Nuclear Magnetic Resonance Spectroscopy of Chlorophylls and Corrins

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I. INTRODUCTION

The role of NMR in the study of compounds of biological importance is widely recognized and appreciated. There can be few categories of such substances, however, to which NMR has made such substantive contributions as it has to the understanding of the chlorophylls and the corrins. The chlorophylls are indispensible agents in the conversion of the energy of light to chemical oxidizing and reducing capacity. The natural corrins are coenzymes for a number of enzyme systems involved in important isomerization and methyl group transfer reactions, and are intimately involved in protein and probably also in lipid and carbohydrate metabolism. Vitamin B_{12} plays a very important part in hemopoiesis (stimulation of red blood cell formation), and together with folic acid participates in the formation of deoxyribonucleotides from ribonucleotides. Applied to the chlorophylls, NMR has provided information relevant to such aspects as the sites of exchangeable hydrogen, keto-enol tautomerism, the biosynthetic pathways of

chlorophyll formation, hyperfine interactions in chlorophyll cations and other paramagnetic chlorophyll species, as well as more conventional NMR information useful in establishing the chemical identities of several chlorophylls of previously unknown structure. Perhaps the most important contribution from NMR to an understanding of chlorophyll behavior is the delineation of the coordination donor-acceptor properties of the chlorophylls that largely determine the state of chlorophyll *in vivo*. For the corrins, clarification of the path of biosynthesis and the variables that affect their coenzyme activities has up to now been the most significant contribution from NMR studies.

II. THE CHLOROPHYLLS

The chlorophylls constitute a small group of closely related compounds whose function in nature is to collect light quanta and to use the subsequent electronic excitation energy to effect charge separation. The oxidizing and reducing power so produced is then used to drive redox reactions that would otherwise not proceed spontaneously. Useful introductions to the role of chlorophyll in photosynthesis have been provided by Govindjee and Rabinowitch (1), by Clayton (2), and by Govindjee (3).

A. Structural Features

The chlorophylls are cyclic tetrapyrroles, and thus belong to the porphyrin family. There are both important similarities and differences between the chlorophylls and the more widely studied porphyrins. The side chains of the tetrapyrrole macrocycle , i.e. methyl, ethyl, vinyl, and propionic acid, are much the same in both porphyrins and chlorophylls. The side chain positions likewise for the most part appear to be identical, arguing for similar biosynthetic pathways. The chlorophylls, however, all have an alicyclic 5-membered ring V (Figure 1), which contains a keto carbonyl function at position C-9. Most of the chlorophylls contain a carbomethoxy group at

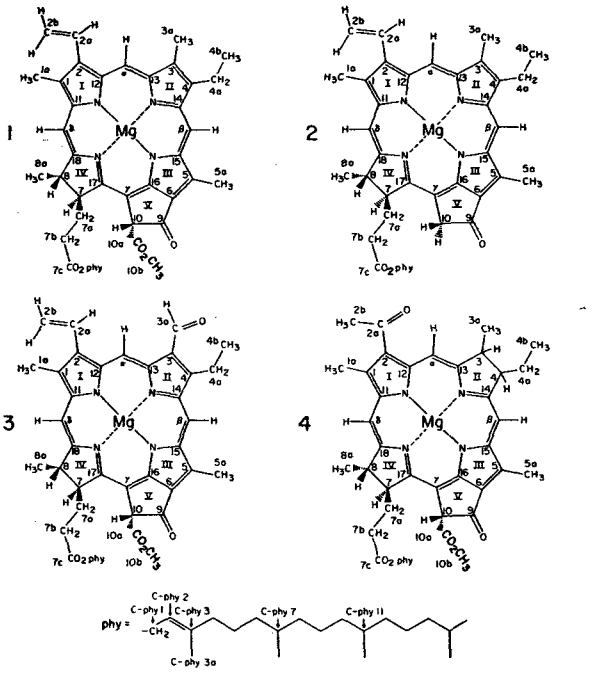

Figure 1. Structure and numbering of the chlorophylls. 1, Chlorophyll *a* (Chl *a*); 2, pyrochlorophyll *a* (Pyrochl *a*); 3, chlorophyll *b* (Chl *b*); 4, bacteriochlorophyll *a* (Bchl *a*). The methyl chlorophyllides have the same macrocycle as the chlorophylls, but the phytyl moiety is replaced by $-CH_3$. The removal of the central Mg and its replacement by 2H from chlorophylls and methyl chlorophyllides forms pheophytins (Pheo) and methyl pheophorbides, respectively. Chlorophylls c_1 and c_2 have an acrylic acid side chain at position 7 in structure 1; in Chl c_2, a vinyl group is also present at position 4. Both Chl c_1 and c_2 lack any esterifying alcohol at position 7C. Bacteriochlorophyll *b* has only an ethylidene group, $=CH-CH_3$ at position 4 in structure 4. Protochlorophyll *a* is identical with 1 except for the absence of protons 7 and 8. The protons at positions 7 and 8 are also missing in Chl c_1 and c_2.

the 10 position, but in several important chlorophylls the carbomethoxy group is replaced by an H atom. Ring V is a structural feature unique to the chlorophylls. It is this feature that is mainly responsible for the rich and complex chemistry characteristic of the chlorophylls. The proton at C-10 in chlorophylls containing a carbomethoxy group is part of a β-keto ester system, which can undergo enolization. Enolization is associated with epimerization at the chiral center at C-10 , and is implicated in a very complicated set of oxidation (allomerization) reactions by molecular oxygen that occur at position C-10, which ultimately results in the rupture of ring V. Excellent reviews on the chemical properties and reactions of the chlorophylls by Seely (4) and more recently by Jackson (5) are available.

The central magnesium atom chelated by the chlorophyll macrocycle is a regular rather than a transition metal ion such as is present in porphyrin-containing respiratory pigments, oxidases, and the like. The Mg atom of the chlorophylls has significant electrophilic properties found to a distinctly lesser extent in the corresponding transition element complexes. The keto C=O group at C-9 in ring V endows the chlorophyll molecule with nucleophilic properties that have no parallel in the porphyrins. Esterification of the propionic acid by phytol, a long-chain aliphatic alcohol, makes for solubility properties different from those of heme, which contains the free acid. The chlorophylls are for all practical purposes insoluble in water and must be studied in organic solvents. There are, of course, many other important differences between chlorophylls and porphyrins, but those indicated here are perhaps the most significant in terms of their consequences for NMR spectroscopy. By far the best studied chlorophyll is chlorophyll a (Chl a) the principal chlorophyll in green plants and blue-green algae (cyanobacteria), and most of the discussion in this review will deal with this chlorophyll.

B. Experimental Aspects

The susceptibility of chlorophylls to oxidation by molecular oxygen necessitates special precautions in recording NMR spectra. Reaction with oxygen in polar organic solvents, particularly methanol , rapidly produces sufficient allomerized chlorophylls to complicate spectral interpretation. The allomerized chlorphylls are similar in chemical structure to chlorophyll itself, and the spectrum of a mixture of closely related but not identical compounds may show broad, poorly resolved resonance peaks. Even 1% by weight of a compound of low molecular weight can produce an equimolar concentration of an impurity resonance. Samples for NMR are preferably dissolved in purified and inert solvents, and the sample tubes sealed off in a high vacuum after thorough degassing. The manipulation of chlorophylls is best carried out in nitrogen-atmosphere gloved boxes. Chlorophyll samples for NMR kept in air may be altered so rapidly that they can no longer be safely used after only a few hours, but NMR samples prepared from pure components in sealed tubes show no changes for months or even years. ^{1}H and ^{13}C chemical shifts are given in δ, ppm, relative to TMS, unless otherwise indicated.

C. Chemical Shift Assignments

Despite the structural complexity of the chlorophylls, chemical shift assignments are straightforward, and indeed, more readily accomplished than in the case of many simpler appearing compounds. There are many protons on the chlorophyll macrocycle sufficiently isolated not to experience spin-spin interactions sufficient to complicate the spectra; the methine protons, the proton at position C-10, the methyl groups (in Chl a) at positions 1a, 3a, 4b, and 10b are well-separated and thus appear as singlets. Where spin-spin interactions occur, as in the vinyl group at position 2, the protons of ring IV, and the ethyl group at position 4, the resonances are still well separated, and their multiplicity contributes to the assignment. Where a high-field resonance originating in a macrocycle side chain is overlaid by

resonances from aliphatic protons in the phytyl chain, the phytyl group can be replaced by transesterification with methanol. The methyl pheophorbides (Mg-free derivatives) and methyl chlorophyllides (obtainable *in situ* enzymatically) have simple spectra in which all of the macrocycle proton resonances are clearly visible.

The highly characteristic features of the chlorophyll ^1H NMR spectra are, however, to a considerable extent the result of interatomic induced fields originating in the highly aromatic macrocycle. Such ring current effects have long been known to be important in the ^1H NMR spectra of aromatic compounds, and were early recognized by Becker and Bradley (6) and Abraham (7,7a) to have particular significance for porphyrin and chlorin NMR. The ring current calculations of Janson et al. (8) for oligomeric silicon and germanium phthalocyanins have been successful in calculating ring current shifts in these compounds, but this method has yet to be applied to the chlorophylls. Abraham et al. (7a) have advanced a double dipole model of the macrocyclic ring current in the dehydroporphyrin ring of chlorophyll derivatives. This model accounts reasonably well for ^1H chemical shift differences between corresponding protons in methyl pyropheophorbide *a* and its porphyrin analog 2-vinyl-phylloerythrin methyl ester, in which the additional hydrogen atoms present in Ring IV of the methyl pyropheophorbide *a* have been removed by oxidation. In a qualitative way ring current effects account for the unusually broad range of chemical shift values typical of the chlorophylls. The ^1H NMR chemical shifts of Chl *a* have a range of 10 ppm, and the Mg-free pheophytins a range of 12 ppm. The methine protons in the plane of the macrocycle are deshielded and appear at unusually low field. The ring methyl, vinyl, and propionic acid protons are likewise deshielded to a significant extent. In the pheophytins (chlorophylls in which the Mg is replaced by 2H) the H atoms attached to the pyrrole N are strongly shielded by the ring current and come into resonance several ppm above TMS. The ring current model of Abraham et al. (7a) suggests that the alicyclic ring V has no appreciable effect on the macrocyclic ring current, but that the keto C=O group at position 9, and the addition of 2H atoms in ring IV in the chlorin both reduce the ring current by about 6 and 10% respectively. The sensitivity of the ring current effects to geometry is primarily responsible for the unusual amount of structural information that can be deduced from NMR data on chlorophyll-nucleophile and chlorophyll-chlorophyll interactions (cf. sections II.D.3 and 4).

1. ^1H NMR Chemical Shifts of Methyl Pheophorbides

All of the macrocycle ring protons of Chl *a* (33 of the 72 protons in the molecule) have been assigned. The complete spectral assignment of Chls *a* and *b* depends to a considerable extent on the assignment of resonances of the corresponding methyl pheophorbides (chlorophylls in which the central Mg atom is replaced by 2H and the phytyl chain by a methyl group). Partial assignment of the ^1H NMR of the chlorophyll derivatives chlorin e_6 (9), and rhodochlorin dimethyl ester (9), and of the methyl pheophorbides of the chlorophylls from green photosynthetic bacteria (10) had been made prior to the full assignment of the methyl pheophorbide *a* and *b* chemical shifts by Closs et al. (11). A review of chlorophyll NMR work prior to 1966 describes the rationale of the chemical shift assignments of the methyl pheophorbides (12), and more recent reviews (13-15) cover subsequent developments.

Table 1 summarizes ^1H NMR chemical shift data for the methyl pheophorbides derived from a number of important chlorophylls. The low field chemical shifts originate from the methine bridge protons and the proton of the formyl group in methyl pheophorbide *b*. The methine assignments for methyl pheophorbide *a* are based on the considerations that the proton lies between a pyrrole and a pyrroline ring and should, therefore, be the most shielded methine proton (6,9), and that the β methine proton, because of its proximity to the ring V keto carbonyl group

Table 1

^{1}H NMR Chemical Shifts[a] of Methyl Pheophorbides in C^2HCl_3(12)

Proton	Methyl Pheophorbide a (0.06M)	Methyl Pyropheophorbide a (0.06M)	Methyl Pheophorbide b (0.08M)	Methyl Bacteriopheophorbide a (0.04M)	Methyl 2-vinyl Bacteriopheophorbide c[b] (0.05M)
α	9.15	9.20	9.76	8.96	9.44
β	9.32	9.32	8.89	8.47	9.49
δ	8.50	8.50	8.47	8.40	-
2a	7.85	7.98	7.75	-	7.90
2b	6.12/6.04	6.25/6.15	6.16/6.08	3.15[c]	6.22/6.09
10	6.22	5.13	6.22	6.08	5.23
8	4.40	4.42	4.45	4.28[d]	4.57
7	4.13	4.23	4.15	4.02[e]	n.r.
δ-CH_3	-	-	-	-	3.86
10b	3.88	-	3.95	3.84	-
5a	3.62	3.58	3.46	3.48	1.96[f]
7d	3.57	3.58	3.62	3.57	3.60
4a	3.48	3.50	3.37	2.20	1.67
1a	3.32	3.35	3.28	3.44	3.48
3a	3.15	3.13	10.58	1.72	-
8a	1.82	1.72	1.88	1.79	1.48
4b	1.60	1.55	1.48	1.10	1.67
N-H[g]	-1.75	-1.85	0.83	0.46	-
			-2.15	-0.96	

a) In δ, ppm, downfield from TMS. b) A mixture, with position 4 occupied by ethyl, n-propyl, and i-butyl, and position 5 occupied by methyl and ethyl. The α-hydroxyethyl group normally present in Bchl d has been converted to vinyl (32). c) The methyl group of the acetyl group at position 2. d) Includes the proton at position 3. e) Includes the proton at position 4. f) The methyl group in an ethyl group at position 5. g) The pyrrole nitrogen atoms. The 2 protons are not equivalent and may appear as two resonances. A minus sign indicates a shift at higher field than TMS.

is more strongly deshielded than the α proton. In methyl pheophorbide b, the assignment of the α and β protons is reversed on the presumption that the formyl group should strongly deshield the α proton so that its resonance in the b series appears at the lowest field for the methine protons. In methyl bacteriopheophorbide a, all three of the methine protons are positioned between a pyrrole and pyrroline ring, and all three resonances are at a relatively high field. The α proton must be the least shielded because of the acetyl C=O group at position 2, and the assignments of the β and δ resonances are based on the arguments used for the assignment in methyl pheophorbide a. These methine assignments are consistent with the results of disaggregation titration experiments described in section II.D.4.

The resonances in the region 5-8 ppm are assigned to vinyl or to other strongly deshielded substituents. The vinyl group at position 2 in methyl pheophorbides a and b and methyl pyrpheophorbide a are easily recognized as an AMX spin-splitting pattern, from which by a standard analysis, the following coupling constants for methyl pheophorbides a and b respectively (in Hz) can be extracted: $|J_{H(x)H(A)}|$ 18.7, 18.3; $|J_{H(x)H(B)}|$ 10.9, 11.2; and $|J_{H(A)H(B)}|$ 1.6, 1.6.

The C-10 proton in methyl pheophorbide a and b, is assigned to a sharp resonance at ~6.2 ppm, which coincides in many solvents with the high field portion of the vinyl proton resonances. Assignment is made on the basis of ring current considerations and the proximity of deshielding functional groups; the exchange behavior of this proton confirms the assignment. In methyl pheophorbide a and b, there are two protons at the C-10 position, and these are not magnetically equivalent, having chemical shifts that differ by about 0.12 ppm. These two protons yield a highly distorted AB pattern ($\Delta\delta$ 6 Hz, J ~ 20 Hz) in which the two central resonances of the expected quartet are the most prominent features. The magnetic non-equivalence of the two proton sites at the tetrahedral C-10 provides important information about the epimers of the natural chlorophylls (section II.C.3).

The low intensity multiplets near 4.35 ppm in all the methyl pheophorbides have been assigned to the protons at position 7 and 8 (and positions 3 and 4 in Bchl a and its derivatives) on the basis of shielding considerations and the complicated splitting patterns expected for these protons. These assignments have been confirmed by decoupling experiments that show the 7-proton is coupled to the methylene protons of the propionic acid side chain at ~2.50 ppm, and the 8-proton to the high field methyl group doublet at ~1.80 ppm. A maximum coupling constant of ~2.8 Hz has been estimated for the spin-spin interaction between the 7- and 8-protons, which is consistent only with a trans relationship between the proton and the alkyl groups in ring IV. The chemical shift difference between the 7- and 8-protons is surprisingly large. Originally it was attributed to the deshielding effect of the adjacent carbomethoxy function at position 10. However, the difference in chemical shifts occurs also in the pyro-derivatives of chlorophyll, in which the carbomethoxy group has been replaced by H. The chemical shift difference between the 7- and 8-protons probably arises from the anisotropy of the macrocyclic ring. As the macrocyclic ring is not planar, the 7- and 8-protons may occupy positions that place them at different distances from the ring and so subject them to differing ring current deshielding. Another possibility is that the chemical shift differences between the ring IV protons are the result of substitution at the γ methine position, as Abraham et al. (16) have shown that the effects of methine substitution in porphyrin NMR spectra are much larger than can be accounted for by the resulting changes in bond anisotropies.

The macrocycle ring methyl groups at positions 1a, 3a, 5a, and 10b have resonances located between 3 and 4 ppm and have been assigned with considerable certainty. In the methyl pheophorbides, the resonance of the CH_3-group on the propionic side chains can be differentiated from the methyl group of the carbomethoxy function at position

10 by the synthesis of $[C^1H_3]$-^2H methyl pheophorbide by transesterification of the phytyl group of fully deuterated Chl a with methanol of ordinary isotopic composition; under the usual transesterification conditions only the propionic ester function undergoes reaction. The isotope hybrid methyl pheophorbide in which the position of the C^1H_3-group is independently established makes possible an unequivocal assignment of the ester methyl groups in the methyl pheophorbides, and illustrates one of the ways in which fully deuterated chlorophylls (17) find use in NMR spectroscopy. The assignment of the remaining macrocycle methyl groups is largely from the disaggregation titration studies described below.

The assignment of the high-field proton resonances of the methyl pheophorbides is completely straightforward and follows directly from double resonance experiments. The elimination of the large group of resonances from the phytyl moiety greatly simplifies the spectra and does not significantly affect the position of the macrocycle proton resonances. The chemical shifts of the pheophytins are to a good first approximation the sum of the methyl pheophorbide and phytol chemical shifts. As the capabilities of modern NMR spectrometers have improved it has become possible to see many more highly resolved phytyl resonances. This is particularly the case when chlorophylls or pheophytins of suitable adjusted isotopic composition are used. In ^2H-Chl a containing 1% ^1H, all the methyl resonances of the phytyl moiety can be seen under ^2H-decoupling, as well as a number of the $-CH_2-$ resonances. These are at present unassigned, but there is no reason to suppose that an assignment will not be forthcoming in the future. The phytyl resonances are given in Table 2. Because of the near identity of the chemical shifts of the pheophytins and the methyl pheophorbides, those of the pheophytins are not tabulated here.

A solvent sometimes employed in NMR studies because it contains no protons is trifluoroacetic acid-d_1, $CF_3CO_2{}^2H$. This is an excellent solvent for the pheophytins, and as it is free of non-exchangeable protons, finds use in ^1H NMR work. It should be pointed out that the pheophytins are di-protonated in this strong acid, and form dications, $pheoH_2^{++}$. Trifluoroacetic acid has also been employed as a solvent for the chlorophylls, on the unfounded assumption that the central Mg atom is retained. In fact, the chlorophylls dissolved in trifluoroacetic acid lose their Mg atom and are protonated and converted to the dication of the corresponding pheophytin. Not only are the optical properties of $pheoH_2^{++}$ remarkably similar to those of the corresponding chlorophyll, but the ^1H chemical shifts of the two are also very similar (18).

Unlike the case of the Mg-containing chlorophylls, the chemical shifts of the Mg-free pheophytins and pheophorbides are strongly concentration dependent. As the concentration increases, π-π stacking occurs to an increasing extent (11,12), but as stacking occurs with only partial overlap, selective ring-current chemical shifts are observed. The coordination interactions between chlorophylls yield products of rather different geometry, and the effects of concentration on chlorophyll NMR spectra are considerably smaller than for the Mg-free derivatives. Brockmann et al. (19) have examined the concentration dependence of the ^1H NMR spectra of methyl pheophorbides possessing an α-hydroxyethyl group (derivatives of Bchl c). At high concentrations (>0.1 M) doubling of many of the resonance lines is observed, which is interpreted by Brockmann et al. to be a consequence of aggregate formation. This conclusion is somewhat suprising, as aggregates produced by either π-π or coordination interactions involving Mg form and disaggregate on a much faster time scale than that of the NMR measurements. Consequently, at ambient temperature only one set of lines has been observed in these systems. Pheophorbides containing α-hydroxyethyl groups, however, appear to show line doubling, and this has been attributed to hindered rotation around the C-C bond attaching the hydroxyethyl group to ring I. Whether hindered rotation is responsible, or whether some other

Table 2

^{1}H NMR Chemical Shifts[a,b] of Monomer Chlorophylls *a*, *b*, c_1, c_2 and Pyrochlorophyll *a* (13)

Proton		Chl *a*[c]	Pyrochl *a*[d]	Chl *b*[c]	Chl c_1[e]	Chl c_1[e]
Methine	α	9.23	9.22	9.87	(9.95)	(10.10)
	β	9.50	9.46	9.55	(9.90)	(10.00)
	δ	8.28	8.37	8.18	(9.80)	(9.92)
3-CHO		-	-	10.92	-	-
2-Vinyl	H_X	7.92	7.99	7.85	8.28	8.33
	H_A	5.97	5.99	5.98	6.34	6.35
	H_B	6.13	-	6.15	6.04	6.06
4-Vinyl	H_X	-	-	-	-	8.33
	H_A	-	-	-	-	6.32
	H_B	-	-	-	-	6.04
7-Acrylic						
	7a	-	-	-	8.89	8.99
	7b	-	-	-	6.61	6.67
10-$H_{(2)}$		6.22	4.33	6.10	6.72	6.84
7		4.14[f]	4.21	4.15	-	-
8		4.27	4.09	4.45	-	-
1a		3.28	3.22	3.22	(3.5-4)[g]	(3.5-4)[g]
3a		3.25	3.16	-	(3.5-4)[g]	(3.5-4)[g]
4b		1.72[d]	1.58	n.r.	1.67	-
5a		3.60	3.22	3.52	(3.5-4)[g]	(3.5-4)[g]
8a		1.78[d]	1.64	n.r.	(3.5-4)[g]	(3.5-4)[g]
10b		3.97	-	3.95	(3.5-4)[g]	(3.5-4)[g]
7a		2.0-2.5	~2.09	~2.35	n.r.	n.r.
7b		2.0-2.5	~2.40	~2.35	n.r.	n.r.
P-1		4.41	4.38			
P-2		4.89	4.97			
P-3		1.42	1.45			
P-4		1.74	1.75			
P-CH_2's		1.18	1.17			
		1.16	1.12			
P-CH_3's		0.78	0.77			
		0.75	0.74			
		0.74	0.70			
		0.71	0.67			
		0.68	0.64			

a) Chemical shifts in δ, ppm relative to internal TMS. b) Chemical shifts enclosed in parentheses have been assigned from intercomparison with other chlorophylls. c) In $C^2H_3Cl/C^2H_3O^2H$ (11). d) In acetone-$^2H_6$. e) In tetrahydrofuran-$^2H_8$ (22,23). f) In pyridine-$^2H_5$ (24,47). g) In TFA (22).

cause must be sought for the line doubling still is not clear.

2. ^{1}H NMR Chemical Shifts of the Chlorophylls

Proton chemical shifts of Chl *a*, Bchl *a* and the important derivative Pyrochl *a* are listed in Table 2. The rationale of the chlorophyll assignments is very much the same as for the methyl pheophorbides. As indicated below, the ^{1}H NMR spectra of the chlorophylls are strongly solvent dependent, and the relationship between the spectra in polar (nucleophilic) and non-nucleophilic solvents provided valuable information for the assignment of the resonances observed in nucleophilic solvents.

It should be noted that phytol is by no means the universal esterifying alcohol in the chlorophylls. While all samples of Chl *a* and *b* so far examined appear to be esterified primarily by phytol, Bchl *a* derived from Rhodospirillum rubrum is esterified principally by geranyl geraniol (a C_{20} alcohol with 4 double bonds) (20). It has long been known that the chlorophylls from green photosynthetic bacteria (Bchl *c*) contain farnesol (a C_{15} alcohol with 3 double bonds). The presence of additional vinylic protons or methyl groups in farnesol or geranyl geraniol produces additional olefinic resonances in the region δ 4-5 ppm and this possibility must be kept in mind in the analysis of pheophytin and chlorophyll spectra.

Some more recently characterized chlorophylls merit comment. Chlorophylls c_1 and c_2 are auxilliary accessory pigments in marine diatoms and brown algae. These chlorophylls are closely related to each other and to Chl *a*. Unlike Chl *a*, however, they are porphyrins, not chlorins, although an intact ring V is present in both. Chl c_1 and c_2 are both free acids, and lack an esterifying alcohol at the position 7 side chain. The side-chain substituent at position 7 is a transacrylic acid group (AX pattern). Chl c_1 and c_2 differ from each other in that c_1 has a vinyl and ethyl group at positions 2 and 4, as does Chl *a*, but Chl c_2 has two vinyl groups at positions 2 and 4 (21,22). Deconvolution and integration of the methine proton region can be used to estimate the relative amounts of Chl c_1 and c_2 in a mixture of the two (23). Chemical shifts of Chl c_1 and c_2 are listed in Table 2.

Bacteriochlorophyll *b* is present in Rhodopseudomonas viridis and a few other photosynthetic bacteria. This chlorophyll is responsible for the extreme long wavelength light absorption in these organisms. The most striking feature of the Bchl *b* structure is the ethylidene side chain at position 4, which replaces the ethyl group present in Bchl *a*. The main differences in the ^{1}H NMR of Bchl *b* compared to Bchl *a* are the resonances of the ring II protons (13,24). Both the 3a and 4a protons give rise to double doublets (J_1 = 2 Hz, J_2 = 7 Hz) at low field (δ = 4.93 and 6.84 ppm). Double resonance experiments show them to be coupled to each other (J = 2 Hz) and to a high field methyl group each (J = 7 Hz). The double bond in the ethylidene group shifts the β-proton resonance to lower field. All other resonances are identical with those ot Bchl *a* (Table 3).

Green photosynthetic bacteria contain very complicated mixtures of chlorophylls whose exact structures are still under active investigation. Referred to in early publications as "chlorobium" chlorophylls because of their isolation from Chlorobium species (25) they are more often called bacteriochlorophylls *c*, *d*, and *e*. These chlorophylls are unique among all natural chlorophylls in that each appears to be a mixture of various homologs. Thus, Bchl *c*, *d*, and *e* are each families of chlorophylls containing homologous alkyl groups at positions 4 and 5, Bchl *c* and *e* in addition contain a CH_3-group at the δ methine position. Recently, another series of Chlorobium chlorophylls isolated from Chlorobium phaeoviroides has been investigated and described by Brockmann and co-workers (26,27). This family of chlorophylls (designated Bchl *e*) contains a formyl group (established by the appropriate CHO resonances in both the ^{1}H and ^{13}C

Table 3

^1H NMR Chemical Shifts[a] of Bacteriochlorophylls a and b, and the Methyl Pheophorbides of Bacteriochlorophylls c, d, and e

Proton	Bchl a[b] (0.06M)	Bchl b[b] (0.06M)	Methyl Bacteriopheophorbide c[c] (0.08M)	Methyl Bacteriopheophorbide d[d] (0.04M)	Methyl Bacteriopheophorbide e[e] (0.05M)
α	9.23[b]	9.41	9.90	9.57	10.58
β	9.50	8.93	9.41	9.28	9.42
δ	8.28	8.39	-	8.45	-
10	6.44	6.43	5.17	5.04	5.20
3	4.10	4.93 (dd)	-	-	-
4	3.86	-	-	-	-
7	4.10	4.10	4.14	4.13	n.r.
8	4.21	4.21	4.55	4.36	4.58
2a	-	-	6.47 (q)	6.31 (q)	6.56
4a	~2.5	6.84 (dd)	3.68 (q)	1.68 (t)	1.72
1a	(3.33)	(3.34)	3.48	3.38	3.53
2b	(3.00)	(2.99)	2.12 (d)	2.08	2.15
3a	1.58 (d)	1.66 (d)	3.26	3.19	11.07
4b	n.r.	2.01 (d)	1.68 (t)	-	1.20
5a	(3.44)	(3.45)	3.61	3.51	4.01
8a	1.41 (d)	1.41 (d)	1.41 (d)	1.75	1.51
10b	(3.66) (d)	(3.66)	-	-	-
δ-CH_3	-	-	3.85	-	3.86
7a	~2.5	~2.5	-	-	-
7b	~2.5	~2.5	-	-	-
7d	-	-	3.58	3.62	3.62
5b	-	-	-	-	1.92

a) Chemical shifts in δ, ppm relative to internal TMS. Chemical shifts in parantheses are assigned by interconversion with other chlorophylls. b) In pyridine-$^2H_5$ (24). c) In C^2HCl_3. This sample contained methyl groups at positions 3 and 5, and an ethyl group at position 4 (30). d) In C^2HCl_3. This sample contains an ethyl group at position 4 and a methyl group at position 5 (31). e) In C^2HCl_3. This sample contained a mixture of ethyl, n-propyl or isobutyl side chains at position 4 (3). d) Includes the proton at position 3.

NMR spectra) and, thus, has the same relationship to Bchl c and d as does Chl b to Chl a. All of the Chlorobium chlorophylls have an (α-hydroxyethyl)-substituent at position 2 characterized by a low-field quadruplet at 6.1-6.6 ppm and a high-field doublet. These chlorophylls all lack a 10 CO_2CH_3 group and are, thus, pyrochlorophyll derivatives. The AB double doublet expected for the 10-CH_2 protons is often only poorly resolved (10). The predominant esterifying alcohol in Bchl c (the chlorobium-650 of Holt (28)) is farnesol, but Strouse et al. (29) have shown that small amounts of at least five other esterifying alcohols are present. Risch et al. (30) have likewise found that the chlorophylls from Chloroflexus aurantiacus contains stearyl ($C_{18}H_{37}$), phytyl ($C_{20}H_{39}$), and geranyl geranyl ($C_{20}H_{33}$), and not farnesol as the esterifying alcohols.

To simplify the application of NMR and mass spectroscopy, it has been customary to eliminate the long-chain aliphatic alcohol by transesterifyication with methanol, a procedure during which the Mg is lost. The methyl pheophorbides of Bchl c, d, and e are more easily separated by chromatography than the chlorophylls themselves (31). Consequently, all of the available NMR data on Bchl c, d, and e is for the methyl bacteriopheophorbides. Selected data for some of the numerous homologs of the bacteriomethylpheophorbides are given in Table 3.

The methyl pheophorbides of Bchl c and d have been used by Trowitzch (32) to assign the methine chemical shifts in methyl pheophorbide a and methyl pyropheophorbide a. In the Bchl c and d derivatives, the α and β protons have distinctly different neighbors, unlike the situation in methyl pheophorbide a or methyl pyropheophorbide a, and assignment of the α and β protons is facilitated. Conversion of the hydroxyethyl group to vinyl converts methyl bacteriopheophorbide d to methyl pyropheophorbide a, and the spectral changes support the original assigment of the methine chemical shifts of methyl pheophorbide a.

Sanders and co-workers (33,34) have reported spin-lattice relaxation times (T_1), nuclear Overhauser enhancements (NOE), and long-range coupling constants for the chlorophylls. The T_1 values for the methyl protons depend largely on distance from the macrocycle and steric crowding, but the T_1 values for the methine protons are dependent on the substitution pattern (Table 4). In the absence of any information on the T_1 error limits it is difficult to judge the usefulness of relaxation times that fall in a narrow range for making chemical shift assignments. Sanders et al. (33) attribute line-width variations in the chlorophyll spectra to unresolved long-range acyclic couplings. The assignments of Sanders et al. made on the basis of T_1, NOE, and long-range coupling effects agree, however, in all particulars with previous chemical shift assignments made from ring-current and disaggregation considerations.

3. Chlorophyll Related Structures

A number of structures related to the chlorophylls have been characterized by ^{1}H NMR. These include the epimers, enol, and the Krasnovskii photoreduction product of Chl a, and this has contributed significantly to the clarification of some longstanding problems in chlorophyll chemistry.

It has long been known that Chl a and b, in the course of column chromatography on sugar, are accompanied by small, faster- running satellite bands, designated a' and b' (35). The two substances are easily interconvertible, and it was suggested by Strain (35) that a and a' were diastereomers, epimeric at carbon-10. Experimental evidence for this interpretation was provided by ^{1}H NMR studies (36)., which showed that the diastereotopic C-10 protons of a and a' had chemical shifts closely resembling those of the two C-10 protons of pyrochlorphyll a.

Table 4

Spin-Lattice Relaxation Times(s) for Chlorophylls

Protons	Chl a	Chl b	Methyl Chlorophyllide a	Methyl Chlorophyllide b	Bchl a
α	1.0	0.8	1.3	1.5	1.0
β	0.9	0.6	1.1	1.1	0.7
ô	1.0	0.8	1.3	1.3	0.6
10	1.4[a]	1.2	1.7[b]	1.6	0.8
7	0.7	-	-	-	0.5[c]
8	0.6	0.6	-	-	0.5[c]
1a	0.7	0.6	0.9	0.9	0.5
2b	-	-	-	-	1.3[d]
3a	0.6	-	0.9	-	0.3
4b	0.7	0.6	0.9	0.08	0.7
5a	0.7	0.7	1.0	1.2	0.5
8a	0.4	0.5	0.5	0.5	0.4
10b	1.0	0.8	1.1	1.3	0.8
7d	-	-	1.8	1.8	-

a) For Chl a', T_1 = 2.0 s. b) For methyl chlorophyllide a', T_1 = 2.0 s. c) Estimated by null point because of signal overlap. d) The methyl group of the acetyl function at position 2.

In Pyrochl a, the two protons at C-10 are in magnetically non-equivalent positions (37) and mixtures of Chl a and a' have peaks in their ^{1}H NMR spectra that can be assigned to analogous diastereomeric C-10 protons. Recently, an alternative interpretation for the structure of Chl a' was revived (38), which claimed that Chl a' is the enol form of Chl a. To resolve the situation, Chl a and Chl a' were separated chromatographically at 0°C, and ^{1}H NMR spectra recorded on the eluted components at low temperatures, where interconversion between a and a' is very slow. Chl a and a' are clearly seen to have C-10 protons with different chemical shifts (Table 5), thus disproving the enol hypothesis and establishing Chl a' as the epimer of Chl a (40). In further work, Hynninen and Sievers (40a) deduced from additional ^{1}H NMR data that conformational changes (puckering) of the whole macrocycle occurs with epimerization at C-10. It is interesting to note that the methine protons (and other resonances as well) in a' are easily resolved from those of a, and can often be clearly distinguished in the ^{1}H NMR spectrum of an equilibrium mixture of Chl a and a'. In an ^{1}H NMR study, Ellsworth and Storm (41) have shown that the Mg-free methyl pheophorbide a' is much less prone to isomerization than is Chl a'. In chloroform solution at room temperature, methyl 10-epipheophorbide a appears to be stable indefinitely. The difference in the rates of epimerization is attributed to conformational differences between the chlorophyll and pheophorbide. As in the case of Chl a and a', the methyl pheophorbides a and a' have significantly different chemical shifts for the methine, C-10, carbomethoxy, and 1a, 3a, and 5a methyl protons.

Ring V in the chlorophylls contains a β-keto ester function and is therefore prone to enolization. In solution, the keto/enol equilibrium in all of the chlorophylls is strongly displaced

toward the keto form, and only a small, stationary concentration of enol appears to be present. The enol has been implicated in the Molisch phase test, which establishes the integrity of ring V (42) as an intermediate in the allomerization reactions of chlorophyll (4,5), and in hydrogen exchange at position C-10 (43). Interest in the enol remains keen, for many models have been advanced involving enol participation in photosynthetic oxygen evolution (44) and in the primary events of photosynthesis (45).

Peripheral complexes are formed from pheophytins or methyl pheophorbides and Mg^{2} (46). Peripheral complex formation with Mg-containing chlorophylls does not occur to a significant extent. ^{1}H NMR shows that peripheral complexation occurs with the enol form of the β-ketoester system of ring V. The C-10 proton is no longer to be seen in the ^{1}H NMR spectrum of the peripheral complex. The ring-current induced shifts in these complexes are smaller than in the free pheophorbides. The 10-$COOCH_3$ becomes more or less coplanar with the macrocyclic systems, and the incremental low-field shift is unusually low, which is a direct result of the movement of this group into a deshielding region of the ring current. A comparison of the chemical shifts of methyl pheophorbide a and its peripheral Mg complex is shown in Table 6.

The enols of Chl a, Pheo a, and methyl pheophorbide a have been trapped as the tetramethylsilyl ethers (40). The silyated enol of Chl a is labile, and easily reverts to the original Chl a, or is converted to the silylated enol of Pheo a. The ^{1}H NMR chemical shifts of methyl pheophorbide a and its enol trimethysilylether are compared in Table 7. The largest changes are observed in the chemical shifts of the methine protons, which again implies a large decrease in the ring current in the stabilized enol.

The Krasnovskii photoreduction of Chl a was the first and is possibly the most widely studied photoreaction of the chlorophylls. Chl a dissolved in pyridine can be reversibly reduced in light by ascorbic acid to a pink photoproduct, which in the dark reverts to Chl a (48). The structure of the photoreduction product remains elusive, however, despite much study (49). ^{1}H NMR studies have now made possible a structure assignment to the photo-product (50). The photoreduction of Chl a is carried out with $^{1}H_2S$ or $^{2}H_2S$ directly in a sealed NMR tube. When $^{2}H_2S$ is used as the reductant, the already simple NMR spectrum of the photo-product is even further simplified. From the ^{1}H NMR spectra it is immediately evident that the photereduction of Chl a results in the loss of the ring current, i.e., the conjugation in the macrocycle is disrupted. Most of the lower field resonances of Chl a are shifted to substantially higher field, while the signals originating in the phytyl moiety remain substantially unchanged. The upfield shift is most pronounced in the resonances of protons closest to the macrocycle. The upfield shifts are of the order of 1.0-1.7 ppm for the vinyl and ring methyl protons, and about 6 ppm for the β and δ methine protons. As the integrated area of these two resonances indicates the presence of 2 protons, it is concluded that the Krasnovskii reaction product is β,δ-dihydro-chlorophyll a. ^{1}H NMR also establishes that the reversal of the photoreaction in the dark restores the original Chl a. The increased sensitivity of modern NMR spectrometers makes it possible to study the effect of light irradiation on chlorophyll solutions sufficiently low in concentration to permit photochemical investigations in the spectrometer probe, and such investigations very likely will open a new chapter in chlorophyll photochemistry.

4. ^{13}C NMR

All 55 carbon atoms in Chl a have had their ^{13}C NMR chemical shifts assigned. General studies of the ^{13}C NMR spectra of chlorins have been reported by Lincoln et al. (51), and Smith and Unsworth (52), and Chl a itself has been studied by a number of research groups (53-59). Assignment of the quarternary carbon atoms was carried out by Boxer et al. (56), while Goodman et al. (60) have assigned all

Table 5

^1H NMR Chemical Shifts[a] of Methine and C-10 Protons in Chlorophyll a and a' (39)

	In Pyridine-$^2H_5$		In Acetone-$^2H_6$	
Proton	Chl a	Chl a'	Chl a	Chl a'
α	9.42	9.39	9.07	9.04
β	9.58	9.56	9.40	9.37
δ	8.28	8.26	8.26	8.22
C-10	6.44	6.30	5.99	5.87

a) δ, ppm, relative to internal hexamethyl disiloxane.

Table 6

^1H NMR Chemical Shifts[a] of Peripheral Mg Complex of Methyl Pheophorbide a (47)

Proton	Peripheral Mg Complex[b]	Methyl Pheophorbide a[c]
α	8.83	9.47
β	9.01	9.75
δ	8.00	8.71
Vinyl H_X	7.77	8.08
H_A	6.06	6.23
H_B	5.87	6.05
10	-	6.61
7	4.65	4.29
8	4.10	4.42
10b	3.83	3.76
7b	3.38	3.52
5a	3.11	3.42
3a	2.95	3.21
1a	2.83	3.08
8a	1.73	1.66
4a	3.29	3.54
4b	1.39	1.53
N-H	2.44	+0.74
N-H	2.04	-1.48

a) Chemical shifts in δ, ppm relative to internal TMS. b) Recorded on a solution of pheophytin or pheophorbide a (7×10^{-3} M) in a saturated solution of $Mg(ClO_4)_2$ in pyridine-$^2H_5$. c) Spectrum of the free methyl pheophorbide a regenerated by addition of 10 μl of ^2H_2O.

Table 7

^{1}H NMR Chemical Shifts[a] of Methyl Pheophorbide a and the Trimethylsilylether of the Enol of Methyl Pheophorbide a (40)

Proton	Methyl Pheophorbide a	Trimethylsilyl Ether of the Enol[c]
α	9.25	8.19
β	9.33	8.24
δ	8.18	7.03
Vinyl H_X	7.71	7.19
H_A	5.98	5.64
H_B	5.78	5.46
10	6.20	-
7	4.16	4.66
8	3.95	3.22
10b	3.37	3.60
7d	3.26	3.07
5a	3.18	2.73
4a	3.13	2.98
1a	2.91	2.46
3a	2.90	2.37
7a	2.29	1.81
7b	2.06	1.73
8a	1.51	1.27
4b	1.42	1.22
N-H	0.79	2.26
	-1.38	2.14

a) Chemical shifts in δ, ppm. b) In benzene-$^2H_6$. Chemical shifts relative to internal hexamethyldisiloxane. c) In benzene-$^2H_6$. Chemical shifts relative to the trimethylsilyl group of the compound.

of the carbon atom resonances in the phytyl moiety. Argonne studies used Chl a enriched to 15-20% ^{13}C and Matwiyoff et al. (58,59) used Chl a of 90% ^{13}C enrichment, in both instances prepared by biosynthesis with $^{13}CO_2$ of the appropriate isotopic composition.

Chemical shift assignments for the carbon atoms of methyl pheophorbide a, methyl pyropheophorbide a, and Chl a are listed in Table 8. The original assignment by Boxer et al. (56) of C-6, C-16, and C-17 have been revised by Smith et al. (61), Wray et al. (62), and Lötjönen and Hynninen (62a). The revisions have been incorporated into Table 8. Insertion of Mg into methyl pheophorbide a produces downfield shifts of carbon resonances in rings I and III, and upfield shifts for the resonances associated with the carbon atoms in rings II and IV. Coupling constants ($J_{^{13}C-H}$) for carbon atoms bearing protons are listed in Table 9. ^{13}C NMR spectra recorded on Chl a and b containing 90% ^{13}C have been made it possible to extract a number of ^{13}C-^{13}C coupling constants, which are listed in

Table 8

^{13}C Chemical Shifts (δ, ppm)[a] of Monomeric Chlorophyll a, Methyl Pheophorbide a, and Methyl Pyropheophorbide a

Carbon No.[b]	Chlorophyll a[c]	Methyl Pheophorbide a[d]	Methyl Pyropheophorbide a[e]
1a	14.9 or 15.0	11.8	11.8
2a	133.4	128.3	128.5
2b	121.2	121.8	121.6
3a	13.5	10.7	10.8
4a	22.2	19.0	19.1
4b	20.2	17.1	17.2
5a	14.9 or 15.0	11.8	11.8
7a	33.6	31.0	51.4
7b	32.6	29.8	31.0
7c	175.1	172.6	29.8
7d	-	51.4	172.8
7	53.3	51.0	51.4
8	51.8	49.9	49.7
8a	26.1	22.8	22.9
9	191.9	189.0	195.2
10	68.2	64.5	47.8
10a	173.1	168.9	-
10b	54.3	52.6	-
α	103.0	96.4	96.4
β	110.1	103.6	103.2
γ	108.4	104.8	105.4
δ	95.2	92.6	92.4
1	137.6 or 136.4	131.1	130.7
2	141.7	135.7	135.1
3	137.6 or 136.4	135.3	135.2
4	146.6	144.2	144.0
5	137.6 or 136.4	128.3	127.4
6	164.5 (130.9)[g]	160.5 (128.3)	129.7
11	156.8	141.3	140.7
12	150.7	135.3	135.3
13	154.1	155.0	154.1
14	148.7	150.7	(149.7)
15	150.6	137.2	136.9
16	158.6 (162.4)[g]	149.0	148.2
17	~174.5 (156.3)[g]	172.6 (160.5)	159.5
18	170.2	171.4	170.4
P-1[f]	63.8		
P-2	122.1		
P-3	144.7		
P-3a	18.5		
P-4	42.4		
P-5	27.7		
P-6	39.4		
P-7	35.4		
P-7a	22.3		
P-8	40.1		

Table 8 (Continued)

^{13}C Chemical Shifts (δ, ppm)[a] of Monomeric Chlorophyll a, Methyl Pheophorbide a, and Methyl Pyropheophorbide a

Carbon No.[b]	Chlorophyll a[c]	Methyl Pheophorbide a[d]	Methyl Pyropheophorbide a[e]
P-9	27.3		
P-10	40.1		
P-11	35.5		
P-11a	22.3		
P-12	40.1		
P-13	27.7		
P-14	42.1		
P-15	30.7		
P-15a	25.2		
P-16	25.2		

a) Relative to hexamethyldisilane for chlorophyll a; relative to tetramethylsilane (TMS) for methyl pheophorbide a. b) See Figure 1 for numbering. c) In benzene/tetrahydrofuran solution. d) In C^2HCl_3 solution (14). The values in parentheses are from the reassignment of Smith et al. (61). e) References 52, 61. f) Phytyl carbon assignments are based on those of Goodman et al. (60) and are relative to internal TMS. g) Lötjönen and Hynninen (62a) have revised the assignments for carbon atoms 6, 16, and 17. Note that their ^{13}C chemical shifts are relative to TMS and for acetone-d_6 solutions.

Table 10.

Smith et al. (61), in connection with efforts to resolve the complicated questions surrounding the structure of the Bchls c, have collected extensive ^{13}C NMR data on the methyl pheophorbides derived from the Bchls c, and have presented more difinitive ^{13}C NMR assignments for methyl pheophorbide a and methyl pyropheophorbide a. Assignment is greatly facilitated by the use of chlorophylls containing 15-20% ^{13}C. For Chl a and b, this is not difficult to accomplish, but for the chlorophylls from photosynthetic bacteria, ^{13}C enrichment is a rather complicated task, which up to now has been fully solved only for Bchl a. Prospects for obtaining the other bacterial chlorophylls sufficiently enriched in ^{13}C to make a full assignment possible do, however, appear to be good.

5. ^{15}N and ^2H NMR

Full assignment of the ^{15}N chemical shifts in Pheo a and Chl a have been made by Boxer et al. (56) (Table 11). The Chl a and Pheo a derived from it contained 95% ^{15}N, incorporated by biosynthesis. The ^{15}N spectrum of Pheo a was recorded directly, but for Chl a the ^{15}N relaxation times were so long as to preclude direct observation of the spectra, and the ^{15}N spectral parameters were obtained indirectly by INDOR. Long-range coupling between ^{15}N and the methine protons was observed in the ^1H NMR spectra of both compounds, as well as the expected ^{15}N coupling with the inner protons in Pheo a. Analysis of the ^{15}N NMR spectrum of Pheo a also yielded all of the ^{15}N-^{15}N coupling constants (Table 12).

^2H NMR spectra (at 15.4 MHz) have been reported by Dougherty et al. (63)

Table 9

Coupling Constants $J_{^{13}C-^{1}H}$ for Chlorophyll a and Methyl Pheophorbide a[a]

Carbon atom	$J_{^{13}C-^{1}H}$ (Hz)[b] Chlorophyll a	Methyl pheophorbide a
Vinyl		
C-2a	150	155
C-2b	158	160
C-Phy-2	152	-
Methine		
α	150	155
β	148	155
δ	152	157
Aliphatic		
-CH		
C-7	-	129
C-8	130	~130
C-10	132	136
$-CH_2$		
C-4a	-	125
C-7a	-	130
C-7b	-	126
$-CH_3$		
C-Phy-1	153	-
C-1a	128	129
C-3a	125	126
C-4b	-	160
C-5a	128	129
C-8a	-	125
C-10b	148	148

a) Table from Janson and Katz (53). b) ±2.5 Hz.

for methyl pheophorbide a-d_{35} and Chl a-d_{72}. The line widths of 2 to 7 Hz for methyl pheophorbide a-d_{35} were broadened by quadrupole relaxation, but were still sufficently narrow to permit assignment of many of the ^{2}H resonances.

C. Applications of NMR

Many applications of NMR to structure determination and conformation studies have been made to the chlorophylls. In addition, NMR has been largely responsible for major advances in the understanding of chlorophyll behavior, and has been particularly

Table 10

Some Coupling Constants $J_{^{13}C-^{13}C}$ for Chlorophylls a and b[a,b]

Carbon atom	Chlorophyll a	Chlorophyll b
1-1a	44	44
2a-2b	68	68
3-3a	45	50
4-4a	42	42
4a-4b	34	34
5-5a	44	44
7b-7c	55	55
8-8a	46	46
10-10a	58	58

a) In Hz. b) Table from Matwiyoff and Burnham (59).

Table 11

^{15}N Chemical Shifts for Chlorophyll a and Pheophytin a[a,b]

Nitrogen	Chlorophyll a[c]	Pheophytin a[d]
N-1	163.6	102.5
N-2	183.5	218.5
N-3	166.4	110.9
N-4	224.0	272.8

a) Table from Boxer et al. (56). b) In ppm relative to external $^{15}NH_4Cl$ in 2 N HCl. c) In acetone-$^2H_6$. d) In C^2HCl_3.

effective in defining the nature of chlorophyll-chlorophyll and chlorophyll-nucleophile interactions. Some of the more significant applications of NMR in chlorophyll chemistry are now considered.

1. Exchangeable Hydrogen in Chlorophyll

A possible role for chlorophyll in the light conversion step in photosynthesis as a cyclic hydrogen donor was for long a subject of speculation. Such an hypothesis implies exchangeable hydrogen in either the ground state or excited states of chlorophyll. ^1H NMR has successfully addressed both of these problems (64,65). Both the C-10 and the δ-methine protons are readily exchangeable in Chl a, Chl b, and Bchl a. With the hydroxyl group of methanol in pyridine, hydrogen exchange at C-10 is rapid, whereas exchange at all methine carbon atoms is much slower. Exchange at the δ position is strongly

influenced by the presence of Mg. Removal of the Mg reduces the exchange rate at the methine bridge positions to very low values. That the "extra" hydrogen atoms in ring IV do not function as reducing agents in a cyclic process in photosynthesis is demonstrated by the failure to observe hydrogen exchange when green algae grown in 99.5% ^2H_2O are transferred to ^1H_2O and allowed to conduct photosynthesis. Chlorophyll extracted from these organisms is found to contain no ^1H by ^1H NMR, arguing against a role for exchangeable hydrogen at the C-7, C-8, and δ-methine positions in photosynthesis (65).

2. NMR of Paramagnetic Chlorophyll Species

The hypothesis that the primary electron donor in photosynthesis is a special pair of chlorophyll molecules was originally based on EPR lineshape analysis of the cation species that remains after electron transfer (66). A comparison of the hyperfine coupling constants of *in vitro* Chl $a^{+\cdot}$ and Bchl $a^{+\cdot}$ and *in vivo* $P700^{+\cdot}$ and $P865^{+\cdot}$ by ENDOR spectroscopy has provided considerable support for the original conclusions (67). ENDOR spectroscopy, however, is not without its problems, and the chemical manipulations required for the preparation of selectively deuterated chlorophyll derivatves required for ENDOR assignments are not trivial (68). Sanders and Waterton have, therefore, undertaken the determination of the hyperfine coupling constants of the chlorophyll cations by NMR line broadening in the fast exchange limit (69,70). NMR in principle is a far superior method for determination of the hyperfine coupling constants as assignment follows immediately from the chemical shift assignment, and no chemical manipulations are required. Only relative hyperfine coupling constants can be deduced by NMR, and a reliable value from ENDOR of at least a few of the coupling constants is required for conversion to absolute values. Waterton and Sanders (70) find that NMR results with Chl $a^{+\cdot}$ and Bchl $a^{+\cdot}$ agree well with ENDOR, but for Chl $b^{+\cdot}$, agreement is poor. The experiments of Waterton and Sanders were carried out at room temperature or above to insure the chlorophyll species were in fast exchange. The Chl $a^{+\cdot}$ cation has a half-life at room temperature of about 20 min. and at 310 K about 5 min. The variation in Chl $a^{+\cdot}$ concentration during the experiment may, thus, complicate interpretation of the line broadening data.

Table 12

^{15}N Coupling Constants in Pheophytin *a*[a]

Coupling Constant	Value (Hz)
$\lvert {}^1J_{^{15}N\text{-}H} \rvert$	98
$\lvert {}^3J_{^{15}N\text{-methine H}} \rvert$	3.0
$\lvert {}^2J_{^{15}N_1\text{-}^{15}N_2} \rvert$	2.0
$\lvert {}^2J_{^{15}N_2\text{-}^{15}N_3} \rvert$	5.7
$\lvert {}^2J_{^{15}N_3\text{-}^{15}N_4} \rvert$	1.4
$\lvert {}^2J_{^{15}N_1\text{-}^{15}N_4} \rvert$	2.5

a) Table from Boxer et al. (56).

Brereton and Sanders (70a) have studied the radical anion of bacteriochlorophyll *a* by observing differential electron transfer line broadening in the ^1H NMR spectrum of diamagnetic Bchl *a* in the presence of a small amount of chemically produced anion, formed by reaction with sodium sulfide. From the observed line broadenings, it was concluded that the protons at the α,β,γ,7,4,5a,1a,2b,3a, and 8a positions have significant hyperfine interactions with the unpaired electron in Bchl $a^{-\cdot}$, but no hyperfine coupling constants were calculated. In these experiments, no chemical evidence is presented that unequivocally establishes the chemistry

of the reaction of Bchl *a* with $S^{=}$ (see reference 50).

Closs and Sitzmann (71) have successfully determined the hyperfine coupling constants of radical cations of chlorophylls and derivatives by time-resolved CIDNP (chemically induced dynamic nuclear polarization) studies. In these experiments, polarized ^1H NMR spectra are recorded on a chlorophyll-benzoquinone system in which electron transfer is induced by a nanosecond laser light pulse. The hyperfine coupling constants determined by this procedure are relative, and require at least one ENDOR-determined value for normalization; the absolute hfc values obtained by CIDNP (after normalization) agree well with the ENDOR values. This procedure should have wide applicability. It would be desirable, however, because of the importance of these studies, to explore in more detail the chemistry of the reaction used. Benzoquinone can oxidize Chl *a* by a dark reaction, and nucleophilic attack on the cation can form alteration products. The presence of methanol in these experiments raises the possibility that 10-methoxy Chl *a* could be forming in the system during the course of the experiment, which would conceivably complicate the interpretation of the observations.

Selective line broadening by light irradiation has been shown by Boxer and Closs (71a) to occur in light-excited molecules in an NMR probe, and they have developed a method that successfully extracts information on spin distribution in photo-excited triplet states from high resolution ^1H NMR data. Some preliminary ^1H NMR data on the relative hyperfine coupling constants in light-excited methyl chlorophyllide *a* have been reported (72). Spin distribution in the Chl $a^{+\cdot}$ cation free radical appears to be considerably different from that of ^{3}Chl *a*. Relatively little spin density is to be found at the methine positions in doublet state Chl $a^{+\cdot}$, whereas the methine positions have the highest spin densities in the chlorophyll triplet.

Paramagnetic chlorophyll species are formed in the primary light conversion event in photosynthesis, and other paramagnetic species appear to be involved in the oxygen-generating side of photosynthesis. Wydrzynski and coworkers (73, 74, 75) have initiated research in which the effects of paramagnetic species on the ^1H and ^{17}O relaxation times of water are being explored in an effort to clarify the oxygen-evolving apparatus in green plants. Proton and ^{17}O relaxation rates ($1/T_1$ and $1/T_2$) have been measured in chloroplast preparations, with results that suggest that manganese in a mixture of oxidation states is normally present in dark adapted chloroplasts (74). The relaxation rates for ^1H and ^{17}O are for the most part determined by loosely bound Mn present in the chloroplast membranes; it is estimated that from one-third to one-fourth of the loosely bound Mn is present in dark-adapted chloroplasts as Mn(II), the remainder being in higher oxidation states. The Mn appears to be located in the interior of the photosynthetic membranes (73). Experiments have also been carried out in which the ^1H spin-spin (transverse) relaxation rate of chloroplast suspensions has been measured after each of a series of 2.4 μsec light flashes. The sequence of relaxation rates oscillates and has a maximum value after every fourth flash. This has been interpreted to indicate that manganese participates in the charge accumulation process during oxygen evolution (75). However, the interpretation of the experiments of Wydrzynski et al. (75) has been questioned by Robinson et al. (75a). These investigators found that the changes in the proton relaxation rate can be abolished by removal of Mn(II) from the chloroplasts with appropriate chelating agents without affecting the evolution of O_2. The manganese that is involved in the relaxation phenomena thus appears not to be the manganese involved in the literature evolution of O_2 during photosynthesis. In any event, the application of NMR techniques would appear to be of considerable promise for the study of the oxygen side of photosynthesis.

3. Chlorophyll-Nucleophile Interactions

Chlorophyll NMR spectra are remarkably solvent dependent, being very different in nucleophilic and non-nucleophilic solvents. This solvent dependence was early recognized to be a consequence of nucleophilic interactions at the central Mg atom of the chlorophylls (11,76). All lines of spectroscopic investigation support the view that Mg with coordination number 4 in chlorophyll is coordinatively unsaturated, and that there is in consequence a driving force to acquire electron donor functions (i.e., lone pair electrons on oxygen, nitrogen, or sulfur) in one or both of the Mg axial positions. Chlorophylls dissolved in typical nucleophilic, polar (Lewis base) solvents such as acetone, diethyl ether, pyridine, tetrahydrofuran and the like occur as monomeric chlorophyll with one or two molecules of solvent, depending on basicity, in the axial positions of the Mg. In weak donor solvents such as acetone or diethyl ether the Mg occurs largely with coordination number 5, and in more basic solvents such as pyridine, the coordination number of the Mg approaches 6.

The coordination interaction at Mg positions the ligand in the center of the chlorophyll macrocycle , where it is subject to the full force of the ring current. Chlorophyll is in effect a natural NMR shift reagent. The chemical shifts of the protons of a ligand bound to Mg will therefore experience an upfield ring current shift to an extent determined by the distance of a particular proton from the center and plane of the chlorophyll macrocycle.

Katz et al. (77) have made a quantitative study of chlorophyll-nucleophile interactions by observing the ring current effect on proton chemical shifts of the ligands bound to chlorophyll. The use of fully deuterated chlorophyll simplifies interpretation of the spectra. Pentacoordinate Mg(II) appears to dominate the equilibrium with aliphatic alcohols, the equilibrium constant for the formation of Chl a•CH_3OH in CCl_4 solution being K_1 = 56 l mol^{-1} (Figure 2). Georghe et al. (78) have studied the chlorophyll-water interaction by ^1H NMR. Water as a nucleophile is observed to have about the same base strength as methanol as measured by coordination to the Mg atom of Chl a.

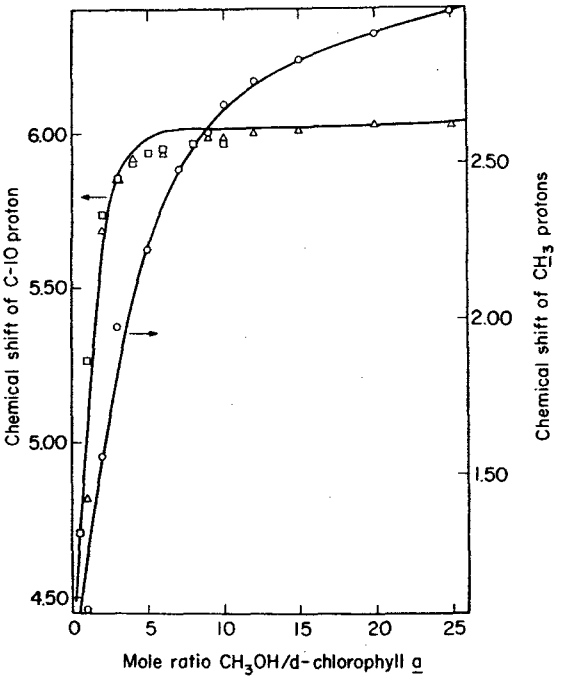

Figure 2. Chlorophyll a-methanol interaction in carbon tetrachloride solution. Chemical shifts of CH_3(O) and C-10 (Δ) protons as a function of $CH_3/^2H$-Chl a (0.064 M) ratio. Additional C-10 points (□) are derived from a methanol titration of ordinary Chl a. The solid lines are calculated curves.

Quantitative observations have been made on coordination interactions between chlorophyll and various compounds present in thylakoids and likely to be near neighbors of chlorophyll (77). As expected, β-carotene does not appear to experience any interaction with chlorophyll that results in placing any of its protons near the chlorophyll macrocycle. The ^1H NMR spectrum of β-carotene in C^2HCl_3 solution is the same in the presence or absence of ^2H-Chl a. With lutein, a dihydroxy-β-carotene, the situation is very different, for there are major

differences in the ^1H NMR of lutein when ^2H-Chl a is present. These differences are consistent with the coordination of the hydroxyl groups to the Mg of Chl a, which results in a marked difference in the magnetic environment of the ring methyl groups of the lutein. Similar changes are observed with other carotenoids carrying nucleophilic groups. Another important class of chloroplast components are the galactolipids, sulfolipids, and phospholipids. These compounds might reasonably be expected to coordinate to Mg by way of ester C=O, -OH, or $-SO_3H$ functions present in these molecules. However, in C^2HCl_3 solution, plant lipids, particularly the sulfolipid characteristically present in green plant photosynthetic membranes, show only a weak tendency to compete for coordination to Mg. This may be due to the presence in chloroform solution of both the chlorophyll and the sulfolipid as inverted micelles, in which the polar regions of both substances are buried in the center of the micelle. With current interest in the photosynthetic membrane, further studies of chlorophyll-lipid interaction would appear to merit attention.

Larry and VanWinkle (79) have made an ^1H NMR study of the interactions of Chl a and b with sym-trinitrobenzene. The interactions were studied in chloroform solution containing methanol so that the chemical shift changes must be attributed to generalized π-π forces rather than to coordination interactions at Mg. At a molar ratio of 1:1, the largest paramagnetic chemical shift differences are observed for the methylene protons bound to the oxygen of the phytyl moiety and the diamagnetic shifts observed for the α- and β-methine protons. The methyl protons at positions 3a and 5a both show diamagnetic shifts while little or no change is observed for the protons at positions 1a, 10b, 10, or δ. The trinitrobenzene protons experience a large upfield shift. These observations are consistent with the formation of a Chl a-trinitrobenzene complex in which the trinitrobenzene lies on the surface of the macrocycle with two of its nitro groups extended over the α- and β-methine protons. The shift in the phytyl may then be due to displacement of the phytyl chain from the diamagnetic zone of the macrocycle.

4. Chlorophyll-Chlorophyll Interactions

^1H NMR spectra of Chl a in non-nucleophilic solvents are very different from those in nucleophilic solvents

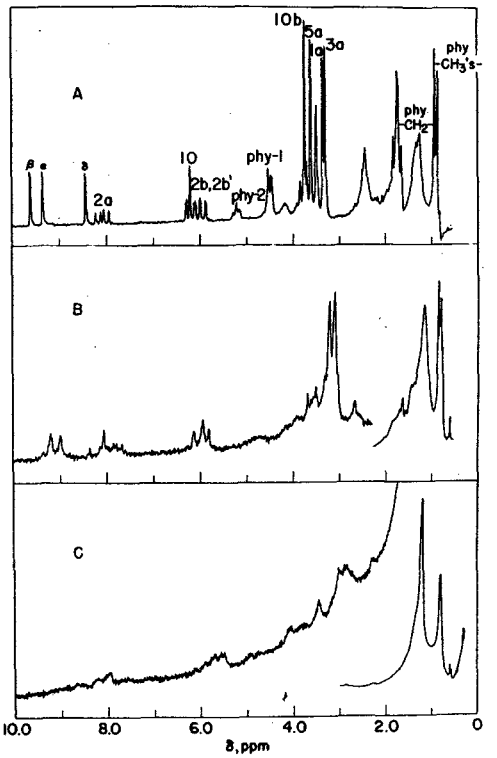

Figure 3. ^1H NMR spectra of chlorophyll a in nucleophilic and non-nucleophilic solvents. (A) in tetrahydrofuran (0.13 M); (B) in carbon tetrachloride (0.06 M); (C), in n-octane-$^2H_{18}$ (0.04 M). The monomer spectrum assignments are shown in A. Spectrum B is the spectrum of $(Chl\ a)_2$.

(Figure 3). In the polar solvent tetrahydrofuran, Chl a occurs as the monosolvate, Chl a•THF, but in CCl_4 or n-octane, Chl a occurs as a dimer or an oligomer, respectively. Evidently, in these solvents a mobile equilibrium $nChl\ a \rightleftharpoons (Chl\ a)_n$ exists. The extent

of aggregation is then determined by solvent, chlorophyll concentration, and temperature. The equilibrium constant for aggregation is very large, probably greater than 10^6 mol^{-1} l for the dimer, so that the concentration of monomer Chl a in systems free of extraneous nucleophiles is very small. In polarizable, non-nucleophilic solvents such as carbon tetrachloride, chloroform, or benzene, Chl a occurs predominantly as the dimer, whereas in difficultly polarizable , non-nucleophilic solvents such as aliphatic hydorcarbons, oligomers, $(Chl\ a)_n$, with n > 20 occur in 0.1 M Chl a solutions. It is, therefore, not surprising that the ^1H NMR spectra reflect the differences in the Chl a species present in the different solvents. ^1H NMR spectra of even the dimer are distorted, and for larger oligomers are obliterated. However, ^1H NMR studies on dimerized solutions yield important information that bears on the structure of the dimer. A detailed review of chlorophyll-chlorophyll interactions can be found in reference 14.

Addition of a nucleophile to a solution of $(Chl\ a)_2$ in CCl_4 changes the ^1H NMR spectra to an extent determined by the molar ratios of Chl a/nucleophile (11). When a molar excess of nucleophile has been added, the ^1H NMR spectrum in a non-nucleophilic solvent becomes identical to that of Chl a in a neat base. Because the chemical shifts are fully assigned in the monomer spectrum, it is possible to ascertain the positions of the corresponding proton resonances in the self-aggregated $(Chl\ a)_2$ by a titration procedure in which ^1H chemical shifts are recorded as a function of Chl a/nucleophile ratio. Such an experiment makes possible structural conclusions about the nature of the chlorophyll aggregate.

A typical titration experiment, in this case on aggregated Bchl a, is shown in Figure 4 (80). The addition of incremental amounts of the strong base pyridine to a solution of $(Bchl\ a)_n$ results in a larger change for the α-methine resonance than for the β and δ protons. The C-10 proton, the protons of the methyl groups at positions 1a, 5a, and 10b, and the CH_3-group of the

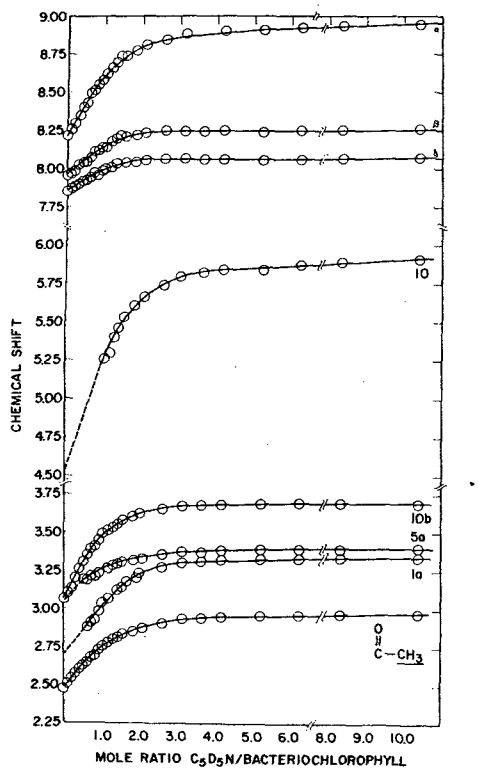

Figure 4. Titration of bacteriochlorophyll a (0.03 M) in benzene solution with pyridine-$^2H_5$. Chemical shifts in δ, ppm relative to internal hexamethyldisiloxane. See Figure 1 for proton numbering.

acetyl function at position 2 likewise experience a large downfield shift as base is added. All of the changes in chemical shift on disaggregation are to lower field, indicating that in the aggregate the resonances of many of the protons are at higher fields than in the monomer. A reasonable hypothesis for this effect is a diamagnetic ring current effect on the protons experiencing a high field shift in the aggregate. The protons fall roughly into two classes, one in which the proton resonances are essentially the same in both monomer and aggregate, and the other in which the protons are shifted to varying degrees upfield. It follows

that the chlorophyll macrocycles are only partially eclipsed in the aggregates. The downfield shifts observed in the titration experiment can, therefore, be interpreted to indicated that the protons experiencing the largest downfield shift are the ones most strongly shielded. The titration results can be presented in the form of an aggregation map (Figure 5), in which

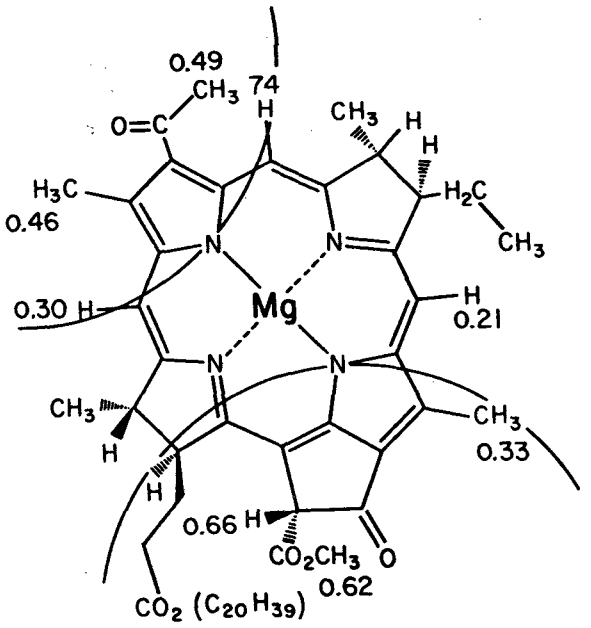

Figure 5. Aggregation map of bacteriochlorophyll a from chemical shift differences between aggregated and monomeric bacteriochlorophyll a. The numbers in the figure show the maximum differences in chemical shift (in ppm) between monomer and aggregate for the indicated protons as deduced from the titration data of Figure 4. The arcs indicate regions of macrocycle overlap in the aggregate resulting from coordination interactions by both the 2-acetyl and 9-keto carbonyl functions with Mg atoms in adjoining bacteriochlorophyll molecules.

the maximum differences in chemical shift between the aggregate and the monomer for given protons are superimposed on a structural formula. For Bchl a aggregates, two regions of overlap are evident, one in the vicinity of the keto C=O function, the other near the acetyl C=O group. Both of these groups must be acting as nucleophiles to the Mg atom(s) of other chlorophyll molecules. The presence of two donor functions in Bchl a considerably complicates matters, and whether there are two populations of dimers in this system is still uncertain.

Although there is convincing evidence from IR spectroscopy that it is the keto C=O group in ring V that is the principal donor in the coordination interaction between Chl a molecules (12,81), Fong and Koester (82,83) proposed a symmetrical (parallel) structure for (Chl a)$_2$ in which the carbomethoxy C=O functions are used as donors to Mg. Additional NMR evidence on the relative donor strengths of the oxygen functions in Chl a is now available from a detailed comparison of the aggregation behavior of Chl a and Pyrochl a (a Chl a derivative lacking a carbomethoxy group at C-10, see Figure 1), from a comparison of the ^{13}C chemical shifts in Chl a•L$_1$ and (Chl a)$_2$, and from an examination of the aggregation behavior of desoxomesochlorophyll a (a Chl a derivative in which the keto C=O function at position 9 is replaced by 2H).

A titration experiment on a chlorophyll dimer or oligomer relates the chemical shifts of the protons (or ^{13}C) in the aggregated species to that in the monomer, where the chemical shifts are fully assigned. The aggregation map so constructed, thus, defines the ring current shifts resulting from aggregate formation. The results of titration experiments with (Chl a)$_2$ and (Pyrochl a)$_2$ are shown in Figures 6 and 7, respectively. The numbers superimposed on the monomer structure give the chemical shift difference between the corresponding proton(s) in the dimer and the monomer. A positive sign indicates that the chemical shift of the particular proton in the dimer is at higher field than in the monomer.

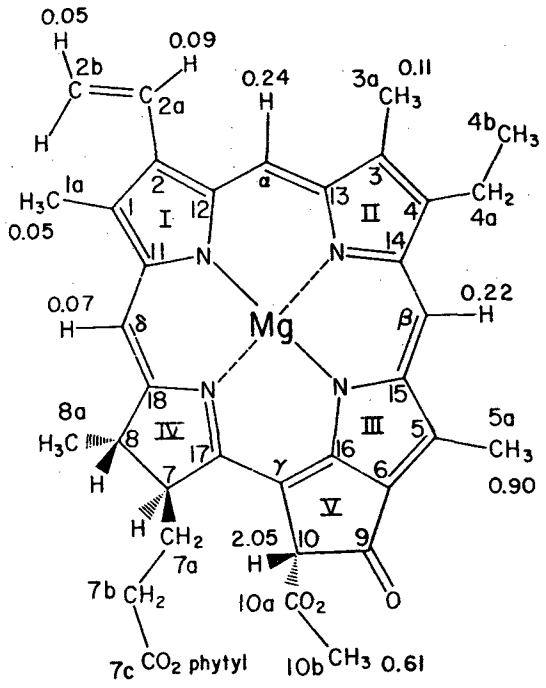

Figure 6. ^1H NMR titration of chlorophyll a (0.06 M) in carbon tetrachloride with pyridine-$^2H_5$. Chemical shift differences (δ, ppm) between dimer and monomer [$\Delta(\delta) = \delta_{monomer} - \delta_{dimer}$] are positive for upfield shifts in the dimer (14).

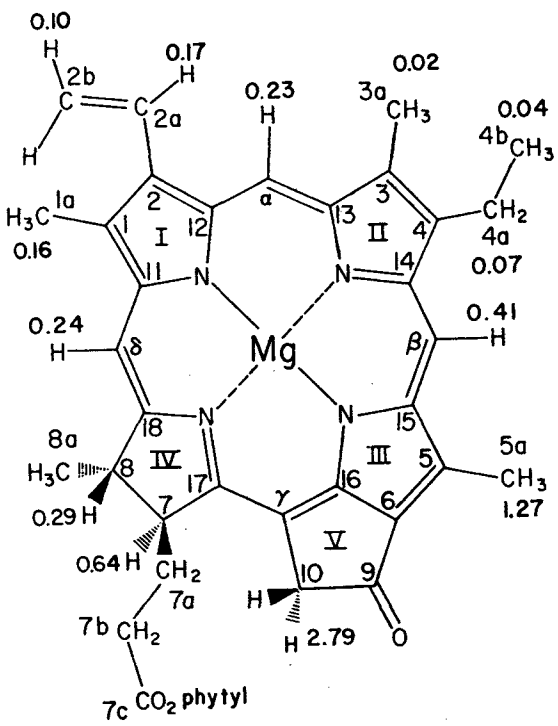

Figure 7. ^1H NMR titration of pyrochlorophyll a (0.06 M) in carbon tetrachloride with pyridine-$^2H_5$. Chemical shift (δ, ppm) differences [$\Delta(\delta) = \delta_{monomer} - \delta_{dimer}$] are positive for upfield shifts in the dimer (14).

In both Figures 6 and 7, all of the shifts in the dimers are to higher field, suggesting that on the average, all of the protons in the dimer are in the shielding region of the partner macrocycle. A carefully constructed model of the Fong dimer structure put together with carbomethoxy C=O interactions shows that for such a structure the 1a, 3a, 4a, 10b, α-methine, and vinyl protons are all in the deshielding zone of the adjacent macrocycle, which is not what is observed. The results from the titration experiments, therefore, provide no support for a dimer with a parallel structure cross-linked by carbomethoxy C=O interactions. A perpendicular structure for the dimer generated by the keto C=O···Mg interactions as suggested by Shipman et al. (84) appears, however, to be consistent with the experimental findings. A similar conclusion has been reached by Georghe et al. (78).

The largest differences in chemical shifts between dimer and monomer are in the vicinity of ring V, and the ring current effects in (Chl a)$_2$ and (Pyrochl a)$_2$ are qualitatively similar. As Pyrochl a has no carbomethoxy group, it is difficult to avoid the conclusion that it is the keto C=O group that is the principal donor function in both cases. The incremental shift in the C-10 protons of (Pyrochl a)$_2$ is substantially larger than in (Chl a)$_2$, which indicates that ring V in (Pyrochl

a)$_2$ comes closer to the center of the binding macrocycle than it does in (Chl a)$_2$. Indeed, from the incremental chemical shifts, (Chl a)$_2$ appears to be less stable than (Pyrochl a)$_2$, and the steric hinderance from the 10-carbomethoxy group is a destabilizing influence rather than the driving force in dimerization.

A Chl a derivative in which the carbomethoxy group is present but the 9-keto group is reduced to -CH_2 has been synthesized by Scheer (85). In this synthesis, the vinyl group and the double bond in the phytyl chain are both hydrogenated, but this is not expected to have any significant effect on the donor properties of the molecule. As judged from the ring-current induced chemical shift of the C-10 protons (Figure 8) the aggregation

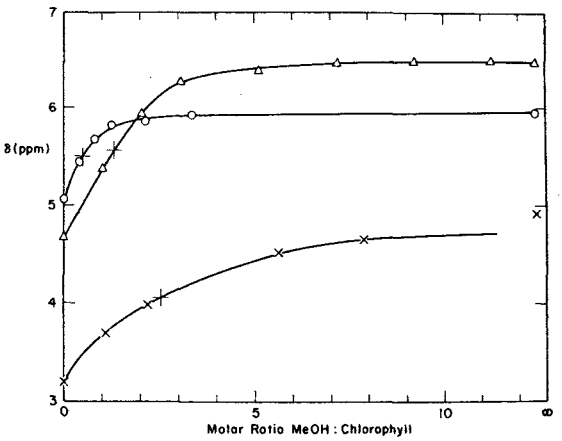

Figure 8. ^{1}H NMR titration experiment that compares aggregation in (Δ), ^{2}H Chlorophyll a (0.15 M); (0), desoxomesochlorophyll a (0.59 M); and (x), ^{2}H-pyrochlorophyll a (0.049 M). Only the chemical shift (δ, ppm) of the C-10 protons is shown. Both deuterio-chlorophylls had ^{1}H at position C-10. The 9-desoxomesochlorophyll a, which lacks a 9-keto function, requires the smallest ratio of nucleophile/chlorophyll for complete disaggregation.

strength is greatest in (Pyrochl a)$_2$, which lacks a carbomethoxy group, and weakest in 9-desoxomesochlorophyll a, which has a carbomethoxy group but no keto C=O function.

^{13}C NMR has provided direct evidence for the participation of the ring V keto function in dimerization (54,55). Figure 9 shows the results of a ^{13}C NMR titration experiment on (Chl a)$_2$. The incremental ^{13}C chemical shifts are again a function of ring current effects in the dimer, but superimposed is a deshielding effect expected for the carbon atom of any carbonyl function participating in a coordination interaction. By far the largest deshielding is observed for the carbon atom in the keto function. The carbonyl carbon in the carbomethoxy group experiences an upfield shift in the dimer. This upfield shift in the carbomethoxy carbonyl carbon effectively excludes this function from consideration as a donor group. The downfield shift in the carbonyl carbon atom of the propionic acid side chain is also observed in all of its immediate neighbors, and this is consistent with a ring current rather than a coordination origin. Although deshielded, the propionic ester C=O resonance is sharp, suggesting that it enjoys freedom of motion and, thus, does not participate to a significant extent in dimer formation.

All of the available evidence thus focusses on the keto group as the principal donor function in Chl a. A similar conclusion has been reached by Rasquain et al. (86) from an ^{1}H NMR study of the aggregation of protochlorophyll a in non-nucleophilic solvents. The relative donor strengths in Chl b, and Bchls a, b, c, d, and e, all of which have donor functions in addition to the 9-keto group still remain to be established. As in the case of Chl a, ^{13}C NMR is expected to be the method of choice.

In a system at room temperature containing both monomer and dimer Chl a, only one set of resonance lines can be observed, which implies an averaging process rapid on the ^{1}H NMR time scale. For Pyrochl a, the ^{1}H NMR spectrum shows comparatively sharp resonances at room temperature and above. Decreasing

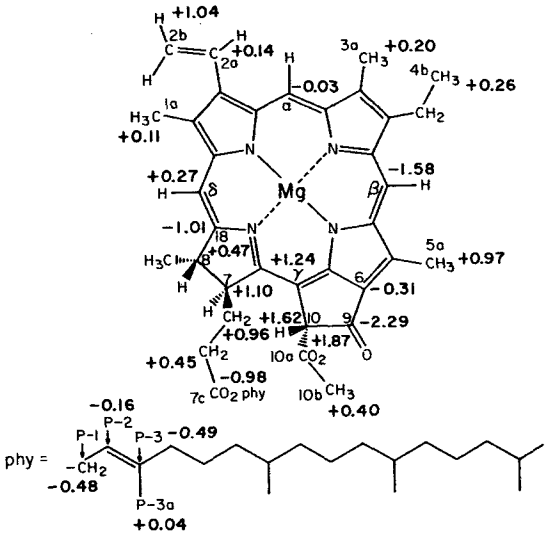

Figure 9. ^{13}C NMR titration of chlorophyll a dimer. The incremental chemical shifts $[\Delta(\delta) = \delta_{monomer} - \delta_{dimer}]$ (δ, ppm) are shown for various carbon atoms in the molecule. A negative sign indicates the ^{13}C chemical shift of the indicated carbon atom is at lower field in the dimer (55).

temperatures lead first to increased line broadening, but below -35° C the lines gradually sharpen and split into a multitude of resonances. Such behavior is typical for an exchange process, and the line broadening at room temperature and somewhat below shows that (Pyrochl a)$_2$ is close to coalescence in this temperature range. The room temperature ^1H NMR spectrum of (Pyrochl a)$_2$ represents an average conformation to which at least two but probably several more conformations contribute. An attempt to define more precisely the structures of the conformers of (Chl a)$_2$ by a lanthanide-induced chemical shift study of Chl a in CCl_4 has met with indifferent success because the shift reagent, itself a good electrophile, seriously perturbs the (Chl a)$_2$ equilibria and the dimer present in the system experiences changes in conformation (87).

5. Photoreaction Center Models

There is considerable evidence that the primary electron donor in the photoreaction center of both green plants and photosynthetic bacteria is a special pair of chlorophyll molecules (88). Possible structures for the chlorophyll special pair have been suggested (89-93) and, despite the many unresolved questions remaining about the structure of the *in vivo* photoreaction center, chlorophyll special pair models have been synthesized from Pyrochl a (91), Chl a (94), and Bchl a (95). These particular models consist of two macrocycles covalently linked through their propionic acid side chains. In the absence of extraneous nucleophiles, the covalently linked pairs occur in an open configuration, but in the presence of nucleophiles such as water or ethanol, which are capable of coordination to Mg and simultaneous hydrogen-bonding to ring V keto function, folding of the linked pair occurs to form species that mimic the salient features of *in vivo* photoreaction centers. ^1H NMR has been used to demonstrate that in the folded configuration the linked pair has the structure suggested in references 91 and 92. Wasielewski et al. (95) have shown that in the folded covalently linked Bchl a special pair model, the 5a-CH_3 and the C-10 proton experience substantial upfield shifts, while the 4b methyl group is shifted downfield. This behavior closely parallels that observed by Boxer and Closs (91) in the Pyrochl a linked pair. As the chemical shift in the methyl group of the acetyl function in Bchl a is not perturbed by folding, it appears that only the keto group is involved in the folding operation (Figure 10). All of the ^1H NMR evidence, thus, supports the structures proposed by Boxer and Closs (91) and Shipman et al. (92).

Recently, Boxer and Bucks (96) have linked through a covalent bond a

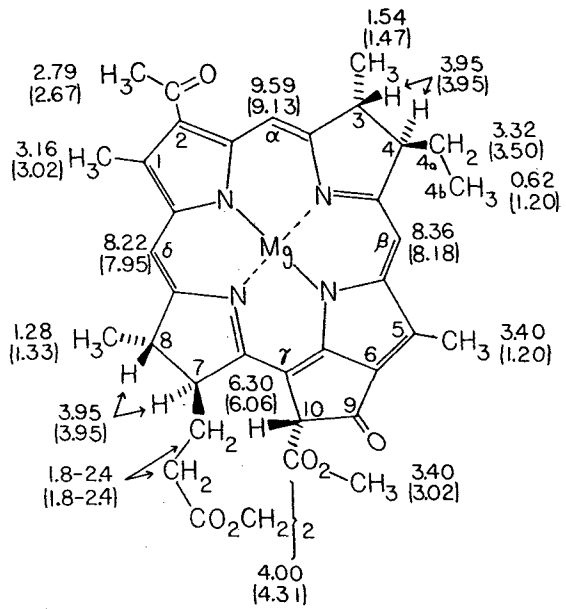

Figure 10. ^1H NMR titration experiment that compares the chemical shifts (δ, ppm indicated in parentheses) in the open (in 10% pyridine-$^2H_5$ in benzene-$^2H_6$ solution) configurations of the photoreaction center bacteriochlorophyllide a special pair model bis(bacteriochlorophyllide a) ethylene glycol diester. The 5a and 10b methyl groups are strongly shielded in the folded configuration (94).

pyropheophytin macrocycle to a pair of covalently linked Pyrochl a molecules. The chemical shift changes observed on folding are consistent with a symmetrical, rapidly-averaging, folded configuration in which the pyropheophytin ring is positioned over the 5a-CH_3 and β-proton of the metal-containing folded pair. ^1H and ^{13}C NMR can be expected to play an increasingly important role in defining the structures and properties of these and other model systems now under development.

6. Biosynthesis Studies

In spite of the current interest in chlorin biosynthesis, NMR methods have not been widely used. Presumably, this reflects the lower sensitivity of NMR methods as compared to ^{14}C tracer techniques. NMR, however, is capable of providing biosynthesis information difficult to obtain by conventional tracer procedures. Applications of ^1H NMR tc Chl a and Bchl a biosynthesis have been described by Katz and Crespi (97).

All higher green plants contain Chl a and b (Figure 1). Although it is generally accepted that Chl a is the precursor of Chl b *in vivo*, the evidence from tracer experiments on this important point is not as firm as might be desired. When green algae are grown in an ^1H_2O-^2H_2O mixture, both hydrogen isotopes are incorporated. The $^1H/^2H$ ratio at different sites in the chlorophylls extracted from such organisms can readily be obtained by integration of the ^1H NMR spectra of the respective methyl pheophorbides. Except for the $^1H/^2H$ ratio in the formyl group of Chl b, and CH_3 group in Chl a in position 3 all other isotope ratios are identical in the two chlorophylls (Figure 11). The identical isotopic composition of Chl a and b at all corresponding sites provides strong evidence for the formation of Chl b from Chl a, as it would be surprising for two independent biosynthetic pathways to have identical isotope effects at all corresponding sites in the two molecules. There appears to be no significant branching in the biogenetic pathways prior to the oxidation of the methyl group at postion 3 in Chl a to the -CHO group in Chl b. The fractionation factors also indicate some possible complications in the biosynthetic pathway. If the isotopic composition of the vinyl group is compared to that of the ethyl group at position 4, no mechanism readily suggests itself whereby an ethyl group of the observed isotopic composition could be generated from a vinyl group of the observed composition even if pure ^1H were used as the reducing agent. Either the vinyl groups at positions 2 and 4 are produced by different reaction mechanisms, or protoporphyrin IX is not

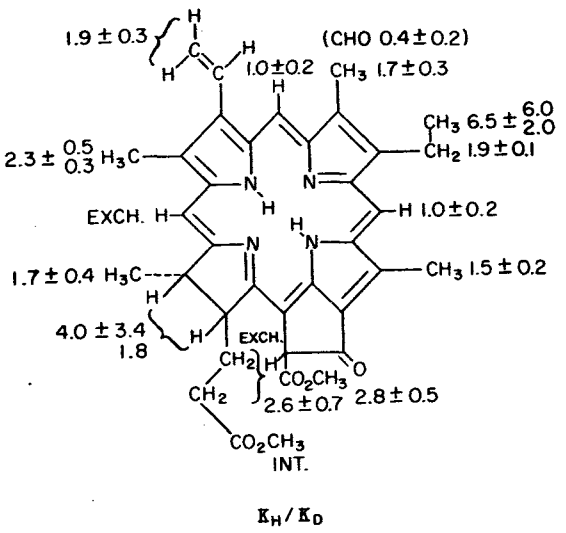

Figure 11. Isotope discrimination factors (K_{1H}/K_{2H}) as determined by integration of the ^1H NMR spectrum of methyl pheophorbides prepared from Chl a and b extracted from green algae grown in a 1:1 mixture (v/v) of ^1H_2O and ^2H_2O (97).

the only intermediate. The isotopic composition of the methyl groups at positions 3 and 8a as compared to the 1a and 3a methyl groups differ to an extent greater than the experimental error, suggesting the possibility that the methyl groups at positions 1a and 5a had a different chemical history from that of the 3a and 8a methyl groups, or that all of the methyl goups were not formed at the same time. Similar NMR experiments with Bchl a in media of mixed isotopic composition indicate similar problems requiring resolution in the biosynthetic pathways to Bchl a (98,99).

Ahrens et al. (100) have used ^{13}C NMR to study the biosynthesis of phytol. ^{13}C-enriched acetate was fed to Euglena gracilis, the chlorophyll was extracted, and the esterifying alcohols obtained by hydrolysis and thin-layer chromatography. ^{13}C incorporation from the acetate precursor was preferentially localized in 8 sites in the phytol backbone. These results demonstrate specific incorporation of [1-^{13}C]acetate, and the results are consistent with the normal terpenoid pathway to geranylgeranyl pyrophosphate, the precursor of phytol.

III. CORRINS

A. Structural Features

A corrin is a partially reduced tetrapyrrole macrocycle in which two of the pyrole rings are bonded directly to each other via their α-carbon atoms. Most research involving the corrins has been performed with vitamin B_{12} and its biologically active analogues, and has had the goal of determining how the corrin ring is biosynthesized and how the chemical and physical attributes of the corrin ring yield the biological functions of B_{12}. For this reason, we shall stress the NMR spectroscopy of this special class of corrins and shall mention the literature relating to the synthetic corrin complexes only when it applies to their biologically important counterparts.

Vitamin B_{12} (Figure 12) has three distinct components. These are the corrin ring, which binds a Co(III) ion via its four pyrrole nitrogens, a side chain that is attached to carbon-17 of the corrin ring and whose 5,6-dimethylbenzimidazole moiety binds an axial coordination position of the Co(III) ion, and a cyanide ion bound to the remaining coordination position of the Co(III) ion. The cyanide is present in the vitamin for the technical reason that it binds to the Co(III) ion more tightly than most other ligands and, thus, permits isolation of the molecule as a single species with cyanide as the only ligand in this coordination position. This substance is properly named cyanocobalamin. The coenzyme form of this vitamin in the body is 5'-desoxyadenosylcobalamin, with a 5'-deoxyadenosyl group substituted for the cyanide. The biological activity of cobalamin arises from its ability to produce a cobalt-carbon bond with a variety of ligands at the coordination position filled by cyanide in Figure

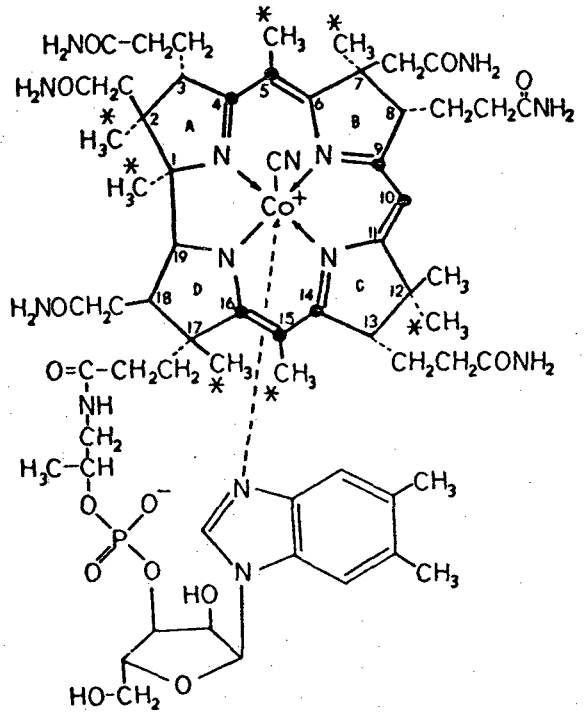

Figure 12. Structure of vitamin B_{12}. The coordination position of the CN above the corrin ring is referred to as the fifth coordination position and that to which the benzimidazole nitrogen atom is coordinated below the corrin ring is the sixth coordination position, in agreement with the nomenclature of Brodie and Poe (112). The ligands in these coordination positions also are referred to in the text as the axial ligands. The indicated carbon atoms of vitamin B_{12} are derived from carbon atom-5 of δ-aminolevulinic acid (0) and from the methyl group of methionine (*).

12. When the 5,6-dimethylbenzimidazole-nucleotide is removed so that both axial coordination position of the Co(III) ion are available for binding exogenous ligands, the complex is called a cobinamide.

B. ^{1}H NMR Chemical Shift Assignments and Applications

The 75 to 100 protons of cyanocobalamin and its various analogues give rise to a relatively large number of well resolved resonances (101-113). The very early experiments were severely limited by the sensitivity and resolution of early spectrometers, and the rather low solubility of the cobalamins and cobinamides in available solvents such as trifluoroacetic acid and deuterated dimethylsulfoxide. By comparing the spectrum of cyanocobalamin with those of its various isolated substituents and of synthetic corrin complexes, it was possible, however, to assign several chemical shift ranges to specific functional groups. The early observations yielded a number of interesting results: i) the chemical shifts of the protons of the 5,6-dimethylbenzimidazole moiety depend on whether this ring is protonated and/or coordinated to the Co(III) ion; ii) the chemical shift of the proton on the methine bridge C-10 depends on the ligands attached to the cobalt; iii) the proton on C-10 exchanges rapidly in trifluoroacetic acid; and iv) the corrin system does not exhibit an aromatic ring current.

The advent of superconducting NMR spectrometers made it possible to record useful spectra on dilute solutions in ^2H_2O, and this made possible the assignment of the entire ^{1}H NMR spectrum and the characterization of the parameters that regulate the binding of ligands to the fifth and sixth coordination positions of the Co(III) ion (Figure 12) (108-113). The proton on C-10 of both cobalamins and cobinamides, which readily undergoes electrophilic substitution reactions (114), was demonstrated to exchange with ^2H_2O under acid conditions at a rate that is intermediate between those of porphyrins and chlorins (107). In addition, it was demonstrated that the chemical shifts of the protons on the axial ligand in the sixth coordination position of the cobalt and on C-10 of the corrin ring depend on the identity of the ligand in the fifth coordination position. The length of the Co-N bond between the Co(III) ion and the dimethylbenzimidazole appears to be a

function of the nature of the other axial ligand, as does the electron density on the cobalt. The electron density of the cobalt appears to be delocalized partially to the C-10 position of the corrin ring, and indeed there is a correlation between the chemical shift of the C-10 hydrogen and the energy of the first electronic absorption band (107,112).

Since the protons on the 5'-carbon of the 5'-deoxyadenosyl moiety of the coenzyme appeared to be involved in hydrogen transfer reaction and reduction of the Co(III) ion of cobalamin appeared to occur in both hydrogen and methyl transfer reactions (115-118), the ^1H NMR spectra of these complexes were studied. The 5'-protons of the 5'-deoxyadenosyl ligand in the coenzyme were found not to exchange readily with ^2H_2O and to be magnetically nonequivalent (108,109,113), but the results of a more recent experiment with [5'-^{13}C]-5'-deoxyadenosylcobalamin raise the possibility that the earlier assignments for the 5'-protons were incorrect (119). In any case, nonequivalence of the protons of the cobalt-bound methylene group has been observed with alkyl ligands other than 5'-deoxyadenosine in the fifth coordination position (112). The cobalamins with cobalt in the Co(I) and Co(III) oxidation states were found to be diamagnetic, but the ^1H NMR spectra were found to be paramagnetically broadened when cobalt was in the Co(II) oxidation state (112). Spectral line broadening appears to be a consequence of the relatively long relaxation time of the unpaired electron of the Co(II) ion (120-122). Furthermore, it was found that the 5,6-dimethylbenzimidazole group does not bind to the sixth coordination position of the Co(I) cobalamin although it does so in the Co(III) cobalamins. The 5,6-dimethylbenzimidazole group of Co(III) coenzyme B_{12} is in a dynamic equilibrium with the coordination and uncoordinated states; the first-order rate constant for the breaking of the Co(III)- benzimidazole coordination was estimated as somewhat larger than 550 s^{-1} (113). This equilibrium is dependent upon pH and temperature, and does not appear to be associated with a paramagnetic intermediate when the benzimidazole is not coordinated (113). Methyl cobinamide appears to bind a molecule of water to its sixth coordination position, whereas 5'-deoxyadenosyl cobalamide may not (112,113). Aquocyanocorrins can exist in two isomeric forms in which the water and cyanide occupy the fifth and sixth coordination positions in either order (123,124). The only effect on the ^1H NMR spectrum of these two isomers appears to be a small splitting of the resonance of the proton on C-10.

The resonance in the ^1H NMR spectra of the cobalamins at ~0.5 ppm from TMS was assigned to the methyl group on C-1 of the corrin ring, and the shielding was shown to arise from the aromatic ring current of the 5,6-dimethylbenzimidazole moiety coordinated to the Co(III) ion (Figure 13) (107). Methyl groups of alkyl ligands bonded to the cobalt were demonstrated to resonate at even higher field than the C-1 methyl group (112,125). Comparison of a large number of cobalamins and cobinamides (112) permitted almost complete assignment of the proton resonances of the methyl groups on the corrin ring (Table 13).

C. ^{13}C NMR Chemical Shifts and Applications

The application of ^{13}C NMR spectroscopy to the investigation of B_{12} and its analogues with natural-abundance ^{13}C did not become feasible until the late 1960's. The detection and assignment of the natural-abundance ^{13}C resonances required large diameter sample tubes and pulse-Fourier transform techniques to increase sensitivity and facilitate measurements of spin-lattice relaxation times (T_1). From chemical shift comparisons, T_1 values, ^{13}C-^{31}P spin-spin coupling constants, and the results of off-resonance single-frequency proton decoupling experiments Doddrell and Allerhand were able to provide surprisingly complete assignments for the ^{13}C NMR spectra of cyanocobalamin, 5'-deoxyadenosylcobalamin and a number of their analogues (126,127). The region of the spectrum

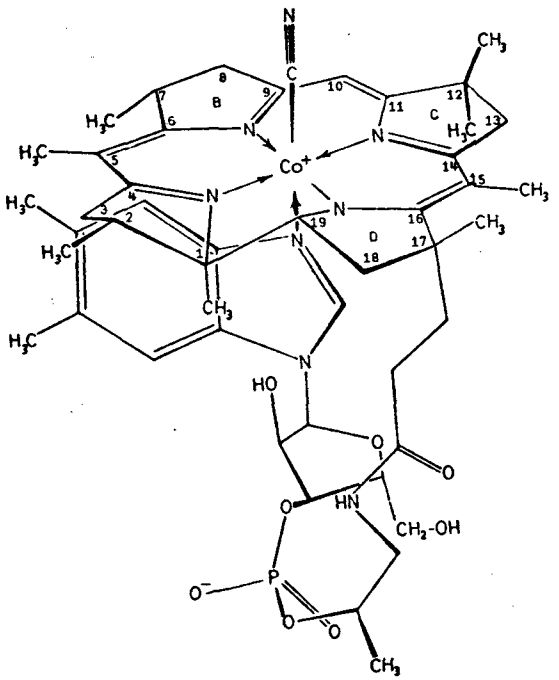

Figure 13. The orientations of the methyl group of vitamin B_{12} with respect to the corrin and 5,6-dimethylbenzimidazole rings. The pro-R methyl group at carbon atom 12 lies below the plane of the corrin ring and the pro-S methyl group lies above. The acetamide and propionamide side chains have been omitted for the sake of clarity.

from about 85 to 100 ppm upfield from CS_2 was assigned to the methine carbons that bridge pyrrole rings A, B, and C. C-10 was assigned to the resonance at 100 ppm, whereas C-5 and C-15 were assigned to the resonances at 86 to 89 ppm because they are bonded to methyl groups. The unsaturated carbon atoms of the corrin ring directly bonded to the pyrrole nitrogen atoms were assigned to a downfield spectral range that overlaps that of the amide carbonyl region, and the methyl groups were assigned to the spectral lines above 170 ppm relative to CS_2. The cyanide carbon atoms of dicyanocobalamin were assigned to the broad resonance centered at 55 ppm. Broadening was suggested to be due to scalar coupling to the ^{59}Co nucleus, which has a quadrupole moment. Unlike the ^1H NMR spectra of the two isomers of aquocyanocorrins, the ^{13}C chemical shifts of aquocyanocobyric acid were found to be very sensitive to the isomeric orientations in which the water and cyanide can bind to the cobalt. The observed differences in chemical shifts between the two isomers were attributed to both electronic differences and conformational changes of the corrin ring.

Historically, the use of specific ^{13}C labelling and ^{13}C NMR for detection of the label that followed the original assignments of Doddrell and Allerhand fell into two categories. One of these was concerned with establishing the biosynthetic pathway to B_{12}, and the other consisted of studies on the effects of axial ligands on the biological activity of B_{12}.

1. ^{13}C NMR of the Corrin Ring

The early steps in the biosynthetic pathway to the corrins and to the porphyrins had been shown to be quite similar (128-130). It was known that the 5-carbon atom in δ-aminolevulinic acid (Figure 14) was the precursor of at least seven carbon atoms in the corrin ring of B_{12}, and of eight carbon atoms in the porphyrins. The mechanism by which pyrrole ring D is incorporated into the corrins and prophyrins was the subject of much speculation, and the origin of the methyl group on C-1 of the corrin was uncertain. It was known that one of the two methyl groups on C-12 of the corrin ring arises from decarboxylation of an acetic acid side chain and that at least six of the seven remaining methyl groups on the corrin ring arise from the methyl group of methionine, but the general concensus was that the methyl group on C-1 should arise from the 5-carbon atom of δ-aminolevulinic acid, as does the δ-bridge carbon atom of the porphyrins (Figure 14). For technical reasons, this latter point could not be proven with ^{14}C-labelling experiments (130).

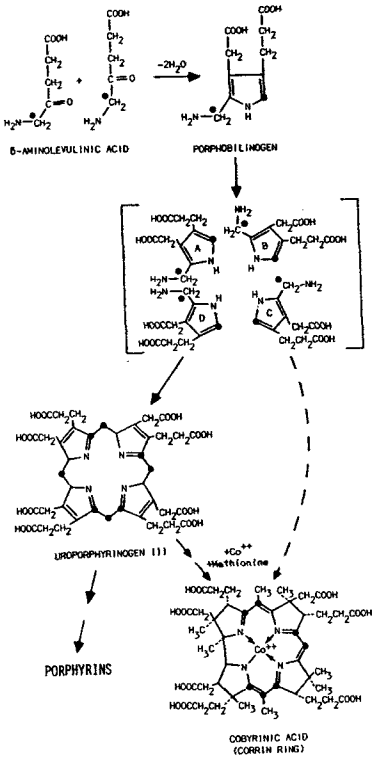

Figure 14. The labelling pattern that is expected when the ring structures of porphyrins and corrins are biosynthesized from δ-aminolevulinic acid that is labelled isotopically in carbon atom 5 (O). The curving dashed arrow represents the early hypothesis that the corrin ring was biosynthesized directly from a linear tetrapyrrole intermediate. It has since been demonstrated that uroporphyrinogen III is an intermediate in the biosynthetic pathway to the biological corrins (48-50).

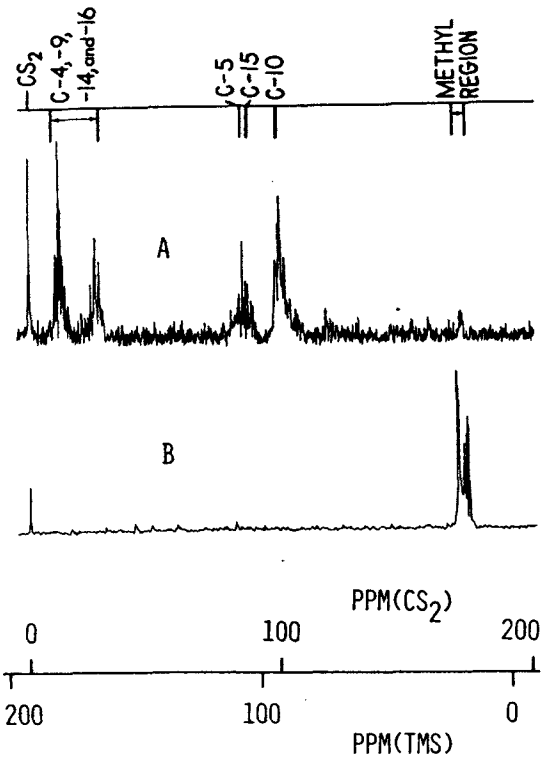

Figure 15. The proton-decoupled ^{13}C NMR spectra of vitamin B_{12} in water. The spectrum of (A) vitamin B_{12} synthesized from δ-amino[5-^{13}C]levulinic acid; and (B) vitamin B_{12} synthesized from L-[methyl-^{13}C]methionine. CS_2 is the external reference, but the chemical shift is presented in ppm relative to both CS_2 (top scale) and tetramethylsilane (bottom scale) to permit easier comparison of spectra in the references (see text).

To overcome this complication, we added δ-amino[5-^{13}C]levulinic acid to a culture of Propionibacterium shermanii, isolated the B_{12} produced as cyanocobalamin, and recorded its ^{13}C NMR spectrum (Figure 15A) (131). This spectrum confirmed the labelling pattern discovered by radiotracer techniques and confirmed the assignments for these carbon atoms by Doddrell and Allerhand (126). From the ^{13}C-^{13}C spin-spin splitting patterns, we distinguished between carbon atoms 5 and 15 of cyanocobalamin and demonstrated that carbon atom 5 resonates at lower field. The complex ^{13}C-^{13}C spin-spin splitting pattern observed for C-15 provided direct evidence that the pyrrole ring is turned over during its incorporation into the corrin ring, as also occurs in porphyrin biosynthesis. This experiment yielded the unexpected result that the methyl group on C-1 of the corrin ring did not contain an appreciable amount of ^{13}C-label. Similar results were

obtained by Scott et al. (132), who also assigned the ^{13}C resonances of the methylene carbon atoms in the acetamide and propionamide side chains of the corrin ring of cyanocobalamin and the methyl group on C-12 of the corrin ring that arises from decarboxylation of an acetic acid side chain (132,133). It is interesting to note that this methyl group on C-12, rather than the methyl group on C-1 as assumed by Doddrell and Allerhand, resonates at abnormally low field compared to the other methyl carbon atoms in cyanocobalamin. This has been ascribed to the lack of a γ effect for this one methyl group (see below) (133). Specific ^{13}C chemical shift assignments were reported recently for the carbon atoms of several derivatives of cyanocobalamin (134).

Realization that the methyl group on C-1 of the corrin ring does not arise from the 5-carbon atom of δ-aminolevulinic acid led to the immediate assumption that seven, rather than six, methyl groups arise from the methyl group of methionine. When Scott et al. (132,133) added L-[methyl-^{13}C]methionine to a culture of Propionibacterium shermanii and isolated the B_{12} produced as cyanocobalamin, the methyl region of the ^{13}C NMR spectrum was found to exhibit only six enhanced ^{13}C resonances. However, addition of excess cyanide to the sample to produce dicyanocobalamin resulted in the appearance of a seventh peak in the upfield ^{13}C NMR spectrum, and this was taken as proof that the methyl group on C-1 of the corrin ring arises from the methyl group of methionine. Brown et al. (135) reproduced this work, and also demonstrated that protonation of the benzimidazole ring and substitution of a water molecule at the sixth coordination position of the cobalt also produces seven enhanced resonances in the upfield region of the ^{13}C NMR spectrum (Figures 15B and 16). In addition, single-frequency proton-decoupled ^{13}C NMR spectra were recorded for all three of these cobalamin complexes, which permitted assignment of all seven enhanced resonances on the basis of the existing assignment of the methyl groups in the ^{1}H NMR spectrum of B_{12} (Figure 17 and Table 13).

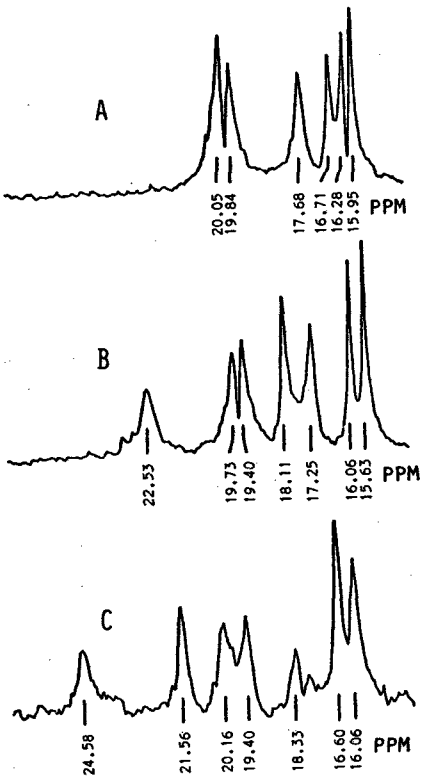

Figure 16. The proton-decoupled ^{13}C NMR spectra of vitamin B_{12} synthesized from L-[methyl-^{13}C]methionine. The spectra were obtained in water (A), in 0.1 M KCN (B), and in aqueous HCl (pH > 1) (C). The chemical shift values are given in ppm downfield from tetramethylsilane.

This latter experiment pointed out one of the apparent anomalies in the ^{1}H NMR spectrum of cobalamins. The laboratories of both Scott and Battersby demonstrated that the methyl group of methionine gives rise to the pro-R methyl group on C-12 of the corrin ring (i.e., the methyl group cis to the propionamide side -chain on C-13, marked by an asterisk in Figure 12). Scott et al. (133) produced dicyanocobinamide and dicyanoneocobinamide, in which the configuration of C-13 in ring C is reversed (136), from cyanocobalamin that had been biosynthesized with L-[methyl-^{13}C]methionine. The ^{13}C resonance of the enriched methyl group of

the dicyanocobinamide was found to resonate at higher field than that of the dicyanoneocobinamide. This shielding was attributed to the steric interactions between the labelled methyl group and the adjacent propionamide side chains, i.e., the γ effect (137,138). Battersby et al. (139,140) performed the same labelling experiment but isolated the imide of ring C by ozonolysis and compared the ^{1}H and ^{13}C NMR spectra of this isolated ring with those of a chemically synthesized standard. By combining the assignment of Scott and Battersby for the ^{13}C resonances of the methyl groups on C-12 with the single-frequency proton-decoupling experiments, it is possible to assign the proton resonances of these two methyl groups, as shown in Table 13. The assignment of the peak at 120 ppm for the protons of the pro-S C-12 methyl group, which lies above the corrin ring in Figure 13, agrees with the observation that this peak does not change position when cyanide is substituted for the benzimidazole ring in the sixth coordination position of the cobalt below the corrin ring. However, this assignment leads to the unexpected conclusion that of the two methyl groups on C-12 of cyanocobalamin, the one lying above the aromatic benzimidazole ring is the less shielded. In the ^{1}H NMR spectrum of dicyanocobalamin, the C-1 methyl group is deshielded by displacement of the benzimidazole from the cobalt, and one other methyl group (which is the C-2, C-7, pro-R C-12, or C-17 methyl group) is more shielded, so that its chemical shift is the same as that of the pro-S C-12 methyl protons (Table 13). These apparent anomalies may be explained by anisotropic shielding properties of the bound cyanide, but this has yet to be demonstrated fully.

The subsequent ^{13}C NMR experiments involving the corrin ring have utilized the spectral assignments presented above for studying the incorporation of various ^{13}C-labelled intermediates into the corrin ring. This work has led to the discovery that the methyl groups of the corrin ring that arise from methionine are incorporated without proton exchange (141-143), and that both

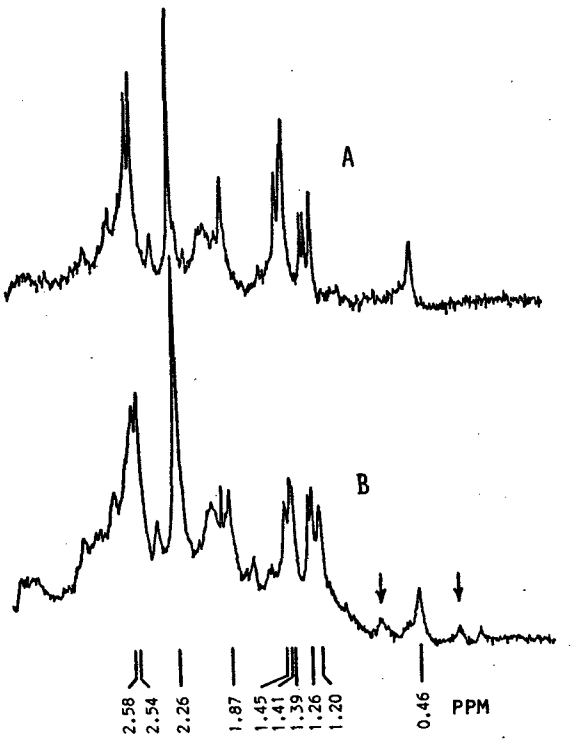

Figure 17. The ^{1}H NMR spectra of vitamin B_{12}. A spectrum of vitamin B_{12} with natural abundance ^{13}C; vitamin B_{12} synthesized from L-[methyl-^{13}C]methionine. The chemical shift values are given in δ, ppm downfield from tetramethylsilane. The two arrows about the resonance 0.46 ppm indicate the satellite peaks due to ^{13}C-^{1}H spin-spin splitting.

uroporphyrinogen III and sirohydochlorin are intermediates in the biosynthetic pathway to corrins (144-147). Since this work has been the subject of recent reviews (148-150), it is not further discussed here.

2. ^{13}C NMR of the Axial Ligands

^{13}C NMR spectroscopy has been used to investigate $-^{13}CN$, $-^{13}CH_3$ (125), $-^{13}CH_2-^{13}COOH$ (151) and [5'-^{13}C]-5'-deoxyadenosine (119,152) bonded to the fifth and sixth coordination positions of coba cobinamide, and

Table 13

^1H NMR and ^{13}C NMR Chemical Shifts of Vitamin B_{12} and ^1H Chemical Shifts at which Nuclear Overhauser Enhancement (NOE) is maximum for ^{13}C Resonances.

	Chemical Shifts in δ, ppm from TMS in H_2O, 0.1M KCN and HCL aq (pH<1)								
	H_2O			KCN			HCl		
	1	2	3	4	5	6	7	8	9
Position of Methyl Group in Vitamin B_{12} (see Figs. 12,13)	Assigned ^1H NMR	^{13}C NMR	NOE	^1H NMR	^{13}C NMR	NOE	^1H NMR	^{13}C NMR	NOE
C-1	0.46[b]	20.05[g]	0.46	1.44	22.53	1.44	1.81	24.58[g]	1.8
C-12 (Methyl group trans[c] to propionamide on C-13	1.20			1.26			1.15		
Methyl of aminopropanol	1.26[b]			1.31			1.20		
C-2[d], C-7[d], C-12, (cis[c] to propionamide on C-13), and C-17[d]	1.39	17.68[g]	1.39	1.22[e]	18.11[g]	1.24	1.31	19.40[g]	
	1.41	16.71[g]	1.41	1.37	17.25[g]	1.41	1.54	18.33[g]	(1.3-1.6)[f]
	1.45	20.05	1.48	1.46	19.40	1.46	1.59	20.16	
	1.87	19.84	1.88	1.74	19.73	1.74	1.81	21.56	1.8[e]
Methyl groups of benzimidazole	2.26[b]			2.36			2.35		
				2.38					
C-5 and C-15	2.54[b]	16.28[h]	2.54	2.26	15.63	2.23	2.38	16.06	2.40
	2.58[b]	15.95[h]	2.60	2.30	16.06	2.59	2.40	16.60	2.44

a) The nuclear Overhauser enhancement data were obtained by single-frequency experiments by Brown et al. (135). b) Chemical shifts assigned by Hill et al. (106,107). c) ^{13}C chemical shifts assigned by Scott et al. (133) and Battersby et al. (139). d) Individual assignments cannot be made among the ^1H NMR peaks of these methyl groups. However, the ^{13}C-resonances are related to the ^1H NMR assignments as determined by NOE. ^1H chemical shifts assigned by Brodie et al. (112). e) We cannot differentiate the assignments of these two proton resonances. f) Three chemical shifts were found in the region of 1.3-1.6 ppm. However, no assignments of the proton resonances to the ^{13}C resonances can be made. g) The ^{13}C resonances of these three methyl groups appear to shift to lower field when the dimethyl benzimidazole group is replaced by a less bulky CN or H_2O ligand in the sixth coordination position. h) Hogenkamp et al. (152) have assigned δ16.28 to the C-5 carbon atom.

epicobalamin (153). The use of ^{13}C-labelled cyanide confirmed the assignment of Doddrell and Allerhand (126) for the cyanide carbon in the natural abundance spectrum of cyanocobalamin and provided evidence that aquocyanocobinamide binds the cyanide and water ligands in two isomeric orientations, as does aquocyanocobyric acid (127). ^{13}C-cobalamin was observed to yield one relatively sharp enhanced ^{13}C NMR resonance in the upfield region of the spectrum, with a chemical shift dependent on the pH of the solution. This pH dependence was attributed to displacement of the 5,6-dimethylbenzimidazole moiety by water at low pH. $^{13}CH_3$-cobinamide gives rise to two enhanced ^{13}C resonances of equal intensity in the upfield region of the spectrum. These have been assigned to methyl groups bound to two different isomers in the fifth and sixth coordination positions of the cobalt, and the fact that the two resonances are of equal intensity has been taken to mean that there is no steric interference against the binding of a methyl group to either coordination site.

In contrast to the observation of Doddrell and Allerhand (126), the ^{13}C resonances of both the $-^{13}CN$ and $-^{13}CH_3$ ligated to cobalamins or cobinamides were found to be relatively narrow. This was attributed to a quadrupolar contribution that dominates the spin-lattice relaxation rate of ^{59}Co and thereby obliterates the cobalt-carbon spin-spin splittings. Chemical exchange of the methyl group of methylcobalamin and methylcobinamides was found not to contribute to the line width of the methyl ^{13}C resonance, but chemical exchange between the "base on" and "base off" forms of methylcobalamin at pH values near the pK_a of the dimethylbenzimidazole moiety was found to have a marked effect on the line width of the methyl ^{13}C resonance.

From the long T_1 value of the methyl group of methylcobalamin it was concluded that rotation about the carbon-cobalt bond is rapid and unrestricted. The same is not true, however, for the carboxymethyl ($-^{13}CH_2-^{13}COOH$) and 5'-deoxyadenosyl ligands. These have very short T_1 values, which indicate severe restriction to rotation about the carbon-cobalt bond for both these coordinated ligands. The pK_a of the carboxymethyl ligand is unusually high ($pK_a = 7.2$), and both the restricted rotation and the high pK_a value have been attributed to hydrogen bonding between the carboxyl group and the acetamide side chains on the periphery of the corrin ring. The restricted motion of the 5'-deoxyadenosyl moiety was attributed to a combination of steric interactions and intramolecular hydrogen bonding. It is of interest to note that the T_1 values of the [^{13}C]methylcorrinoids became shorter when the benzimidazole moiety is removed from the coordination sphere of the cobalt.

The chemical shifts of the axial ligands were found to be highly dependent on the identity of the other axial ligand (the trans effect) in both the cobalamins and cobinamides. Replacement of the dimethylbenzimidazole moiety of methylcobalamin by water at low pH causes the methyl ^{13}C resonance to shift to higher field as noted above. By investigating a number of substituted methylcobinamides it was possible to make the more general observation that substitution of a weak ligand by a strong field ligand in the sixth coordination position leads to a substantial downfield shift and reduction of the $^{13}C-^{1}H$ coupling constant of the methyl group in the fifth coordination position. The highest chemical shift and J_{C-H} of the methyl ligand were observed with a molecule of H_2O in the sixth coordination position, and were found to decrease in the order H_2O > pyridine > benzimidazole > CN^- > CH_3 upon substitution of these ligands in the sixth coordination position. Both the chemical shift of the ^{13}C resonance and the $^{13}C-^{1}H$ coupling constant of the methyl group in the fifth coordination position are linearly correlated with the energy of the β-vibrational component of the first electronic absorption band.

The chemical shift of the proton on C-10 of the corrin ring (the only protonated methine bridge) correlated with the energy of the first electronic absorption band as discussed above

(107,112), and this was taken to suggest that the charge density of the cobalt might be delocalized to this methine bridge. Delocalization of charge from the cobalt has been demonstrated by ^{13}C NMR to occur with all three methine bridges (cis effect). When a weak field ligand in the sixth coordination position is replaced by a strong field ligand, all of the methine ^{13}C resonances shift to higher field. In the methylcobalamide series the methine ^{13}C resonances shift to high field in the order H_2O < pyridine < cyanide, and similar correlations have been noted with cyanocorrinoids and alkylcobalamins. The chemical shifts of the β-vibrational component of the first electronic absorption band, as in the case of the ^{13}C NMR resonance of the methyl ligand in the fifth coordination position. However, introduction of ligands in the sixth coordination position that cause the ^{13}C resonance of the methine bridges to shift to higher field cause the ^{13}C resonance of the methyl ligand in the fifth coordination position to shift to lower field.

In the epicobalamins the 5,6-dimethylbenzimidazole moiety is coordinated more strongly to the cobalt, the cobalt is more electronegative, and the carbon-cobalt bond in the fifth coordination position is stronger than in the cobalamins. These differences have been attributed to both steric and electronic changes that may result from reversing the configuration by which the propionamide side chain is bonded to C-13 of the corrin ring, but the exact mechanism appears to be sterically blocked since the intensities of the two methyl resonances arising from the two isomers of aquo[^{13}C]-cyanocobinamide are in a ratio of 95:5.

In contrast, the chemical shifts of the ^{13}C nuclei on the remainder of the periphery of the corrin ring appear to be much less affected by the charge state of the cobalt, or at least other variables appear to play more prominent roles. Axial ligands do affect the chemical shifts of the ^{13}C atoms on the periphery of the corrin ring, but the mechanism by which the effects arise may be different. For example, substitution of a small ligand for the bulky dimethylbenzimidazole ring in the sixth coordination position of the cobalamins results in downfield shifts of the ^{13}C resonances of four of the methyl groups on the periphery of the corrin ring. The most shifted of these resonances arises from the C-1 methyl group (Table 13) (135) and the least shifted has been assigned to the methyl group on the C-5 methine bridge (152). The two remaining shifted resonances (Table 13) may arise from the other two methyl groups that extend from the corrin ring on the same side as the dimethylbenzimidazole moiety (i.e., the methyl groups on C-7 and C-12 (pro-R) in Figure 13). These downfield shifts resulting from substitution of a less bulky ligand in the sixth coordination position have been attributed to a reduction in steric compression (the γ effect) (152).

On the basis of comparisons of the ^{1}H and ^{13}C NMR spectra of the various cobalamins and cobinamides that have been studied to date, it appears that there are at least three parameters that determine the biological activity of coenzyme B_{12} (5'-deoxyadenosylcobalamin) and methyl cobalamin. These are as follows: i) the charge density on the cobalt and its delocalization over the two axial ligands and the methine bridges of the corrin ring (154-160); ii) steric interactions between the dimethylbenzimidazole nucleotide and the propionamide side chains at C-8 and possibly C-13, and the methyl groups at C-1, C-5, C-7, and C-12 (pro-R) that project from the periphery of the corrin ring on the same side as this nucleotide (133,135,152,161); and, iii) hydrogen bonding between the axial ligand in the fifth coordination postion and the acetamide side chains on this side of the corrin ring. When the dimethylbenzimidazole group of the cobalamins is replaced by a weaker field ligand, the overlap between the sp^3 orbitals of the carbon atom in the fifth coordination position and the 4s and $3d_{z^2}$ orbitals that are localized in the axial bonds of the cobalt ion is expected to decrease (155). Decreased overlap is expected to increase the polarizability

of the cobalt-carbon bond, with electron density shifted from the cobalt ion to the carbon atom, and thereby weaken the cobalt-carbon bond. Mechanisms that have been suggested to cause the observed change in chemical shifts of the axial ligand in the fifth coordination position to higher field include increased electron density on the carbon atom, variations in spin pairing between p electrons, the extent of p orbital occupation, changes in radial distance of electrons from screened nuclei, and variations in excitation energies for mixing ground with excited state wave functions (125). The decrease in electron density on the cobalt ion that is expected to occur upon substitution of the dimethylbenzimidazole group by a weaker field ligand increases the demand by the cobalt ion for the electron density that is delocalized over the methine bridge carbon atoms of the corrin ring, which in turn decreases the charge density of these methine carbon atoms and shifts their ^{13}C NMR resonances to lower field. Steric interactions between the dimethylbenzimidazole nucleotide and the side chains are expected to reduce the strength of the cobalt-nitrogen bond with the benzimidazole ring, and, by the mechanism described above, to weaken the cobalt-carbon bond to the ligand in the fifth coordination position. Thus, a reason for the methylation of seven carbon atoms on the corrin ring may be to provide sufficient destabilization of the cobalt-carbon bond to the trans ligand in the fifth coordination position to make possible the biological roles of B_{12}. This possibility is supported by the observation by ^{1}H and ^{13}C NMR that such steric factors have a rather pronounced effect on the rate of benzimidazole dissociation in methyl cobalamin (162). Hydrogen bonding between the ligand in the fifth coordination position of the cobalt and the acetamide side chains that project on this side of the corrin ring appear to be negligible in methylcobalamin, but can be expected to play some role in determining the stability of the cobalt-carbon bond in coenzyme B_{12}.

D. NMR Studies with Other Nuclei

We are aware of only one investigation of cobalamins by ^{15}N NMR (163) and three with ^{31}P NMR (164-166). The ^{15}N NMR spectrum of cyanocobalamin exhibits seven resolved amide nitrogen resonances in the region 256.8-268.2 ppm, and a cyano group nitrogen resonance at 80.9 ppm upfield from external 0.1 M $^{2}H^{15}NO_3$ in $^{2}H_2O$. The four pyrrole ring nitrogen atoms were not observed, possibly because of long relaxation times, small nuclear Overhauser enhancement values, and coupling to cobalt.

The first ^{31}P NMR studies (164,165) are an attempt to use the ^{31}P atom in the cobalamins as a probe of events that occur at the corrin site of relatively low molecular weight, B_{12}-dependent enzymes such as the ribonucleotide reductases. The chemical shift of the ^{31}P atom of cobalamins in solution was found to depend on the coordination of the dimethylbenzimidazole moiety to the cobalt atom, but it is relatively insensitive to the identity of the ligand in the fifth coordination position. The line width of the ^{31}P resonance increases when the cobalt atom is reduced with d,l-penicillamine. It was demonstrated that ^{31}P NMR can be used to investigate the pH-dependent properties of cobalamin and the coordination state of cobalamins when bound to bovine serum albumin and the detergent SDS.

^{31}P NMR spectroscopy also has been used (166) to characterize a recently discovered isomeric form of vitamin B_{12}. The UV-visible absorption spectrum of this new isomer is the same as that of native B_{12}, but Mössbauer and ^{1}H NMR results (167) suggest that the new isomer has a small out-of-plane displacement of the cobalt and concomittant change in the conformation of the corrin ring and benzimidazole group. Spin-lattice relaxation measurements of the ^{31}P nucleus in both isomeric forms of the paramagnetic cob(II)alamins and diamagnetic cob(III)alamins appear to corroborate these conformational differences (166). The spin-lattice relaxation times of the ^{31}P nucleus in the cob(II)almin isomers are dominated by dipolar interaction with the

paramagnetic cobalt(II) ion. Differences in the measured T_1 values indicate that the Co(II)-^{31}P distance becomes longer when the new isomer is formed. In the diamagnetic cob(III)alamins, the spin-lattice relaxation time of the ^{31}P nucleus is determined mainly by dipolar interaction with protons on the neighboring ribose and aminopropanol groups. Variations in T_1 values of the ^{31}P nucleus in these diamagnetic complexes support the contention that the "puckering" of the corrin ring is different in the new isomer. These conformational differences appear not to alter the electronic environment of the ^{31}P nucleus since its chemical shift in the new isomer is the same as that in native B_{12}.

The ^{59}Co NMR spectrum of a cobalamin has been reported (165). Furthermore, it has been suggested that ^{59}Co nuclear quadrupole resonance spectroscopy could have great value in probing the environment about the cobalt in vitamin B_{12}, methylcobalamin, and coenzyme B_{12} (169).

E. Summary

The investigations of the biosynthetic pathway to B_{12} and the chemical and enzymatic activities of B_{12}, which stimulated the application of NMR spectroscopy to the corrins, cannot be considered complete. Now that the basic NMR techniques have been developed, the assignments of the ^{13}C resonances of the corrin ring have been made, and the chemical synthesis of potential metabolic intermediates with specific ^{13}C labels is possible (148,167), rapid advances in our understanding of his biosynthetic pathway can be expected. There continue to be reports and reviews in which chemical analogues of cobalamins are characterized with NMR spectroscopy (171-174). Complete interpretation of the ^{13}C NMR spectrum of dicyanobyrinic acid heptamethyl ester has been reported (175). A more flexible form of cobalamins with a different conformation of the corrin has been discovered (167), and analogues of cobalamins have been characterized in which various metal ions have been substituted for cobalt (104,148,176,177). Furthermore, the red and yellow, metal-free corrins that were first isolated from photosynthetic bacteria by Toohey (178,179) have now been subjected to NMR analysis (180-182). The predominant metal-free corrins that are excreted into the culture media by Rhodopseudomonas spheroides are hydogenobyrinic acid c-amide and hydrogenbyrinic acid a,c-diamide (180). These descobaltocorrinoids are formed when there is a deficiency of cobalt in the culture medium and have no known function. Broken cell preparations of Propionibacterium shermanii and R. spheroides do not insert cobalt into hydrogenobyrinic acid a,c-diamide (180). However, chemical insertion of cobalt into hydrogenobyrinic acid c-amide yields, in addition to cobyrinic acid c-amide and 13-epi-cobyrinic acid c-amide, small amount of a blue cobalt-containing corrin. This blue corrin has been identified to be 18,19-didehydrocobyrinic acid c-amide (181) and may be an intermediate in the biosynthesis of vitamin B_{12} (180).

Newer departures include characterization of the chemical reactivities of vitamin B_{12} and its derivatives. The effects of various ligands on the photolability of alkylcobinamides and coenzyme B_{12} have been determined (183-185). It has been demonstrated that mercuric ion and platinum complexes such as cis-diaminodiaquoplatinum(II) displace the benzimidazole ring from the cobalt of alkylcobalamins and/or coenzyme B_{12}. Methylcobalamin has been shown to be capable of methylating mercuric ion and $PtCl_6^{-2}$ (186-188). No chemical reactivities of various corinoid complexes were compared in a recent review (189).

Another area of ongoing interest is activity of B_{12} as an enzyme cofactor. The relative binding of coenzyme and substrate to ethanolamine ammonia-lyase has been investigated (190). Nonenzymatic modelling of the coenzyme B_{12}-dependent isomerization of methylmalonyl coenzyme A to succinyl coenzyme A has been demonstrated (191-193) and the reversible cleavage of the cobalt-carbon bond of coenzyme B_{12} by methylmalonyl CoA mutase has been observed

(194). Coenzyme B_{12} that is stereospecifically deuterated in the 5'-position (194,195) was found to lose deuterium to the solvent and to undergo scrambling of deuterium between the two diastereotopic 5'-positions in the presence of the mutase (195). The reaction appears to involve cleavage of the cobalt-carbon bond and conversion of the 5'-carbon atom into a torsiosymmetric group. The exchange reaction is catalyzed by the methylmalonyl-CoA mutase but occurs without the participation of the substrate. It has been suggested that the mechanism of diol-dehydrase might also be investigated with the use of coenzyme B_{12} stereospecifically deuterated in the 5'-position (196). These enzyme studies depend on the recent assignment of the two 5'-protons of coenzyme B_{12} to ^1H resonances at 0.6 ppm and 1.5 ppm (194,195). These assignments were made possible by the improved resolution of the new higher-field superconducting NMR spectrometers. One might expect that these investigations of enzyme mechanisms will be facilitated by other technical advances, such as, for example, the recent report that the ^1H NMR spectrum of as little as 2.5 mM vitamin B_{12} can be measured in 95% H_2O with a multipulse sequence called the 2-1-4 pulse (197). It seems safe to say that many more applications of NMR spectroscopy to the investigation of the corrins can be expected.

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