

The Effect of Magnesium Coordination on the ^{13}C and ^{15}N Magnetic Resonance Spectra of Chlorophyll a. The Relative Energies of Nitrogen $n\pi^*$ States as Deduced from a Complete Assignment of Chemical Shifts¹

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Abstract: The 55 ^{13}C and 4 ^{15}N nmr transitions in chlorophyll a and its magnesium-free derivative pheophytin a have been assigned. Emphasis is placed on the quaternary carbon atoms of the macrocycle which carry the π network. A modification of the customary heteronuclear indor technique was developed to permit these assignments. On complexation with magnesium, large downfield chemical-shift changes are observed for carbon and nitrogen atoms in rings I and III (see Figure 1), accompanied by upfield shifts in rings II and IV. An analysis of these results reveals that the magnesium atom selectively perturbs low-lying $n\pi^*$ states involving promotion of nonbonding electrons localized on the nitrogen atoms into the π system. It is concluded that introduction of magnesium produces a redistribution of charge density within the molecule, accompanied by a reduction in the energy separations among low-lying $n\pi^*$ states. Magnitudes of the changes observed in the chlorophyll system are compared with parallel changes in various salts of pyrrole, indole, and carbazole to obtain a qualitative picture of the character of the magnesium–nitrogen bonds. ^{13}C nmr spectra of chlorophyll a dimers and oligomers, species considered relevant to the structure of *in vivo* antenna chlorophyll a, are presented and discussed.

The question as to why Nature has chosen magnesium as the coordinating metal in chlorophylls is an intriguing one, and possible answers must occupy a central position in any useful model of photosynthesis. Research begun more than a decade ago in these laboratories led to the assignment of an important structural function to the magnesium atom.³ Applications of proton nuclear magnetic resonance,^{3–7} infrared^{8–11} and visible¹² spectroscopy, and direct molecular weight determinations¹³ showed chlorophyll a to exist in a strongly solvent-dependent monomer–dimer–oligomer equilibrium. In polar, donor solvents, such as tetrahydrofuran, acetone, and methanol, chlorophyll a is monomeric with solvent molecules occupying one or both of the axial coordination sites on the magnesium.¹⁴ In the soft, nonpolar solvents benzene and CCl_4 , chlorophyll a forms a dimer in which the ring V keto–carbonyl of one chlorophyll molecule serves as an electron donor

to relieve the coordination unsaturation of the central magnesium atom in its partner. In hard, nonpolar solvents, such as cyclohexane or *n*-octane, a high molecular weight oligomer containing at least ten chlorophyll a molecules is the most stable species.^{6,7,12,14} By analogy with the dimer, it has been postulated that in the oligomer each chlorophyll acts both as an electron donor *via* the carbonyl function and as an electron acceptor through the central magnesium atom to produce an array of pigment molecules linked together by keto $\text{C}=\text{O} \cdots \text{Mg}$ interactions.

The structural features of chlorophyll a dimers and oligomers have been used in recent years as a model for the structure of antenna or bulk chlorophyll in the plant.^{15,16} The great majority of the chlorophyll a molecules in the photosynthetic unit in the plant is conceived to be photochemically inert and is generally assumed to serve as an antenna to collect and funnel light quanta to an energy trap where the primary photochemical step occurs.¹⁷ Evidence exists that the photochemical center contains a special pair of chlorophyll molecules, possibly bridged by water coordinated to a magnesium atom, but the exact structure of the two special chlorophyll molecules in the plant is at present uncertain.¹⁸

Besides these structural functions, it is conceivable that the magnesium atom also plays an important electronic role in the excited states. At first sight, there seems to be only a minor influence on the spectroscopic states as evidenced by the very similar visible absorption characteristics of chlorophyll a and its magnesium-free

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derivative pheophytin a. However, visible light absorption spectroscopy might not be a particularly sensitive tool to probe the electron distribution in states which carry little oscillator strength and which can therefore be obscured by much more intense absorption bands.

It is well known that ^{13}C and ^{15}N chemical shifts are strongly determined by paramagnetic contributions from excited states. It occurred to us that a comparison of the ^{13}C and ^{15}N magnetic resonance spectra of chlorophyll a and pheophytin a might well give some information on how magnesium complexation influences the electronic properties of excited states, especially those which are not directly observable by absorption spectroscopy.

^{13}C spectra of chlorophyll a have been recorded on enriched and natural abundance samples, and partial assignments of the transitions have been made using the C-H coupling patterns as the guiding principle.^{6,7,19,20} Unfortunately, this method fails for the quaternary carbon atoms constituting the majority of the atoms carrying the π network. It is the chemical shifts of these atoms together with those of the nitrogen atoms which are expected to show the largest sensitivity to electron distributions in the excited states. It became necessary, therefore, to attempt the nontrivial task of a complete assignment of the chemical shifts of all 55 carbon and 4 nitrogen atoms in both chlorophyll a and pheophytin a. A convenient numbering system for all carbon atoms is presented in Figure 1; nitrogen atoms are numbered corresponding to the pyrrole ring in which they are located. In this paper, we report the results of double resonance and indor experiments which complete these assignments. A discussion of chemical-shift trends and comparisons with model compounds will show that they indeed reflect interesting electron distributions in the excited states of these molecules.

Experimental Section

Chlorophyll a was isolated by the method of Strain²¹ from the blue-green alga *Synechococcus lividus*, biosynthetically enriched either with ^{13}C to an extent of 15–18%, according to the procedure of Uphaus,^{22a} or with ^{15}N to an extent of 95%.^{22b} Pheophytin a and methylpheophorbide a (pheophytin a where CH_3 replaces phytol) were prepared by standard procedures.²¹ Phytol was obtained commercially (Koch-Light Laboratories) and used without further purification. Samples for nmr analysis were dissolved in the appropriate dry, deuterated solvents and carefully degassed by the freeze-pump-thaw technique at 10^{-6} Torr prior to sealing. Hexamethyldisiloxane (HMS) served as an internal standard for all ^{13}C chemical-shift measurements (CH_3 at 2.00 ppm relative to the carbon of tetramethylsilane), and external 5 M $^{15}\text{NH}_4\text{Cl}$ in 2 N HCl was used to standardize ^{15}N chemical shifts.

Di(pyrrole)magnesium²³ was synthesized by the reaction of freshly distilled, degassed pyrrole with diethylmagnesium in diethyl ether²⁴ under oxygen-free, anhydrous conditions. The pyrrole salt was transferred in an inert atmosphere to an nmr tube, degassed, sealed, and used immediately to avoid decomposition. Lithium and

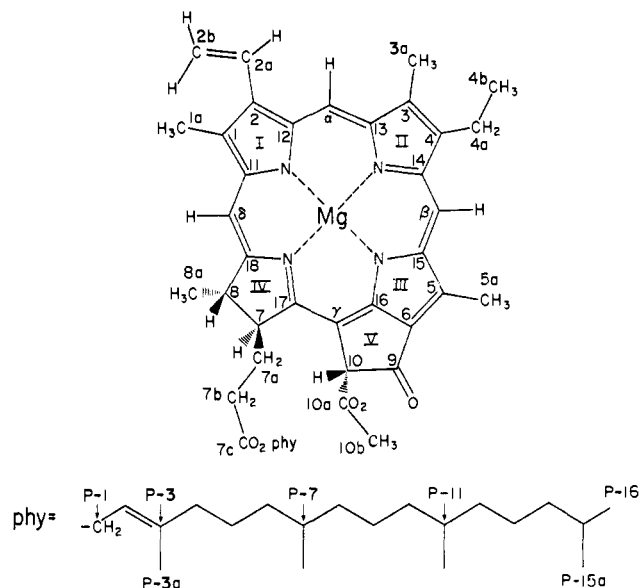


Figure 1. The structure and numbering system used for chlorophyll a, phytol, and methylpheophorbide a. Methylpheophorbide a has 2 H, instead of Mg, and CH_3 in place of phytol at 7c.

sodium salts of pyrrole, indole, and carbazole were prepared by reaction with LiH and NaH in dry THF.²⁵

^{13}C and ^{15}N nmr spectra were obtained at 22.63 and 9.12 MHz, respectively, in pulse Fourier transform mode on a Bruker HFX-90E spectrometer, employing a deuterium lock and broad-band proton decoupling. The temperature was maintained at $40 \pm 2^\circ$ for all measurements. Resolution was limited by the size of the computer memory to 1.2 Hz for ^{13}C spectra and 0.5 Hz for ^{15}N spectra. FT- ^1H nmr spectra were obtained on a Varian HA-220 spectrometer and at 90 MHz on the Bruker instrument. Heteronuclear indor spectroscopy was performed on a Varian HA-100 spectrometer fitted with a matching network double tuned for 100 and 25 MHz. The 25 MHz rf field was provided by a Micro-Now Instrument Co. sweep generator driven by the output ramp from a Varian 1024 CAT. The sweep covered about 2500 Hz in 50 sec, corresponding to the region of the ^{13}C spectrum from 100 to 200 ppm, and about 100 scans were accumulated to obtain good signal-to-noise ratios. Power levels in both rf channels were adjusted carefully to obtain good indor spectra. The indor spectrum was collected in 1024 channels, and peak positions were calibrated with a Hewlett-Packard 5327C frequency counter. Though peak positions could be ascertained with an accuracy of ± 5 Hz, nonlinearities in the response of the sweep generator made meaningful integration of peaks impossible.

Results

^{13}C Spectra. The ^1H nmr spectra of methylpheophorbide a (15% ^{13}C), phytol (natural abundance ^{13}C), and chlorophyll a (18% ^{13}C) are shown in Figure 2. The pigments are in monomeric form and give rise to rather well-resolved resonances, while phytol exhibits the rather featureless spectrum characteristic of long-chain aliphatic hydrocarbons. The proton assignment follows the original work of Closs, *et al.*³ It is observed that most of the chlorophyll a protons give rise to singlets or simple multiplets and are well separated, a fact that greatly facilitates assignment of the ^{13}C spectra.

Proton-decoupled pulse Fourier transform ^{13}C spectra of methylpheophorbide a, phytol, and chlorophyll a at 22.63 MHz are presented in Figure 3 for the same samples whose spectra are shown in Figure 2. The small lines found at the base of each resonance arise from ^{13}C - ^{13}C couplings, expected as a superposition of

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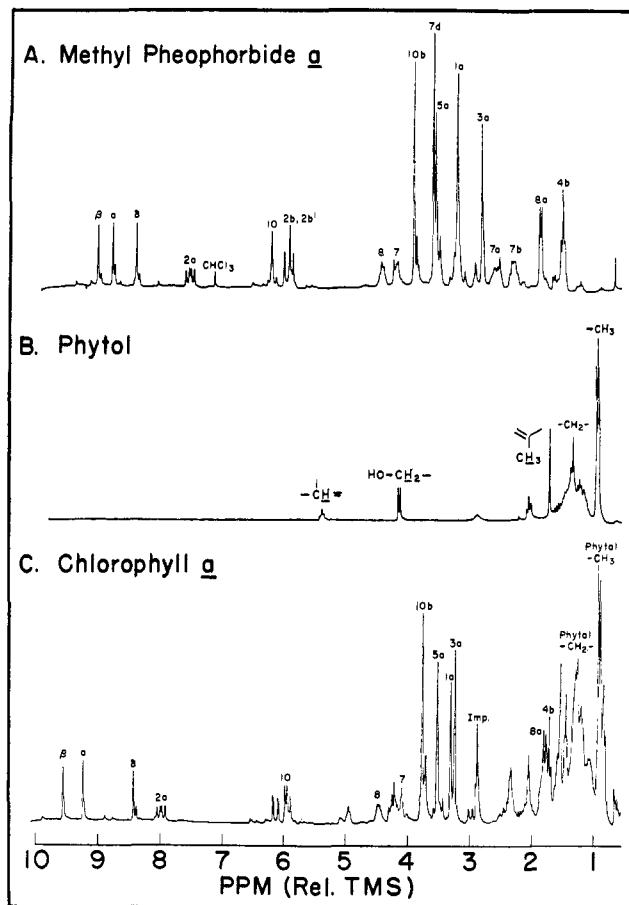


Figure 2. The 220-MHz ^1H nmr spectra of (A) 15% ^{13}C methylpheophorbide a, 0.1 M in CDCl_3 ; (B) 50% (v/v) phytol in CDCl_3 ; (C) 0.1 M chlorophyll a in acetone- d_6 .

AB- and AX-type doublets in the ^{13}C -enriched pigments. Unfortunately, most of these couplings are not sufficiently resolved to be useful. For comparative purposes, the ^{13}C nmr spectrum of phytol in Figure 3 is shown using a wide sweep width; however, all 20 carbons in the esterifying alcohol can be resolved using a narrow sweep and solutions of low viscosity. It should be noted that ^{13}C chemical shifts of methylpheophorbide a and pheophytin a are identical for all macrocyclic and side-chain carbons.

The assignment of the upfield protonated carbons in methylpheophorbide a was recently completed by Katz and Janson.^{6,7} The assignments were based on single-frequency decoupling experiments and take advantage of the excellent resolution of the assigned proton spectrum. The ^{13}C spectrum of free phytol and phytol as the esterifying alcohol in chlorophyll has recently been published by Goodman, *et al.*¹⁹ The phytol assignment largely depends on the empirical parameters developed by Grant²⁶ for calculating the chemical shifts of alkane carbon atoms, and on the observation of "segmental motion" in phytol, which by analogy with the behavior of neat 1-decanol,²⁷ permits definitive assignment of all resonances. As expected, carbon atoms adjacent to the alcohol group experience a shift upon esterification, while the remainder of the chain is unaffected. We had

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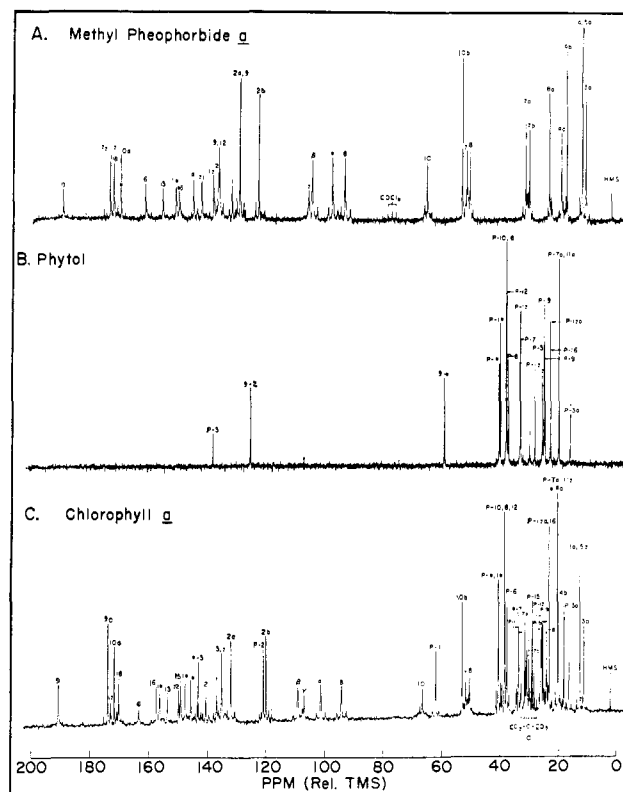


Figure 3. The 22.63-MHz ^{13}C nmr spectra of (A) 15% ^{13}C methylpheophorbide a, 0.1 M in CDCl_3 ; (B) natural abundance phytol, 50% (v/v) in CDCl_3 ; (C) 18% ^{13}C chlorophyll a, 0.1 M acetone- d_6 .

independently performed the identical nmr experiments as Goodman, *et al.*,¹⁹ and obtained the same results.

With the exception of the C-9 keto-carbonyl carbon,^{6,7} no progress had been made in the works quoted above toward the assignment of low-field quaternary carbons of methylpheophorbide a or chlorophyll a. Reference to the structure in Figure 1 shows that there are eight carbon atoms bonded to nitrogen, six bonded to other carbons, and two ester carbonyl carbons at C-7c and C-10a. The quaternary region of the ^{13}C nmr spectrum of methylpheophorbide a has only 13 resolved resonances. The line at 135.9 ppm is about twice as large as the others, indicating overlapping peaks, and the proton-coupled spectrum reveals a singlet under the 2a vinyl carbon doublet. Arguments advanced below suggest that the resonance at 173.3 ppm is also composed of two overlapping lines. In chlorophyll a, one resonance is missing, but the intensity of the peak at 134.4 ppm again argues for the presence of two lines. Proton-coupled spectra of both compounds show considerable line broadening arising from long-range C-H couplings, but efforts at assignment by analysis of line narrowing during single-frequency decoupling were unsuccessful.

Before the wide availability of pulse Fourier transform methods, ^{13}C nmr spectra of small molecules were often obtained by internuclear double resonance (indor).²⁸ This technique consists of partially saturating the ^{13}C satellite of a proton resonance with a fixed rf field (H_1) and sweeping through the frequency range of expected ^{13}C transitions with a second rf field (H_2). Because the

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Table I. Indor Spectra from Proton Resonances used in Assignment of Quaternary Carbon Atoms in Methylpheophorbide a and Chlorophyll a

Proton	Indor lines ^a , ppm		Coupled quaternary carbons	
	Methylpheophorbide a	Chlorophyll a	² J	³ J
1a	132.2, 136.4, 142.5	136.7, 140.1, 155.7	C-1	C-2, C-11
2a	131.3, 135.8	136.7, 140.5, 149.2	C-2	C-1, C-12
2b	136.6	139.5		C-2
3a	136.2, 145.0, 155.3	134.9, 145.2, 153.0	C-3	C-4, C-13
4b	145.2	145.2		C-4
5a	128.6, 137.7	134.9, 148.6	C-5	C-6, C-15
7a	173.5			C-7c, C-17
7d	173.2	<i>b</i>		C-7c
8a	172.3	169.4		C-18
10	150.2, 160.8, 169.6	140.3, ^e 156.5, 162.6, 171.0	(C-γ, C-9) ^d 10a	C-6, C-16, C-17
10b	169.6	170.1	C-10a	
α	135.8, ^e 155.0	135.1, 140.2, 149.4, 153.2	C-12, C-13	C-2, C-3
β	129.1, 138.0, 144.9, 150.6	135.2, 145.4, 147.3, 148.8	C-14, C-15	C-4, C-5
δ	131.7, 142.1 ^f	136.6, 155.6, 169.5	C-11, C-18	C-1

^a Average of two runs. The maximum variation in chemical shifts was one channel, *i.e.*, ± 3 Hz. For calibration method for parts per million, see text. ^b Proton 7d is not present in chlorophyll. Replacement of the methyl group by phytol should not result in a chemical-shift change for C-7c. ^c The proton 10b singlet occurs in the middle of the AB part of the ABX vinyl multiplet; thus C-2 also appears in the indor spectrum. C-2 is unambiguously identified by irradiating the multiplet away from the ten-proton singlet. ^d Indor confirms previous assignment. ^e This indor signal is very strong and results from the near-degeneracy of carbons 2, 3, and 12. Note that all are resolved in chlorophyll. ^f C-18, assigned unambiguously from proton 8a indor spectrum, does not appear.

proton and ¹³C nucleus are spin-coupled, they have an energy level in common, and a change in the intensity of the monitor proton line occurs whenever the rf field (H₂) passes through a coupled carbon transition. As our chlorophyll a and methylpheophorbide a samples are enriched with ¹³C, one-bond ¹³C satellites are easily detected in the ¹H nmr spectra (see, for example, Figure 2a). However, no long-range carbon-proton couplings are observed, except as a slight broadening at the base of each proton peak, suggesting that the long-range coupling constants are, as expected, at most a few hertz.²⁹ (Some line broadening is present owing to the rather long correlation time—about 2×10^{-9} sec estimated from ¹³C T₁ measurements—of these high molecular weight pigments.)

A modification of the standard indor technique was adopted to assign the quaternary carbons. The monitoring proton frequency (H₁) was fixed at the center of a particular proton transition, while sweeping the carbon (H₂) frequency at a high-power level through the low-field region of the carbon spectrum. Though the pigments are enriched with ¹³C to about 15%, the probability that any given molecule contains two ¹³C nuclei coupled to the monitor proton is very small; thus all long-range C-H couplings constitute a superposition of two-spin AX systems, and the ¹³C satellites are closely spaced around the strong uncoupled line. As H₂ passes through the frequency of a coupled quaternary carbon transition, the corresponding carbon satellites collapse into the central line, increasing its intensity by the per cent of ¹³C enrichment. Four-bond C-H couplings are known to be negligible, and as they were rarely observed in these experiments, only two- and three-bond couplings (²J and ³J) are considered in making the assignments. The greatest source of noise under these conditions is slight field fluctuations which shift the proton line leading to an apparent decrease in its intensity. For this reason, a very strong field fre-

quency lock is mandatory. "Center-line indor" as described above should be quite generally applicable to the assignment of quaternary carbons, even with samples containing ¹³C at natural abundance.

The details of the method described above will be illustrated for several typical cases: Carbon-1 should appear in the indor spectrum of proton 1a (²J), proton 2a (³J), and proton δ (³J). Indeed, for methylpheophorbide a, the indor spectra give a transition at 132.2, 131.3, and 131.7 ppm, respectively, with the average value of 131.7 ppm corresponding to the line at 131.6 ppm in the ¹³C nmr Fourier transform spectrum. (Chemical shifts were calibrated relative to the midpoint of the well-resolved doublet in the normal indor spectrum of the one-bond ¹³C satellite of proton δ whose chemical shift is known in the normal ¹³C spectrum.) This transition is therefore assigned to C-1. The 1a and δ proton indor spectra share another transition (142.5 and 142.1 ppm, respectively) whose average value of 142.3 ppm corresponds to the resonance at 141.9 ppm observed in the normal ¹³C spectrum; this must be carbon C-11. Similarly, the only quaternary carbon expected to couple with the 8a methyl protons is C-18, and the indor spectrum gives a single resonance at 172.3 ppm, corresponding to the line at 172.0 ppm in the ¹³C nmr Fourier transform spectrum. Because all quaternary carbons are coupled to more than one proton, it should be possible to assign unambiguously all resonances by this approach, and this turns out to be the case.

Table I summarizes the indor spectra for each proton and possible coupled carbons, two and three bonds removed. Table II demonstrates that the indor spectra of various protons can be combined to assign all the carbon atoms by the line of reasoning demonstrated above for C-1, C-11, and C-18. The agreement between the composite ¹³C spectrum obtained by indor and the Fourier transform spectrum is good in all cases, and the self-consistency of the data assures a high confidence level for the resulting assignment.

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Table II. Assignment of Quaternary Carbon Atoms from Composite Indor Spectra and Comparison with Fourier Transform Spectra

Quaternary carbon	Methylpheophorbide a				Chlorophyll a			
	FT, ppm, ¹³ C	Indor, avg, ppm	Indor data, ppm	Δ_{ppm}^a	FT, ppm, ¹³ C	Indor, avg, ppm	Indor data, ppm	Δ_{ppm}^a
1	131.6	131.7	131.3, 132.2, 131.7	-0.1	136.1	136.6	136.7, 136.7, 136.6	-0.5
2	136.3	136.2	136.6, 135.8, 136.4, 135.8	0.1	139.8	140.1	139.5, 140.3, ^d 140.5, 140.1, 140.2	-0.3
3	135.9	136.0	135.8, 136.2	-0.1	134.4	135.0	135.1, 134.9	-0.6
4	144.9	145.0	145.0, 145.2, 144.9	-0.1	144.9	145.3	145.2, 145.2, 145.4	-0.4
5	128.8	128.9	129.1, 128.6	-0.1	134.4	135.0	135.2, 134.9	-0.6
6	161.2	160.8 ^b	160.8	-0.3	162.6	162.6 ^b	162.6	0.0
7c	173.3	173.4	173.2, 173.5	-0.1	173.3 ^c			
10a	169.7	169.6	169.6, 169.6	+0.1	171.0	170.5	170.1, 170.0	+0.5
11	141.9	142.3	142.5, 142.1	-0.4	155.5	155.7	155.7, 155.6	-0.2
12	135.9	135.8	135.8, 135.8	0.1	149.0	149.3	149.2, 149.4	-0.3
13	155.3	155.2	155.0, 155.3	0.1	152.8	153.1	153.2, 153.0	-0.3
14	150.7	150.7	150.8, 150.6	0.0	146.8	147.3	147.3	-0.5
15	137.8	137.9	138.0, 137.7	-0.1	148.4	148.7	148.6, 148.8	-0.3
16	149.6	150.2 ^b	150.2	-0.6	156.5	156.5	156.5	0.0
17	173.3	173.5 ^b	173.5	-0.2	172.2	<i>b</i>		
18	172.0	172.3	172.3	-0.3	169.5	169.5	169.4, 169.5	0.0

^a Difference between Fourier-transform ¹³C spectrum and composite indor spectrum. ^b See text for assignment of C-6, -16, and -17. ^c See footnote b, Table I. ^d See footnote c, Table I.

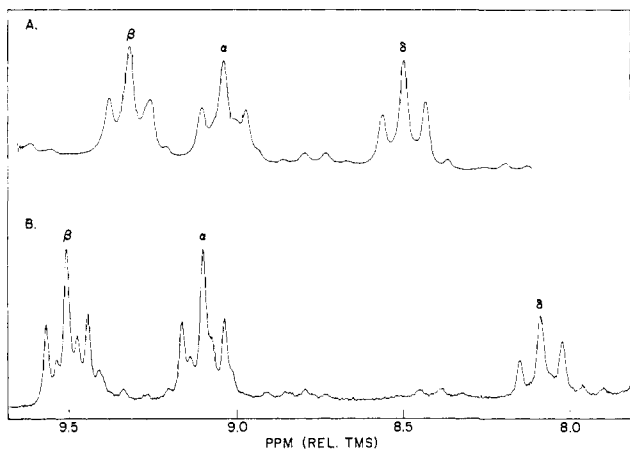


Figure 4. Low-field regions of 90-MHz ¹H nmr spectra of (A) 95% ¹⁵N pheophytin a, 0.22 M in CDCl₃; (B) 95% ¹⁵N chlorophyll a, 0.18 M in acetone-d₆.

The only difficulties arise for carbons 6, 16, and 17. In principle, the indor spectra of protons 5a, 10, and 7 should resolve any ambiguities, but the resonance of proton 7 is an unresolved lump due to coupling with adjacent protons, and repeated attempts to obtain an indor spectrum from proton 7 failed. In methylpheophorbide a proton 7a gives rise to a single indor signal at 173.5 ppm, which could be C-17 or C-7c or both, and C-7c is known to resonate here from the unambiguous indor spectrum of proton 7d. It had been noted while measuring ¹³C relaxation times (*T*₁) that the recovery of the magnetization for the resonance at 173.3 ppm using the standard (180- τ -90-*T*-)_{*n*} pulse sequence could not be fitted with a single exponential as a function of τ , and that for certain intermediate values of τ two resonances, 180° out of phase, were observed. This is a characteristic of overlapping lines with different *T*₁'s. In fully relaxed FT spectra (pulse interval greater than 30 sec), this resonance is equal in intensity to the sum of C-10a and C-18, suggesting the presence of two lines. Also, the chemical shift of C-17 is expected to be near that of

C-18 because of the similarity of their chemical environments. Thus, the bulk of evidence strongly suggests that the C-17 transition is degenerate with C-7c at 173.3 ppm in methylpheophorbide a. For chlorophyll a, C-17 is separated from other lines and is assigned by analogy to its position in the spectrum of methylpheophorbide a.

The remaining two resonances at 156.5 and 162.6 ppm for chlorophyll a and at 149.6 and 161.2 ppm for methylpheophorbide a must arise from the C-6 and C-16 atoms. Though both appear in the indor spectrum for proton 10, nothing appears in this region upon irradiation of proton 5a. Though nitrogen-bonded carbon atoms are generally found at lower field than the adjacent C-C carbon atom, deshielding effects at C-6 from the nearby keto-carbonyl and consistency among the chemical-shift changes which accompany addition of Mg (see below) lead us to assign the upfield resonance in both molecules to C-16.

¹⁵N Spectra. The proton nmr spectra of the low-field methine region of 95% ¹⁵N pheophytin a and chlorophyll a are shown in Figure 4. It is apparent that each methine proton in pheophytin a is coupled to the two ¹⁵N atoms, three bonds removed, with very similar coupling constants, leading to triplets for the X part of the AMX system ($|^3J_{^{15}N-H}| = 3$ Hz). In addition, the upfield N-H protons appear as a doublet ($|^1J_{^{15}N-H}| = 98$ Hz), typical of sp²-hybridized nitrogen³⁰ and virtually identical with the coupling constant reported for pyrrole.³¹ No coupling with other protons is observed. In the proton spectrum of 95% ¹⁵N chlorophyll a, additional couplings are observed as small peaks on both sides of the central lines of the methine triplets, apparently resulting from the possibility of five-bond N-H coupling across the central Mg atom.

The 9.12-MHz proton decoupled ¹⁵N nmr spectrum of 95% ¹⁵N pheophytin a is shown in Figure 5; all

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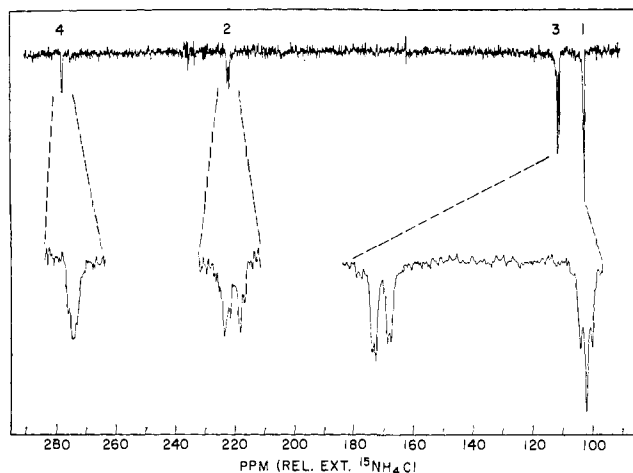


Figure 5. The 9.12-MHz ^{15}N nmr spectrum of 95% ^{15}N pheophytin a, 0.22 M in CDCl_3 .

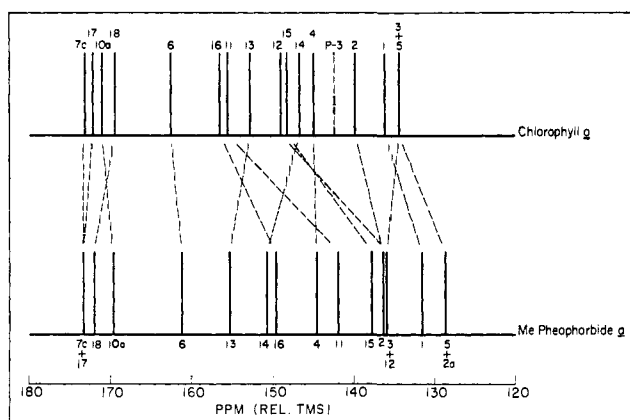


Figure 6. Correlation of ^{13}C resonance positions for low-field, quaternary carbon atoms in chlorophyll a and methylpheophorbide a.

resonances are inverted owing to the expected negative Overhauser enhancement. To obtain good signal-to-noise ratios for the two low-field resonances, a pulse interval of 30 sec was required. Even at the maximum-pulse interval (53 sec) available to our instrument and after 2 days of signal averaging, no ^{15}N spectrum of chlorophyll a was observed. Apparently, the only efficient relaxation mechanism for the nitrogen nuclei is eliminated when Mg replaces the two central protons. The expanded view of each resonance in the spectrum of pheophytin a in Figure 5 reveals that each ^{15}N nucleus is spin-coupled to two others. The multiplet patterns are easily analyzed as the X part of the AMX spectrum and give the following ^{15}N - ^{15}N coupling constants: $|^2J_{12}| = 2.0$ Hz; $|^2J_{23}| = 5.7$ Hz; $|^2J_{34}| = 1.4$ Hz; $|^2J_{14}| = 2.5$ Hz.

The ^{15}N spectrum of pheophytin a is easily assigned by standard double-resonance techniques. This was accomplished by observing the proton spectrum, while applying a single frequency ^{15}N decoupling field at the known positions of ^{15}N resonances. For example, irradiation of the lowest field ^{15}N transition collapses the triplet of proton $\delta(\text{AMX})$ to a doublet (AX), while the α and β triplets are unaffected, clearly assigning this transition to N-4. For the case of chlorophyll a, where no direct ^{15}N spectrum was observed, the ^{15}N decoupling frequency was varied in 50-Hz steps through

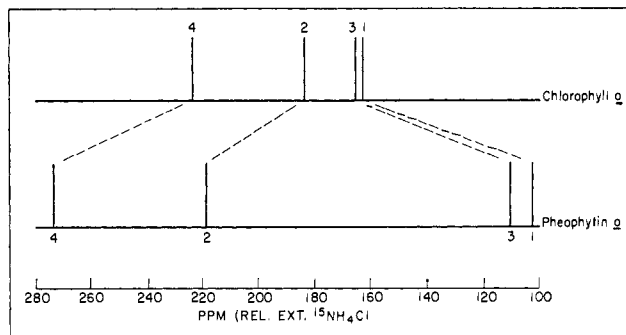


Figure 7. Correlations of ^{15}N resonance positions for nitrogen atoms in chlorophyll a and pheophytin a.

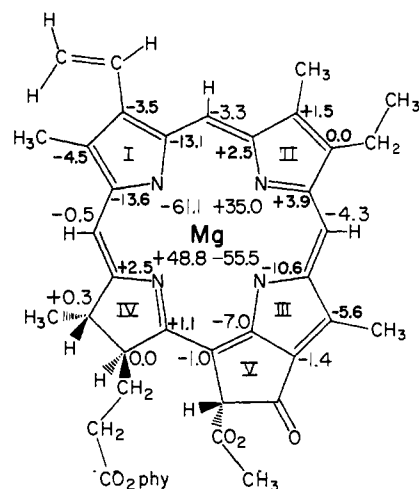


Figure 8. Magnitudes of ^{13}C chemical shift changes upon addition of magnesium to methylpheophorbide a. Negative numbers represent downfield shifts.

the range of anticipated ^{15}N transitions, until the methine proton multiplets were observed to collapse. The complete ^{15}N spectrum can be constructed in this manner to within ± 0.6 ppm. ^{15}N chemical shifts for both molecules are summarized in Table III.

Table III. ^{15}N Chemical Shifts for Chlorophyll a and Pheophytin a

Nitrogen	Chemical shift ^a		Δ_{ppm}^b
	Chlorophyll a	Pheophytin a	
N-1	163.6	102.5	-61.1
N-2	183.5	218.5	+35.0
N-3	166.4	110.9	-55.5
N-4	224.0	272.8	+48.4

^a Ppm relative to external 5 M $^{15}\text{NH}_4\text{Cl}$ in 2 N HCl. Chlorophyll a, ± 0.6 ppm. Pheophytin a, ± 0.1 ppm. ^b $\Delta_{\text{ppm}} = \delta(\text{pheophytin a}) - \delta(\text{chlorophyll a})$.

Discussion

Effect of Magnesium Coordination on Chemical Shifts. The full assignment of the methylpheophorbide a and chlorophyll a ^{13}C and ^{15}N chemical shifts allows analysis of the effects of coordination with magnesium on the chemical shifts of the macrocycle. In Figure 6 the low-field portion of the ^{13}C spectra of chlorophyll a and methylpheophorbide a is shown as a stick diagram, correlating the chemical shift of corresponding carbon atoms in the two molecules. The analogous diagram for nitrogen chemical shifts is presented in Figure 7. Figure 8 gives the actual chemical-

shift differences at each position in the macrocycle; negative numbers represent downfield shifts resulting from the introduction of magnesium. The most notable consequence of the replacement of 2 H by Mg is that carbon atoms bonded to the nitrogen atoms in rings I and III experience large downfield shifts, while nitrogen-bonded carbon atoms in rings II and IV are shifted upfield. The β -pyrrole carbon atoms follow a similar trend but to a lesser extent. These changes are paralleled by large downfield shifts for nitrogens 1 and 3 and upfield shifts at nitrogens 2 and 4.

^{13}C chemical-shift differences similar to those described above for rings I and III have previously been observed in the study of the pyrrolate anion.³² A great deal of effort has been devoted to the interpretation of the proton and ^{13}C chemical-shift changes that accompany formation of the pyrrolate anion and the pyridinium cation and chemical shifts in various aromatic organometallic compounds.³³⁻⁴¹ Of particular relevance is the observation by Pugmire and Grant³² of an 8.6-ppm downfield shift for the α carbon of the pyrrolate anion relative to pyrrole and a 5.6-ppm upfield shift at the α carbon upon protonation of pyridine.³³ Also, Witanowski⁴² finds an upfield shift of 121 ppm for the nitrogen of pyridine on protonation.

Role of Excited States in Determining ^{13}C and ^{15}N Chemical Shifts. Aromatic nitrogen heterocycles are known to possess low-lying $n\pi^*$ states, attained by promotion of a nonbonding electron localized on nitrogen into the lowest antibonding level of the π system.⁴³ In the Ramsey formulation of the theory of chemical shifts,⁴⁴ paramagnetic terms (leading to downfield shifts) arise from magnetic mixing of excited states with the ground state, where the summation over all excited states is replaced by an average excitation energy ΔE ;^{45,46} the magnitude of the paramagnetic contribution is inversely proportional to ΔE . Pugmire and Grant³² and Karplus and Pople⁴⁶ conclude that the observed changes in carbon chemical shift are explained by this mechanism, on the grounds that detailed calculations of

the pyridine and pyrrole systems demonstrate that deprotonation of pyridinium ion and formation of pyrrole anion are accompanied by a decrease in the average excitation energy. Alternatively, the removal of a proton from pyrrole leaves a negative charge, destabilizes the nonbonding electrons on nitrogen, and leads to a decrease in the energy gap between the n and π^* levels.

The salts of several nitrogen heterocycles were prepared in an effort to perturb selectively the nonbonding and π^* levels and to determine in greater detail what effects these perturbations might have on ^{13}C chemical shifts at the α carbon. The lithium, sodium, and magnesium salts of pyrrole exhibit a downfield change in chemical shift at the α carbon relative to pyrrole of -0.97 , -6.15 , and -9.50 ppm, respectively. Since $\Delta E(n\pi^*)$ diminishes as the nitrogen atom becomes more negative, these results may be interpreted to demonstrate an increase in the ionic character of the metal-nitrogen bond in the order $\text{Mg} > \text{Na} > \text{Li}$.

The free-electron theory model for π systems predicts that a lowering in energy of the π^* relative to the nonbonding level is expected to accompany an increase in the length of the conjugated pathway. The sodium salts of pyrrole, indole, and carbazole give rise to downfield chemical-shift changes at the α carbon relative to the protonated species of -6.15 , -9.67 (quaternary α carbon), and -12.57 ppm, respectively. Since a constant perturbation is applied to the n level by virtue of a common counterion, these results may be taken to reflect the effect of increasing the size of the π systems. These results are entirely consistent with the hypothesis that the observed paramagnetic shifts originate in the mixing of low-lying $n\pi^*$ states with the ground state and provide a qualitative framework for interpreting the magnitudes of this effect.

Implications of Chemical Shifts for Excited States in Chlorophyll. Before the model for paramagnetic shifts can be applied to the chlorophyll a system, some comments are in order concerning the positions of the central hydrogens in pheophytin a. In symmetrical metal-free porphyrins, the central hydrogens have been shown to exchange sufficiently rapidly at room temperature to maintain fourfold symmetry on the nmr time scale.⁴⁷ This contrasts with the observation of two distinct transitions for these protons in methylpheophorbide a.³ The ^{15}N - ^{15}N coupling constants provide direct evidence capable of resolving this often debated question. If the coupling mechanism involves electron density shared by the nitrogens *via* the central hydrogens, the magnitudes of the coupling constants may be taken to reflect substantial sharing of a N-H proton between nitrogens 3 and 2, less between nitrogens 1 and 4, and still less between nitrogens 1 and 2, and 3 and 4.

The ^{15}N chemical shifts provide further support for this argument. The N-1 and N-3 transitions in pheophytin a at 102.5 and 110.9 ppm are close to the chemical shift of 118.8 ppm reported for pyrrole,⁴⁸ while the N-4 transition at 272.8 ppm is close to the chemical shift of pyridine at 292.8 ppm.⁴² The ring II nitrogen resonates at somewhat higher field strength, as expected if the proton primarily found on N-3 is somewhat delocalized onto N-2. This picture helps to clarify the interpreta-

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(42) M. Witanowski, *J. Amer. Chem. Soc.*, **90**, 5683 (1968).

(43) The expression " $n\pi^*$ state," though accurately describing low-lying excited states in pyridine or carbonyl functions, is not strictly applicable to pyrrole or metal-bound nitrogen heterocycles where lone pair electrons are absent. For these cases, " $\sigma\pi^*$ state" may be a more precise description. In fact, the chemical shift data discussed in the text demonstrate that neither definition is satisfactory for the intermediate cases observed in metal-heterocycle bonds. To avoid additional confusion, the expression " $n\pi^*$ state" will be used, noting this qualification. For further discussion of this point see, *e.g.*, W. Hug and I. Tinoco, Jr., *J. Amer. Chem. Soc.*, **96**, 665 (1974).

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tion of the crystal structure of methylpheophorbide a. Fischer, *et al.*,⁴⁹ found "partial" hydrogens, with the largest share on rings I and III, a smaller amount on ring II, and the least on ring IV, which is completely consistent with and explained by the ¹⁵N results discussed above.

On the basis of this discussion, we are led to the conclusion that the observed changes in chemical shift on complexation with magnesium are a direct consequence of the perturbation caused by magnesium on the nonbonding levels of the four central nitrogen atoms. The π^* level is assumed to remain fixed since the main red band in the visible absorption spectra of chlorophyll a and pheophytin a (the first $\pi\pi^*$ transition) differs by only 5 nm (λ_{\max} 662 and 667 nm, respectively). Since the majority of nonbonded electron density is localized on the nitrogen atoms, the effect is more pronounced in the ¹⁵N spectra. The large downfield chemical-shift changes in rings I and III indicate the formation of an anion in these rings, destabilizing the nonbonding level and decreasing the energy difference between the *n* and π^* states. The opposite effect is operative in rings II and IV.

The ¹³C chemical-shift changes parallel those observed for the nitrogens. A very ionic structure is indicated for rings I and III by comparison with the model compounds. Because the carbon nuclei experience the $n\pi^*$ state *via* the remaining nonbonded orbital density not localized at the heteroatom,⁴³ the changes in chemical shift are, as expected, smaller. It is apparent that the nonbonded orbitals in rings I and III, from which the protons are removed, are perturbed to a much greater extent than rings II and IV. This effect is most apparent at the meso carbons, which come under the combined influence of nonbonded density from adjacent rings, and which experience downfield shifts from the dominance of the perturbation on rings I and III.

The $n\pi^*$ states invoked in the preceding discussion have never been observed directly in the visible absorption spectrum of chlorophyll or its derivatives because of the much more intense $\pi\pi^*$ bands that dominate this region of the spectrum. Enormous theoretical and experimental effort has been devoted to locating the probable position of the $n\pi^*$ states in symmetrical porphyrins⁵⁰ with mixed results. We are unable to specify the absolute energy of the $n\pi^*$ states in chlorophyll a on the basis of our results, because this would require knowledge of the difference in orbital energy between the nonbonded orbitals and the highest filled π orbital, but we are able to comment on the relative energies of these states. In pheophytin a, two closely spaced low-energy $n\pi^*$ transitions should be available originating in rings II and IV, as well as two at much higher energy originating in rings I and III. The order of transition energy should be as follows: ring IV < II << III ~ I. In chlorophyll a, the same sequence is predicted, but the difference between highest and lowest energy states is much smaller, and all states should be found closer to a central value. This follows from the observation that, though the difference in ¹⁵N chemical shift between the lowest and highest field transition changes from 170.3 ppm for pheophytin a to 60.4 ppm for chlorophyll

a, the *average* chemical shift only increases from 176.0 to 184.4 ppm. Precisely the same effect is noted for the ¹³C chemical shifts with an overall average increase of 4 ppm for the carbon atoms adjacent to nitrogens. To summarize, the introduction of magnesium results in a substantial rearrangement of charge density in the nonbonded orbitals on the nitrogen atoms, in effect making the density in the four rings more nearly equivalent.

A very recent report of ESCA spectra for the nitrogen 1s orbitals in free-base tetraphenylporphyrin (H₂TPP) and Ag^{II}TPP is relevant to this discussion.⁵¹ The ESCA time scale is many orders of magnitude faster than nmr, and the authors observe two nitrogen 1s binding energies separated by 2.1 eV, corresponding to protonated and nonprotonated nitrogens. On addition of the metal, the two peaks coalesce into a single line midway between the free-base nitrogen peaks. This is precisely the behavior expected from the ¹⁵N nmr results (and indirectly from the ¹³C nmr spectra) where nitrogens which are more positive in the free-base become more negative on addition of Mg and *vice versa*. The lower symmetry of the chlorophyll a systems leads one to predict the possibility of four resolvable lines for the nitrogen ESCA spectrum of pheophytin a, which would partially coalesce on addition of magnesium.

Finally, the downward trend of the *average* chemical shifts of the nitrogen and carbon atoms observed upon introduction of magnesium reflects a raising of the average energies of the *n* orbitals. This is predicted to result in a reduction of the electronegativity of the σ core and may be the basis of the difference in redox potentials of chlorophyll and its magnesium-free derivative.⁵²

Whether or not the $n\pi^*$ states have a direct function in photosynthesis remains an open question. One might speculate that mixing of these states with the photochemically accessible $\pi\pi^*$ states will facilitate singlet-triplet intersystem crossing because of contributions by the well-known one-center terms in the spin-orbit coupling matrix of $n\pi^*$ states.⁵³ This is a particularly attractive hypothesis because recent X-ray results⁵⁴ have shown the magnesium to be located substantially outside of the molecular plain. This geometry removes the orthogonality of the *n* and π orbitals and leads to increased mixing between $n\pi^*$ and $\pi\pi^*$ states.

Application of ¹³C Nmr to Aggregated Chlorophyll Species. The aggregated species of chlorophyll a provide strong motivation for the application of ¹³C nmr. As mentioned in the introductory section, chlorophyll a

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(52) It is well known that electrochemical oxidation of porphyrins and chlorophylls to the π -cation radical is more difficult by about 0.3 V for the free base than for the metal complex; see, e.g., J. R. Harbour and G. Tollin, *Photochem. Photobiol.*, **19**, 69 (1974). It is interesting to note that the ¹³C chemical-shift differences in parts per million between Mg-TPP (or Zn-TPP) and free-base TPP are: α -pyrrole, -3.12; β -pyrrole, -0.70; meso carbon, -1.38. Since the central hydrogens are free to exchange (on the nmr time scale) in this symmetrical porphyrin, up- and downfield chemical-shift changes originating in the perturbation of $n\pi^*$ states cancel, and the observed differences require explanation by a different mechanism. The striking coincidence between these changes and the observed *net* change in the chlorophyll a system indicates an additional aspect of the role of metal complexation which might explain the important difference in redox properties discussed above.

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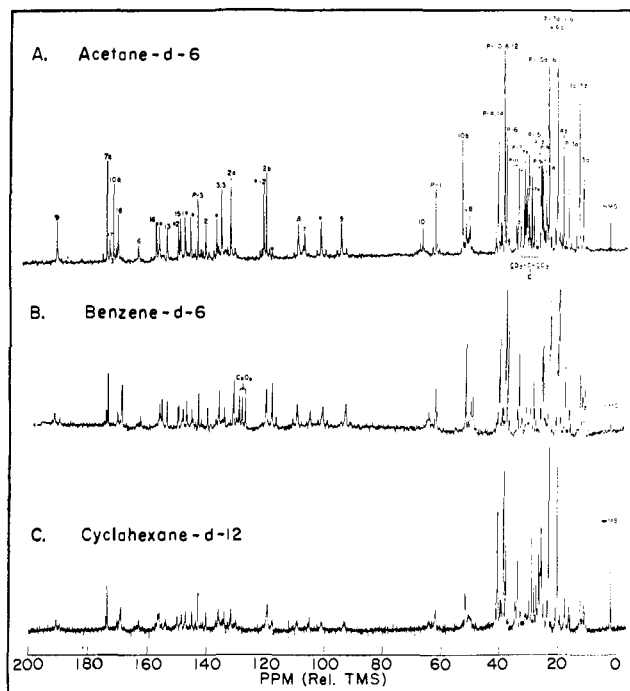


Figure 9. The 22.63-MHz ^{13}C nmr spectra of 18% ^{13}C chlorophyll a, 0.1 M in (A) acetone- d_6 ; (B) benzene- d_6 ; (C) cyclohexane- d_{12} .

is known to form dimers in benzene and CCl_4 , where the C-9 keto-carbonyl function of one chlorophyll a molecule donates electron density to the magnesium atom of its partner. In solvents such as cyclohexane and *n*-octane, it is postulated that these same interactions lead to the formation of chlorophyll a oligomers; these large entities cannot be analyzed directly by ^1H nmr. From the preceding discussion, it is apparent that this interaction will lead to at least three competing influences on the ^{13}C chemical shifts: (1) the π^* level is shifted by the interaction between the two systems; (2) the nature of the ligand interacting with magnesium should indirectly influence its perturbation on the non-

bonded orbitals of the nitrogen atoms; and (3) the overlap of the two ring systems will lead to a change in the ring current experienced by the macrocyclic carbon atoms.

Figure 9 shows ^{13}C nmr spectra of chlorophyll a monomer in acetone, dimer in benzene, and oligomer $[(\text{Chl}_2)_n, n \sim 4]$ in cyclohexane. Though the details of the contributions arising from each effect proposed above have not yet been analyzed, several important deductions can be made from these preliminary data. In contrast to ^1H nmr, line broadening, even in the oligomer, does not prevent observation of resolved resonances for all carbons. The phytol carbon resonances remain very sharp and unshifted, implying that the long aliphatic chains are moving freely in the solvent. The transitions for the macrocycle and side-chain carbon atoms are broadened as the molecular weight of the aggregate increases, but not to an extent sufficient to reduce the utility of the spectra. While there are significant changes in chemical shifts in going from monomer to dimer, no further chemical-shift changes in the ^{13}C nmr spectra of the oligomer relative to the dimer are apparent. This observation provides strong evidence that the geometry of the dimer is repeated in the oligomer; *i.e.*, no radically different chlorophyll-chlorophyll orientations are to be found in the oligomer. That the chlorophyll-chlorophyll orientations are very similar in kind (but not in number) is additional experimental evidence for the hypothesis that identifies antenna chlorophyll in the plant with the chlorophyll oligomers $(\text{Chl}_2)_n$ present in concentrated solution in aliphatic hydrocarbon solvents.^{15,16}

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