnosky, among others, have modified the Varian spectrometer to give the required large frequency sweep ranges and sweep offsets in the field-frequency controlled mode. The details of this modification will be described elsewhere.

[7] Approximate values for J_{cis} (= 11.0 Hz) and J_{trans} (= 17.5 Hz) derived from the splitting pattern are in the range found for other vinyl substituents (Bothner-By, 1965).

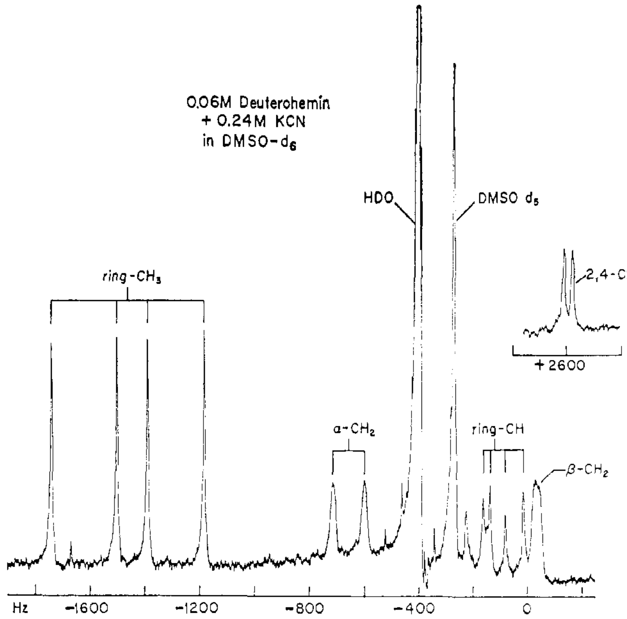

FIGURE 4: 100-MHz nuclear magnetic resonance spectrum of 0.06 M deuterohemin in Me_2SO-d_6 with *ca.* 0.24 M KCN added. Scale is in hertz from Me_4Si.

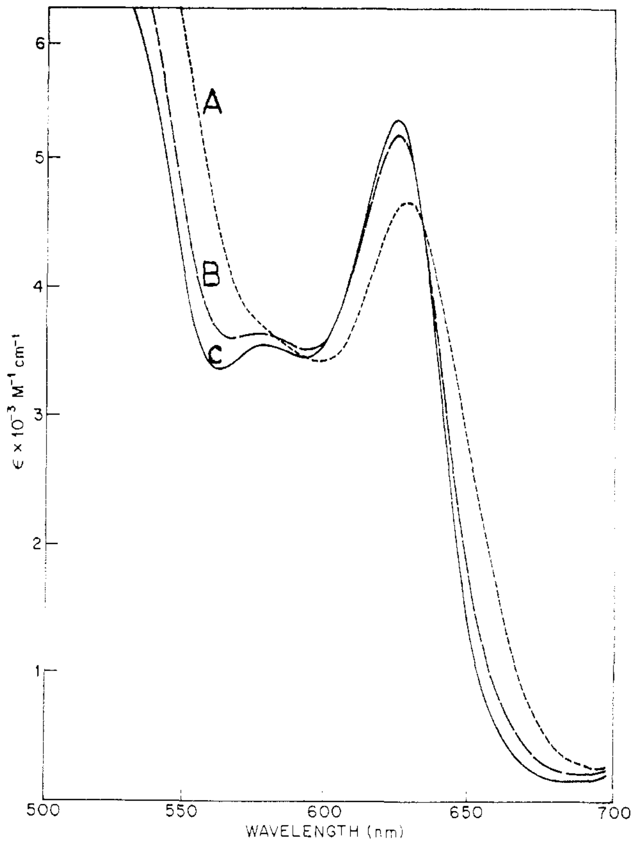

FIGURE 6: The absorption spectra of chlorohemin in Me_2SO as a function of concentration: (A) 3.06×10^{-2} M, (B) 3.06×10^{-3} M, and (C) 3.06×10^{-4} M. The spectrum of a 2.66×10^{-4} M bromohemin solution is identical with part C.

corresponding to the high-spin form (chlorohemin in Me_2SO) remained, although diminished in intensity after an excess of imidazole was added. Secondly, as additional imidazole is added, the broad doublet at *ca.* 800 Hz from Me_4Si grows in intensity, but the intensity of the other lines remain essentially constant. Lastly, it should be noted that the lines of the hemin complex with imidazole are considerably broader than those of the cyanide complex or of imidazole in a solution of the cyanide complex, which may indicate that exchange with hemin uncomplexed to imidazole is faster than that for the cyanide complex. A tentative assignment of lines to positions of the heme group has been made on the basis of relative intensities and by analogy with the scheme for the cyanide complex; these assignments are given in Table I.

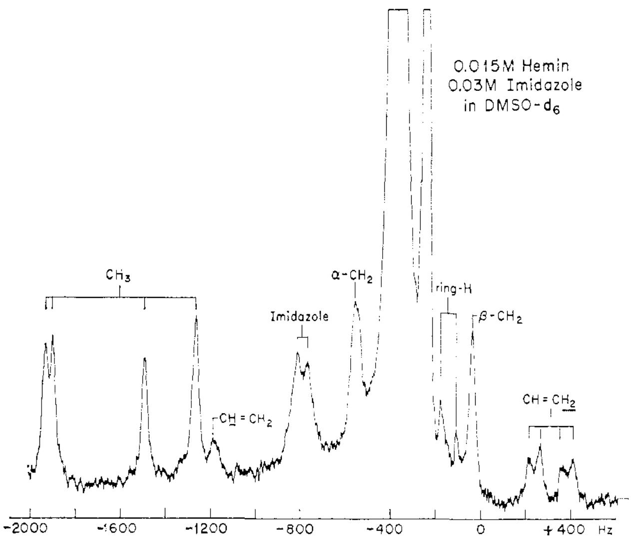

FIGURE 5: 100-MHz nuclear magnetic resonance spectrum of 0.015 M hemin in Me_2SO-d_6 with *ca.* 0.03 M imidazole added. Scale is in hertz from Me_4Si.

Optical spectra of chlorohemin and bromohemin in Me_2SO show that Me_2SO replaces the halide ion as a ligand, in accord with earlier spectroscopic and conductivity measurements (Brown and Lantzke, 1969). Figure 6 shows the effect of dilution in going from a Me_2SO solution 3.06×10^{-2} M in chlorohemin to 3.06×10^{-4} M. At a 3.06×10^{-4} M chlorohemin concentration, the absorption spectrum is virtually identical with that of a 2.66×10^{-4} M bromohemin solution. In contrast, dilution of a similar set of bromohemin solutions gives no shifts in the positions of the absorption maxima and only a slight decrease in the overall extinction coefficients. The greater stability of the chloride complex in Me_2SO compared to the bromide is also demonstrated in Figure 7, where it can be seen that addition of 3.24×10^{-2} M bromide ion to a 1.33×10^{-4} M bromohemin solution gives no observable change in the spectrum, although addition of 3.24×10^{-2} M chloride ion gives large shifts in the 450–700-nm region. The effect of dilution in the chlorohemin solution (or, equivalently, the more concentrated bromohemin solution) could also be reversed by the addition of bromide ion; however, bromide ion concentrations much higher than the chloride were required to give similar shifts.

The proton magnetic resonance spectra of the halide hemin derivatives give additional evidence for the presence of the equilibria demonstrated by the optical spectra. Fine structure in the proton magnetic resonance spectrum of chlorohemin disappears when a large excess of LiCl is added to the Me_2SO

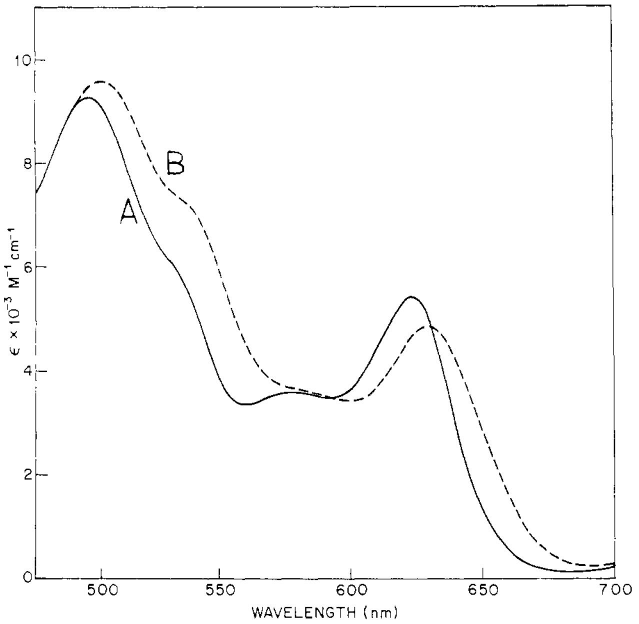

FIGURE 7: The absorption spectra of Me_2SO solutions 1.33×10^{-4} M bromohemin which have been made 3.24×10^{-2} M in added tetraethylammonium bromide (A), and 3.24×10^{-2} M in added tetramethylammonium chloride (B), respectively. The absorption spectrum of a 1.33×10^{-4} M bromohemin solution without added bromide is identical with part A.

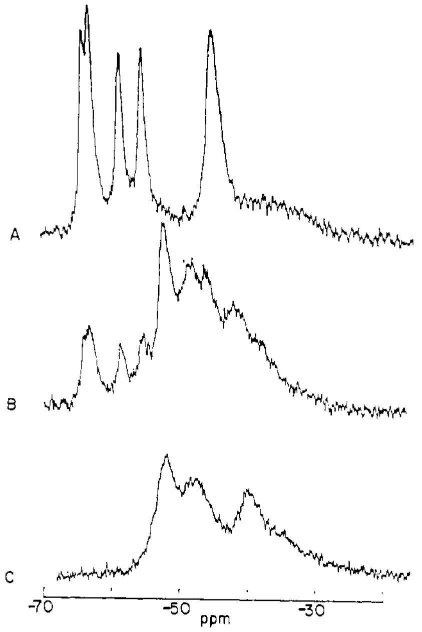

FIGURE 8: Low-field portion of the 100-MHz nuclear magnetic resonance spectra of hemin with varying amounts of Cl^- in Me_2SO-d_6; (A) 0.06 M hemin with 0.12 M $AgNO_3$ added, (B) 0.06 M hemin in Me_2SO-d_6, and (C) 0.03 M hemin in a LiCl-saturated solution of Me_2SO-d_6.

solution (Figure 8). When an excess of $AgClO_4$ or $AgNO_3$ is added to a solution of chlorohemin (taken as the free acid in Me_2SO-d_6), the spectrum becomes essentially identical with those of bromohemin or iodohemin dissolved in Me_2SO (Figure 8); chloride ion is precipitated as AgCl. The spectrum of chlorohemin, taken as *ca.* 10^{-2}–10^{-1} M of the free acid in Me_2SO-d_6 at 27°, is evidently the superposition of spectra corresponding to hemin with coordinated chloride (*i.e.*, corresponding to an excess of added chloride ion) and that for the Me_2SO complex with hemin (*i.e.*, that for the bromohemin solution or equivalently, chlorohemin solution with added silver ion). Furthermore, dilute chlorohemin (10^{-3} M) in Me_2SO-d_6 gives the same proton magnetic resonance spectrum as that of 10^{-2} M hemin with $AgNO_3$ added. The precise nature of the species present with the excess of Cl^- ion is not known; there may be aggregates of the type hemin–Cl–hemin present. However, the general spectral pattern is similar to that observed for high-spin deuterohemin dimethyl ester in $CDCl_3$ (Caughey and Johnson, 1969) which is, presumably, a monomeric species.

An approximate lower limit for the average exchange lifetime of chlorohemin and the Me_2SO complex of hemin can be obtained from the minimum separation of corresponding lines (*ca.* 500 Hz at 100 MHz); this estimate is *ca.* 10^{-3} sec at 27°. Moreover, the fine structure in the 27° spectrum of chlorohemin in Me_2SO (Figure 9) disappears as the sample is heated to 40°, and above: at *ca.* 70°, the spectra of chlorohemin dissolved in Me_2SO and chlorohemin with a large excess of chloride ion present are essentially the same. This behavior is to be contrasted with the temperature dependence of the hemin–Me_2SO complex, as exhibited by the bromohemin solution: the form of the spectra of the Me_2SO complex remains the same, even though the lines shift, as the temperature is raised to 90°. The quantitative interpretation of the temperature variation for the superimposed spectra is difficult since the shifts, the equilibria, and the exchange rates involved are all temperature dependent. Nevertheless, one may argue qualitatively that the stability constant for the chloride coordination to hemin increases

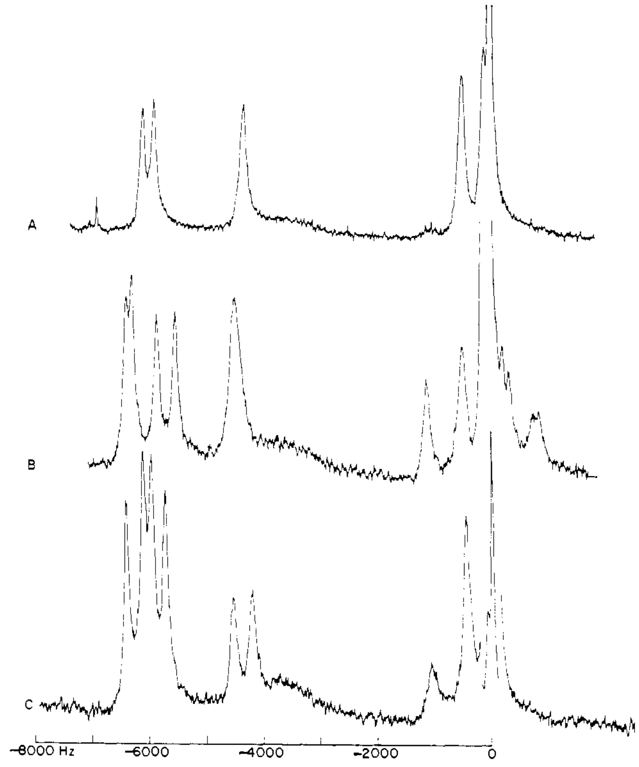

FIGURE 9: 100-MHz nuclear magnetic resonance spectra of 0.06 M high-spin hemin derivatives in Me_2SO. (A) 0.06 M mesohemin chloride with *ca.* 0.12 M $AgNO_2$ added, (B) 0.06 M hemin bromide, and (C) 0.06 M deuterohemin bromide. Scale is in hertz from Me_2SO-d_6.

TABLE II: Line Positions and Assignments in High-Spin Paramagnetic Hemin Derivatives.[a]

Compound	Ring CH_3	α-CH_2	β-CH_2	Ring H[b]	X[c]
0.06 M Bromohemin in Me_2SO-d_6[d]	−66.7	−47.5	−8.0	−40	−1.1[e]
(Me_2SO complex)	−65.9				+0.1
	−62.4				+4.7
	−60.1				+5.9
0.06 M Bromodeuterohemin in	−67.2	−48.2	−7.0	−40	−63[g]
Me_2SO-d_6[f] (Me_2SO complex)	−64.1	−45.3			
	−62.7				
	−60.5				
0.06 M Bromomesohemin in	−65.0	−45.5[i]	−6.4	−40	*i*
Me_2SO-d_6[h] (Me_2SO complex)	−62.9				
0.06 M Hemin in Me_2SO-d_6[j]	−55	−43		*l*	
	−51				
0.06 M Deuterohemin in Me_2SO-d_6[k]	−53	−43			−72
0.06 M Mesohemin in Me_2SO-d_6[k]	−52	−43			

[a] Shifts measured in ppm relative to Me_4Si (at 100 and 60 MHz and at an ambient temperature of 32°); (−) and (+) denote downfield and upfield shifts, respectively; precision, except where noted, *ca.* ± 0.2 ppm. [b] Precision, ±2 ppm. [c] Substituents in 2,4 positions; hemin: X = CH = CH_2; mesohemin: X = CH_2CH_3; deuterohemin: X = H. [d] Positions same as for 0.06 M hemin in Me_2SO-d_6 + $AgClO_4$ and 0.06 M iodo in hemin in Me_2SO-d_6. [e] C*H* = CH_2 and CH = CH_2 positions cannot be distinguished. [f] Positions same as for 0.06 M deuterohemin in Me_2SO-d_6 + $AgClO_4$ and 0.06 M iododeuterohemin in Me_2SO-d_6. [g] Precision, ±2 ppm; line masked by ring-CH_3 lines. [h] Positions same as for 0.06 M hemin in Me_2SO-d_6 + $AgClO_4$. [i] CH_2 lines at 2,4 positions not distinguishable from α-CH_2 lines. [j] Line positions determined from spectra of 0.06 M hemin in Me_2SO-d_6 + LiCl and from comparison of spectra of bromohemin in Me_2SO-d_6 and hemin in Me_2SO-d_6; precision, ±2 ppm. [k] Line positions determined from comparison of spectra of bromohemin derivative in Me_2SO-d_6 and hemin derivative in Me_2SO-d_6. [l] A broad and weak resonance at *ca.* +50 ppm. A similar high-field resonance of the ring protons in deuterohemin ester in CCl_4 has been observed by Caughey and Johnson (1969).

with temperature and that the rate of chloride exchange between hemin coordinated and uncoordinated to chloride increases with temperature. Thus, at temperature above *ca.* 60° the spectra evidently correspond to the fast exchange limit, *i.e.*, to the weighted average of the chlorohemin complex and the Me_2SO complex, with the proportion of chlorohemin increasing as the temperature is raised.

Assignments of line positions for the several high-spin hemin derivatives are given in Table II. These assignments have been made, with the exceptions noted below, on the basis of relative intensities and by comparison of the spectra of the several derivatives. The line at *ca.* −10 ppm from Me_4Si can be attributed to protons in the carboxylic acid group of the propionic acid side chain on the basis of the following evidence: addition of a small amount of H_2O to the bromohemin solution causes this line to disappear (due to rapid exchange of the carboxylic proton with water protons); the line also disappears when $AgClO_4$ (which has a small amount of water present) is added to the chlorohemin solution. The considerations used in the assignment of the 2,4 proton line of deuterohemin–Me_4SO deserve special comment. This line was masked by the methyl group lines in the 100-MHz spectrum (Figure 9c), but its presence is indicated by the unequal relative intensities of the four methyl peaks. Moreover, these relative intensities change as the temperature is raised: at 90°, the lowest field methyl line becomes the most intense. In addition, a spectrum at 250 MHz at 28°, reveals the 2,4 proton line as a shoulder on a methyl peak (Figure 10).[8] Finally, we note that a search under conditions sufficiently sensitive to find the 2,4 proton line of deuterohemin dimethyl ester (Caughey and Johnson, 1969) did not reveal lines (other than those indicated in the assignments, Table II) within ±100 ppm from the Me_2SO-d_6 peak.

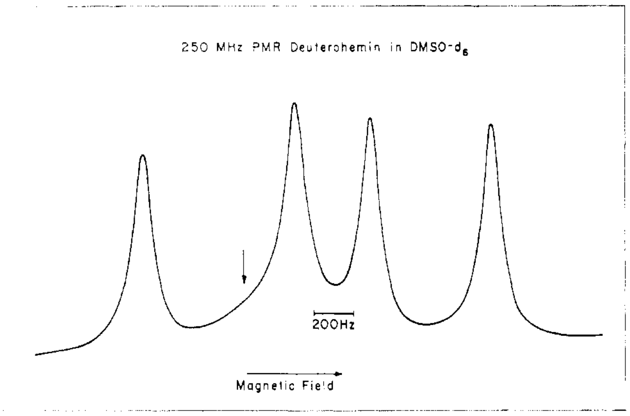

FIGURE 10: 250-MHz nuclear magnetic resonance spectrum of 0.06 M bromodeuterohemin in Me_2SO-d_6 at 28°. Scale is in hertz. Only a portion of the spectrum is shown.

Assignments for the chlorohemin derivatives can be made either directly from the spectra of the solution samples to which excess chloride ion has been added, or indirectly, by

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[8] For detailed information on the principles of the construction and operation of the 250-MHz nuclear magnetic resonance spectrometer, refer to: A. A. Bothner-By, J. Dadok, R. Sprecher, and T. Link, the Proceedings of the Fourth International Conference on Magnetic Resonance in Biological Systems, St. Catherine's College, Oxford, England, Aug 26 to Sept 1, 1970.

comparison of spectra of the pure Me_2SO complexes (Figure 9) with those of the mixture of Me_2SO and chloro complexes (Figure 8). The assignments for the chlorohemin derivatives must, at best, be regarded as tentative, however; the nature of the species present in excess chloride has not been definitely established; moreover, for the mesohemin and deuterohemin chloro complexes, where the assignments were determined indirectly, several lines of the chloro complex were obscured by lines of the Me_2SO complex or diminished in intensity, so that they were not observed.

We have not yet been able to observe a separate line which could be assigned to Me_2SO complexed with hemin. Over the range 20–90°, the temperature variations in line width and chemical shift (with respect to an internal reference of *t*-butylbenzene) of the Me_2SO solvent peak are too small to yield accurate values for exchange lifetimes or for the chemical shift of the complexed Me_2SO line. Nevertheless, these variations suggest that the limit of slow exchange between solvent and complexed Me_2SO holds at 30°, and the fast-exchange limit at 90°;[9] moreover, at 90° an approximate upper limit for the shift of the complexed Me_2SO line from that of the solvent is −3 ppm. Inasmuch as the concentration of hemin is limited by solubility (less than *ca.* 0.1 M), it is probable that the line of the complexed Me_2SO is obscured by that of the solvent.

The temperature dependence of the methyl- and α-methylene proton lines of the cyano–hemin complexes and the high-spin Me_2SO–hemin complexes was measured over the range 20–90° at 60 MHz. We were not able to measure accurately the temperature variation of proton lines for other positions because of overlap with the solvent line (Me_2SO-d_5) or, as in the case of the methine proton lines for the high-spin complex, because of intrinsic line width and overlap with other lines in the hemin spectra. The observed temperature variations follow a Curie law dependence to within experimental error (*i.e.*, showed a linear variation with $1/T$, Figure 11). The paramagnetic proton magnetic resonance shifts for the cyanohemin derivatives, when extrapolated to infinite temperature, fall in the expected range; for the high-spin Me_2SO complexes, the infinite temperature shifts are at higher fields, by about 5–20 ppm, than would be expected for corresponding diamagnetic porphyrins (Alben *et al.*, 1968; Abraham *et al.*, 1961).

Discussion

The principal mechanism by which unpaired electrons induce isotropic nuclear resonance shifts is *via* a Fermi contact interaction. Additional contributions to the isotropic shifts may be grouped under the heading "pseudocontact."[10] We should note first that formulas customarily used (McConnell and Robertson, 1958) to calculate the contact and pseudocontact contributions to the isotropic proton magnetic resonance shifts do not strictly apply to the hemin derivatives.[11]

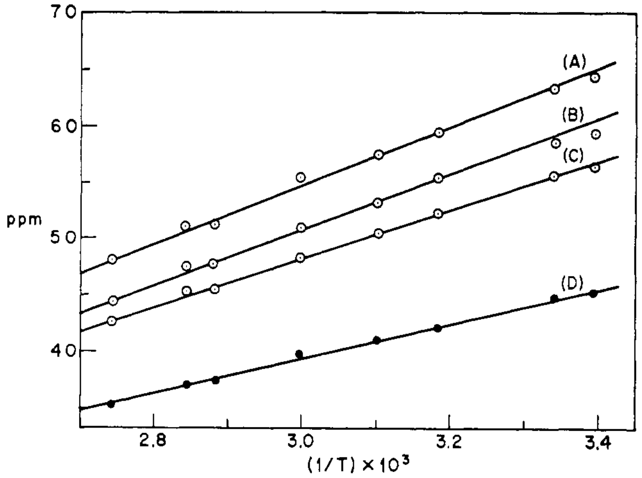

FIGURE 11: Temperature variation of hemin–Me_2SO proton magnetic resonance shifts. (A, B, C) Methyl groups; (D) α-methylene group.

For the high-spin hemin derivatives, the Fermi contact and pseudocontact shifts can be given in terms of the susceptibility components (Kurland and McGarvey, 1970) as shown in eq 1a and 1b. In eq 1a, ΔH_f is the Fermi contact shift at an

$$(\Delta H_f/H_0) = -A\bar{\chi}/\beta g_e(\gamma_n/2\pi) \qquad (1a)$$

$$(\Delta H_D/H_0) = -1/(2R^3)(\chi_{zz} - \bar{\chi})(3\cos^2\Omega - 1) \qquad (1b)$$

applied field H_0, A is the Fermi contact term (in hertz), g_e the free-electron g factor (= 2.0023), $\bar{\chi}$ is the rotationally averaged susceptibility (in erg $(G)^{-2}$ $(\text{molecule})^{-1}$), β is the Bohr magneton (in erg $(G)^{-1}$), and γ_n is the gyromagnetic ratio (in radians $(\sec)^{-1}$ $(G)^{-1}$) of the nucleus. In eq 1b, ΔH_D is the pesudocontact shift at field H_0, R is the distance from the metal ion to the nucelus (in centimeters); χ_{zz} is the component of the susceptibility tensor along the z axis, the approximate fourfold axis of the porphyrin (perpendicular to the heme plane); Ω is the polar angle between the z axis and R, the radius vector from the metal ion to the nucleus. In deriving eq 1b, we have assumed the susceptibility tensor components along the principal axes x and y (in the heme plane) are equal.

The susceptibility tensor may be put in terms of the parameter $\delta = D/kT$ (where D is the zero-field-splitting constant, k the Boltzmann constant, and T the absolute temperature; Kotani, 1961). We do not give these formulas explicity here, but note the following behavior for the susceptibility: in the high-temperature limit ($D/kT = 0$), the susceptibility is that for an ion with spin quantum number $S = {}^5/_2$ and, accordingly, the pseudocontact shift vanishes. Moreover, the average susceptibility, $\bar{\chi}$, corresponds to this value to within a few per cent over a wide range of δ (from $\delta = 0$ to 0.2), whereas the pseudocontact term increases rapidly and nonlinearly over the same range. This latter behavior would yield a nonlinear variation of the total shift *vs.* $1/T$ if the pseudocontact term contributes significantly, although this nonlinearity

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[9] These conclusions are consistent with the results found for the stable complex $Fe(Me_2SO)_6{}^{3-}$ in Me_2SO solutions (Selbin *et al.*, 1961).

[10] In the case of proton magnetic resonance shifts, the pseudocontact contribution can be related to dipolar fields from unpaired spin localization on the central metal ion (Marshall, 1961; Kurland and McGarvey, 1970).

[11] First, for both high- and low-spin forms of hemin, the rotational reorientation rate, 2×10^9 $\sec^{-1}$ (Pitt and Smyth, 1959), is approximately 100 times less than the electron-spin Zeeman anisotropy (in frequency units) of fields at 14,000 G or greater. Accordingly, the Fermi contact and the pseudocontact contributions to the isotropic nuclear magnetic resonance shifts would be calculated as for a polycrystalline solid. Second, for the high-spin form of hemin, there are three thermally populated doublets (at zero magnetic field); the energy separations between the doublets are of the order 10–10² cm⁻¹ (Richards *et al.*, 1967). The large contribution to the isotropic nuclear magnetic resonance shift from mixing of the zero-field doublets by the magnetic field must be accounted for in the calculation of the Fermi contact shift.

TABLE III: Fermi Contact Terms for High- and Low-Spin Hemin Complexes.[a]

				2,4 positions	
Complex[b]	Ring CH_3	α-CH_2	Ring H	$-H$[c]	α-CH_2[d]
Me_2SO (high spin)	+0.2	+0.1	+0.1	+0.2	+0.1
	+0.2*	+0.1*	+0.03†	+0.1†	+0.1*
Cyanide (low spin)	+0.4	+0.08	−0.4	−1	+0.08
	+0.4*			−1*	
Imidazole	+0.5	+0.06	−0.3		

[a] Fermi contact terms, A, given in megaHertz and derived from relative shifts *via* eq 1a (text) for the high-spin Me_2SO complex and *via* eq 2 for the low-spin values; nonstarred values are derived from the shifts, at 300°K, relative to diamagnetic Fe(II) heme complex with pyridine (Alben *et al.*, 1968) and with respect to diamagnetic prophyrin bases (Abraham *et al.*, 1961). Values with an asterisk are derived from the slope of the relative shift *vs.* $1/T$; values with a dagger are calculated taking into account the assumed maximum pseudocontact shift (see text). [b] Taken from the average of shifts for a given substituent over the various derivatives and positions (other then 2,4 positions). [c] Taken from the deuterohemin derivative. [d] Taken from the mesohemin derivative.

might not be noticeable over a narrow temperature range. In order to calculate the pseudocontact shift accurately, an accurate value of D, the zero-field-splitting constant is required. Even though D for the Me_2SO–hemin complex has not been measured, from values of the zero-field splittings for several other hemin derivatives (Richards *et al.*, 1967) we can estimate that a reasonable upper limit for D is about 20 cm^{-1}. Corresponding to this value for D, an upper limit to the pseudocontact shift in the Me_2SO complex may be calculated from approximate molecular geometries for other porphyrin and heme derivatives (Hoard, 1968). At 300°K, this calculated upper limit is −22.1 ppm for the methine protons, −15.4 ppm for the 2,4 protons, and about −10 ppm for methyl or methylene protons α to the porphyrin ring. For a more likely value of D (10 cm^{-1}) the calculated pseudocontact shifts are approximately one-half those corresponding to the upper limit for D. It should be noted that the methine proton pseudocontact shift calculated for $D = 10$ cm^{-1} shows (approximately) a linear variation with $1/T$ over the temperature range $T = 300$ to 375°K; moreover, from this approximately linear plot, the pseudocontact shift extrapolated to $1/T = 0$ is *ca.* +8 ppm. It can be shown theoretically that the temperature-dependent factor in the pseudocontact term in 1b must vanish at infinite temperature. However, if the theoretical values of $\chi_{zz} - \bar{\chi}$ for likely values of D are extrapolated to $1/T = 0$ from a plot in the temperature range 300–400°, the intercepts are not zero. In fact the extrapolated values are of the same order of magnitude as those at room temperature, but of opposite sign. In principle this behavior might be used to estimate the value of D, and thus the pseudocontact shift if other factors, such as solvent effects, can be taken into account. Experiments along these lines are now in progress.

Two procedures may be used to derive the Fermi constant "A" in eq 1a: (1) it may be obtained from the difference (at some given temperature) between the shifts for the high-spin complex and the corresponding ones for a diamagnetic model compound (*e.g.*, a Fe(II) low-spin heme derivative); (2) the slope of the proton magentic resonance shift *vs.* $1/T$ may be used to give A. In Table III we list values for the Fermi constants obtained, where possible, by both of the above procedures. For the methine and 2,4 protons we also give two limiting values for A: one calculated with an assumed zero pseudocontact shift (for $D = 0$), the other with the assumed maximum value for the pseudocontact shift (*i.e.*, for $D = 20$ cm^{-1}); for the α-methyl and α-methylene protons, this maximum pseudocontact shift can be neglected, to the accuracy indicated in Table III. It is evident, in view of the uncertainty in the actual value of the pseudocontact shifts, that we have only an order of magnitude estimate for the Fermi constants.

In the case of low-spin hemin, general equations have been given (Kurland and McGarvey, 1970) which could, in principle, be used to derive the Fermi contact term.[12] We feel, in view of the uncertainties which might be involved in a purportedly accurate calculation for the Fermi constants, that the most appropriate formula to use to estimate these shifts is that in eq 2 (McConnell and Robertson, 1958), where the

$$\Delta H_F/H_0 = \frac{-g\beta S(S + 1)A}{3(\gamma_n/2\pi)kT} \tag{2}$$

isotropic g value, g is taken as 2 and the spin quantum number, S, as $^1/_2$.[13] We emphasize, however, that eq 2 can give at best only an order-of-magnitude estimate of A.

The expression for the pseudocontact shift in low-spin hemin is given in eq 3 (Kurland and McGarvey, 1970). In

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[12] Detailed application of these equations to specific cases—*e.g.*, that of trigonal Co(II) complexes (McGarvey, 1970)—have shown that in situations where there may be multiplet levels lying less than 2 or 3 kT energy units above the ground level multiplet, then the contribution of second-order Zeeman terms to the contact shift may be appreciable, even though the excited levels are populated to only a few per cent. Moreover, the effect of the orbital contribution to the magnetic moment of the complex must also be taken into account. Moreover, in order to use these general expressions one must know (1) the energies of the excited multiplets; (2) the form of the wave functions for each multiplet; (3) if, as in the case of low-spin hemin derivatives, the wave functions do not contain terms which explicitly put s-electron spin density on the nucleus in question, then one must assume some mechanism (*e.g.*, an appropriate spin polarization mechanism) to relate spin density in the various molecular orbitals to s-orbital spin density at the nucleus. Although (1) or (2) can, in principle, be deduced from values of the g-tensor components or susceptibility-tensor components, such data are not available for hemin cyanide.

[13] Equation 2 would be an appropriate approximation for low-spin hemin in the limiting case, where kT is much greater than the energy separation of the three multiplet levels, a condition which does not hold at ordinary temperatures. This high-temperature approximation is used as an attempt to account for the second-order Zeeman term.

eq 3 χ_{yy} and χ_{xx} are the principal axis components of the

$$\Delta H_D/H_0 = \frac{1}{2R^3}[(\chi_{zz} - \chi)(1-3\cos^2\Omega) + (\chi_{yy} - \chi_{xx})\sin^2\Omega\cos^2\psi] \quad (3)$$

susceptibility tensor (along x and y axes in the porphyrin plane) and ψ is the azimuthal angle between the x axis and the projection of R on the xy plane; the other symbols have the same significance as in eq 1a and 1b.[14] Since the location of the principal axes, x and y, in the heme plane, are not known, only approximate limits to the pseudocontact shifts can be calculated. Thus, the estimated pseudocontact shifts (at 306°K) for the methine protons can vary from +3.9 to −2.4 ppm; for the 2,4 protons, from +2.7 to −1.7 ppm; and for the α-methyl or α-methylene protons, from +1.8 to −1.1 ppm, depending on the value of ψ in eq 3. We note that the maximum differences in the observed shifts for the methine protons are somewhat less than the calculated maximum differences in the pseudocontact shifts, whereas the maximum differences in the observed shifts for the α-methyl or α-methylene protons are somewhat greater than those given by the spread in the pseudocontact shift.

In Table III are given the estimated contact terms, A, for the low spin species. We have taken a zero pseudocontact contribution here, inasmuch as the errors in the values of A obtained by use of eq 2 are probably greater than those due to neglect of the pseudocontact shifts. As in the high-spin case, the tabulated values represent only an order of magnitude estimate.

If the mechanism giving rise to the Fermi contact interaction is the same in the heme complexes as it is in aromatic free radicals, then the contact term, A, may be related to the density of unpaired electrons in π-type orbitals centered at the peripheral carbons of the conjugated porphyrin ring system. For protons directly bonded to these carbons (*i.e.*, for the methine protons and, in the case of deuterohemin, also for protons at the 2,4 positions), the contact term, A, is proportional to the π-electron spin density ρ_C^π at the adjacent carbon atom by the McConnell Relation (McConnell, 1956) where

$$A = Q\rho_C^\pi \quad (4)$$

Q is a proportionality constant. Theory and experiment both yield a value for Q of about −63 MHz (Weissman *et al.*, 1957; Bernal *et al.*, 1963; Berthier *et al.*, 1964).

A similar relation holds for methyl group protons adjacent to an aromatic carbon; here Q is found to be approximately +75 MHz (McLachlan, 1958). The above value for Q would be appropriate for freely rotating methyl groups. Where, as in the case of methylene protons, there may be a preferred conformation with respect to the symmetry plane of the π-electron system, the proportionality constant, Q, can be written in the form

$$Q = Q_0 + Q_2\langle\cos^2\theta\rangle \quad (5)$$

where, in eq 5, θ is the dihedral angle between the π orbital on the aromatic carbon and a C–H bond of the methylene group and $\langle\cos^2\theta\rangle$ denotes the average of $\cos^2\theta$ over the various conformations. (Note that for a freely rotating methyl group, $\langle\cos^2\theta\rangle = {}^1/_2$). Approximate values for Q_0 and Q_2 are 10 and 1.3×10^2 MHz, respectively (Carrington and McLachlan, 1967).

The appropriate assignment of chemical shifts to the α- and β-methylene protons is indicated by these considerations. We note first that an examination of molecular model indicates that the α-methylene groups are strongly hindered and that the preferred conformation is such that θ is 60° and, correspondingly, $\langle\cos^2\theta\rangle = {}^1/_4$. Thus, the contact shift for these protons should be approximately one-half that for protons on a freely rotating methyl group bonded to a carbon bearing the same π-electron spin density. Moreover, the β-methylene protons are, in this suggested preferred conformation, pointing directly into the π-electron cloud of the porphyrin ring. Accordingly, the contact shifts of these β-methylene protons might be expected to be larger than those found for the β-methylene protons undergoing free rotation.

In attempting to correlate and to interpret the above results, we note the following significant points. First, the α-methyl and methylene protons are shifted to low field for both high- and low-spin species; the shifts are scaled (very roughly) by the factor $S(S + 1)$. Second, in the low-spin cyanohemin species, the shift of the 2,4 protons (deuterohemin) is opposite in sign to that of the 2,4-methylene protons (mesohemin); however, in the high-spin Me_2SO complex, both the 2,4 protons and the 2,4-α-methylene protons are shifted to low field. Third, in the low-spin complexes the methine protons are shifted to high field, whereas in the Me_2SO complexes, they are shifted to low field.

The first result above suggests that the hyperfine interaction mechanism for the α-methyl and α-methylene proton contact shifts is essentially the same in both high- and low-spin species, and probably involves hyperconjugation (McLachlan, 1958). If this conclusion is correct, then in both high- and low-spin hemin, the ring carbons adjacent to these methyl and methylene groups each bear positive spin density (about 10^{-2} in magnitude).

The second result (and the conclusion above) suggest that some mechanism other than (or in addition to) one involving solely the π-electron system is responsible for the contact interaction of the 2,4 protons in the high-spin deuterohemin–Me_2SO complex. If a π-electron mechanism were dominant, eq 4 would predict that the shifts of the 2,4-ring protons (in deuterohemin) would be of opposite sign, but of roughly the same magnitude, as those of the 2,4-α-methylene protons (in mesohemin). This condition is met in the low-spin cyanide complexes, but not in the high-spin Me_2SO complexes.[15] The third result above (downfield shifts for high-spin methine protons, upfield shifts for the low-spin methine protons) also might make one suspect that, in the case of protons lying in

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[14] Values of the g-tensor components obtained for the cyanide derivative of horseradish peroxidase (Blumberg *et al.*, 1968)—g_{xx} = 1.20, g_{yy} = 2.1, g_{zz} = 3.05—may be used to estimate the susceptibility components, in a procedure analogous to that outlined by Weissbluth (1967).

[15] We do not see how any other set of assignments, consistent with the observed relative intensities and general pattern of shifts, can be produced which would contravene the latter anomalous result. Moreover, it cannot be argued, we feel, that the large downfield shift of the 2,4 protons in the Me_2SO–deuterohemin complex is due solely to a pseudocontact contribution. The value of the zero-field constant, D, required for such a large pseudocontact shift would be much greater than that observed for other Fe(III) heme derivatives; similar large downfield shifts for the 2,4 protons in other deuterohemin derivatives have been observed (Caughey and Johnson, 1969); for and these derivatives, the measured values of D (Richards *et al.*, 1967) do not yield an appreciable pseudocontact shift.

the porphyrin plane, the contact interaction mechanism for the low-spin complexes is different from that for the high-spin Me_2SO complexes.

In order to rationalize the anomalous relative signs of the shifts at the 2,4 position in the deuterohemin– and mesohemin–Me_2SO complexes, we would like to invoke a contact interaction mechanism which involves the σ-electron system. Evidence for such a mechanism, *via* spin delocalization in the σ-electron system, has been proposed for the contact shifts of pyridine, taken as an adduct to metal β-diketonate complexes (Garito and Wayland, 1969; Ke *et al.*, 1970). On *a priori* grounds one might expect that if a σ-electron mechanism were to be a factor, it would more likely be so for the high-spin complexes rather than for the low spin: in the low-spin complexes the unpaired spin on the central metal ion occupies essentially d_{xz} and d_{yz} orbitals, which interact with π-type orbitals of the porphyrin, rather than σ. On the other hand, in the high-spin complexes the unpaired spin is distributed in all five metal ion d orbitals and interactions with both porphyrin π- and σ-type orbitals can take place; interactions *via* the metal ion $\mathrm{d}x_2{-}y_2$ orbital and lone pair orbitals on the pyrrole nitrogens may be of particular importance here. We emphasize that the arguments above are not intended to imply that in the high-spin Me_2SO complexes all, or even a major portion, of the unpaired spin density occupies porphyrin σ-type orbitals. Nor do we propose that the π-electron system is not involved at all in the mechanism for the contact interaction of the high-spin complexes.

We remark finally that the high-spin Me_2SO complexes may be appropriate model compounds for high-spin metmyoglobin and methemoglobin, since a similar range of large downfield shifts occurs for the latters (Kurland *et al.*, 1968).[16] However, before the paramagnetic nuclear magnetic resonance shifts of the hemin–Me_2SO complexes can be given a secure interpretation in terms of an unpaired spin density distribution and then related to that for high-spin hemoproteins, a theoretical examination of the role played by the σ-electron system in the contact interaction is required. For the low-spin hemin complexes, the paramagnetic nuclear magnetic resonance shifts can be interpreted (subject to the various approximations noted above) to give a qualitative picture in which *ca.* 10^{-2} positive spin density is located on each peripheral carbons of the porphyrin ring (*i.e.*, on carbons adjacent to the α-methyl or methylene groups, and on the methine carbons). Any more quantitative assay of spin densities at the various positions is unwarranted at this time, in view of uncertainties in the values for and orientation of the *g* tensor.

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[16] On other grounds, the hemin–Me_2SO system has been proposed as a useful model for the study of hemoproteins (Brown and Lantzke, 1969).