Spin-lattice Relaxation, Nuclear Overhauser Enhancements, and Long Range Coupling in Chlorophylls and Metalloporphyrins

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Methyl groups in the title series have proton spin-lattice relaxation times (T_1) which depend largely on the distance of the group from the macrocycle and their steric crowding whereas T_1 values for *meso*-protons are dependent on substitution pattern. Nuclear Overhauser enhancements and long-range coupling constants can be used to construct connectivities around the macrocycle. Together, these techniques provide unambiguous assignments of resonances and structures. Systematic linewidth variations are due to predictable contributions from unresolved couplings, short T_1 and electron-transfer effects. A non-seasonal preparation of methyl chlorophyllides is described.

We have been using n.m.r. line-broadening to determine spin-density distributions in the radical-cations of chlorophylls and metalloporphyrins.[1,2] We needed, therefore, reliable assignments of the n.m.r. spectra. It was particularly important to distinguish within (i) sets of methyl groups and (ii) sets of *meso*-protons which are in similar molecular environments and have similar chemical shifts but experience different unpaired spin densities. Conventional methods of chemical-shift argument using series of closely related compounds are insufficiently rigorous for shift differences of less than 0.1 p.p.m., whilst selective deuteriation is possible only rarely. Recent observations on aggregation shifts may have general utility in assignments.[3]

We report here [4] that spin-lattice relaxation times (T_1) and nuclear Overhauser enhancements (n.o.e.) provide the necessary rigour as a consequence of the rigidity of the porphyrin nucleus. We also describe some small long-range coupling constants which we discovered during the n.o.e. work and which can be crucial for assignments even when not resolved. These couplings, together with the T_1 effects lead to predictable and consistent linewidth variations between resonances for protons in the same molecule.

THEORY

N.m.r. absorption is a transition from a lower to an upper energy level and requires electromagnetic radiation of the correct frequency. The radiationless return to equilibrium is spin-lattice relaxation: this is characterised by a relaxation time T_1 and requires fluctuating magnetic fields of the same correct frequency.[5] These fields can arise from many sources but for protons under normal solution conditions they arise mainly from the intramolecular dipole–dipole interaction, *i.e.* other protons in the same molecule. The stronger the inter-

[1] J. K. M. Sanders and J. C. Waterton, *J.C.S. Chem. Comm.*, 1976, 247; J. C. Waterton and J. K. M. Sanders, *J. Amer. Chem. Soc.*, in the press.
[2] J. K. M. Sanders, *Chem. Soc. Rev.*, 1977, **6**, 467.
[3] R. J. Abraham, F. Eivazi, H. Pearson, and K. M. Smith, *J.C.S. Chem. Comm.*, 1976, 698; *Tetrahedron*, 1977, **33**, 2277.
[4] Preliminary communication: I. S. Denniss, J. K. M. Sanders, and J. C. Waterton, *J.C.S. Chem. Comm.*, 1976, 1059; 1977, 192.
[5] L. D. Hall, *Chem. Soc. Rev.*, 1975, **4**, 401.

action, which depends on distance and correlation time, then the greater the relaxation rate R_1 ($= 1/T_1$). Thus, the relaxation rate of a proton s caused by a proton i is given by equation (1) where r_{is} is the is distance and τ_{is} is the correlation time of the is vector (effectively the tumbling time).

$$R_1(i \longrightarrow s) \propto \tau_{is} r_{is}^{-6} \qquad (1)$$

The total relaxation rate of proton s relaxed by n neighbouring protons is therefore given by (2).

$$R_1(s) \propto \sum_{i=1}^{n} (\tau_{is} \cdot r_{is}^{-6}) \qquad (2)$$

There are two useful limiting cases. (i) For protons which have the same τ because they are attached to the same rigid framework. Equation (2) then simplifies to (3) and differences in relaxation rate between these protons depend only on interproton distances. (ii) For

$$R_1(s) \propto r_{is}^{-6} \qquad (3)$$

methyl protons, relaxation is dominated by the other protons on the carbon to which they are attached, so all r_{is} values are the same. Equation (2) then simplifies to (4) so that methyl T_1 values depend on mobility only.

$$R_1(s) \propto \tau \qquad (4)$$

Where relaxation of s is dependent only on i, then saturation of the i resonance will change the intensity of the s resonance. This is the nuclear Overhauser effect (n.o.e.), and for the proton–proton case in rapidly tumbling molecules the maximum increase in s is 50%.[6,*] If s is relaxed by more than one proton, then their relative n.o.e. contributions are roughly proportional to r^{-6} and the total enhancement is limited to *ca.* 50%. It is possible, in particular geometries of 3 (or more) spin systems, for nearby protons to show little or no n.o.e.[6] Thus the absence of an n.o.e. does not necessarily imply that the nuclei concerned do not relax each other. The presence of an n.o.e. proves that they do.

The use of T_1 values and n.o.e. values to assign spectra is most appropriate where τ and r can be predicted in advance, *i.e.* in molecules where a rigid conformation leads to well-defined inter-proton distances and where the motional freedom of a group can be estimated from inspection of the structure. Porphyrins and chlorophylls are ideally suited to this approach since their substituents are anchored to a rigid macrocycle and the protons of the substituents experience increasing mobility as their separation from the macrocycle increases.

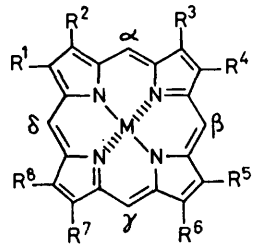
Methyl protons in molecules of this size actually relax slightly non-exponentially in a way which enables details of internal rotation to be elucidated.[8] This effect is insignificant if *initial* relaxation rate after the perturbing pulse is measured.

* In large molecules n.o.e.s can become negative.[7]

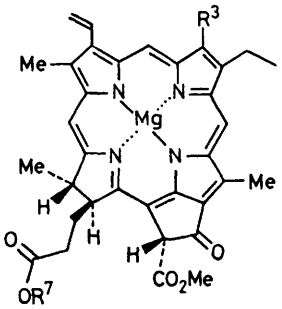
[6] J. H. Noggle and R. E. Schirmer, ' The Nuclear Overhauser Effect ', Academic Press, New York, 1971.

[7] D. H. Williams and J. R. Kalman, *J. Amer. Chem. Soc.*, 1977, **99**, 2768.

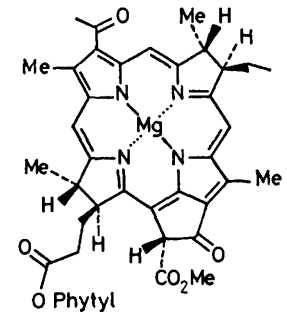
The spin–spin (transverse) relaxation time, T_2, contributes to the resonance linewidth, W, according to



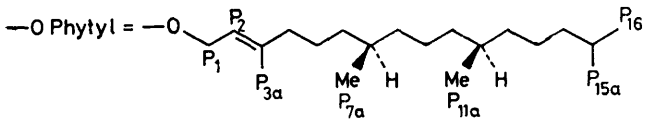
(1) R^1–R^8 = Et, M = Mg
(2) $R^1 = R^3 = R^5 = R^8$ = Me; $R^2 = R^4$ = H; $R^6 = R^7 = CH_2CH_2CO_2Me$; M = Mg
(3) As (2) but $R^2 = R^4 = CH{=}CH_2$
(4) As (3) but M = Zn



(5) R^3 = Me, R^7 = Phytyl
(6) R^3 = CHO, R^7 = Phytyl
(7) $R^3 = R^7$ = Me
(8) R^3 = CHO, R^7 = Me
(10) R^3 = Me, R^7 = H
(11) R^3 = CHO, R^7 = H



(9)



(12) Phytyl OH
(13) Phytyl O.CONH–

equation (5) and is equal to or less than T_1.[6] Resonances with short T_1 will, therefore, have linewidths greater than those with long T_1.

$$W \geqslant 1/\pi T_2 \qquad (5)$$

RESULTS AND DISCUSSION

Porphyrins (1)—(4), chlorophylls-a (5) and -b (6), methyl chlorophyllides-a (7) and -b (8), and bacteriochlorophyll-a (9) were used in this work. For the isolation and purification of large quantities of (5), (6), and (9) the dioxan precipitation method [9] was invaluable as a

[8] E. Haslinger and R. M. Lynden-Bell, *J. Magnetic Resonance*, in the press.

[9] K. Iriyama, N. Ogura, and A. Takamiya, *J. Biochem* (*Tokyo*), 1974, **76**, 901.

preliminary to chromatography. We have developed a new 'non-seasonal' preparation of (7) and (8). In aqueous acetone, the chlorophyllase of spinach beet (perpetual spinach) hydrolyses the chlorophyll-phytyl ester group to give the free acids (10) and (11) which can equilibrium amount, *ca.* 15%, of (5'). This is the C-10 epimer with the carboxymethyl group in the sterically more hindered position *cis* to the 7-propionate.

Spin-lattice Relaxation Times.—Table 1 summarises T_1 values for compounds (1)—(4) at 310 K. As expected

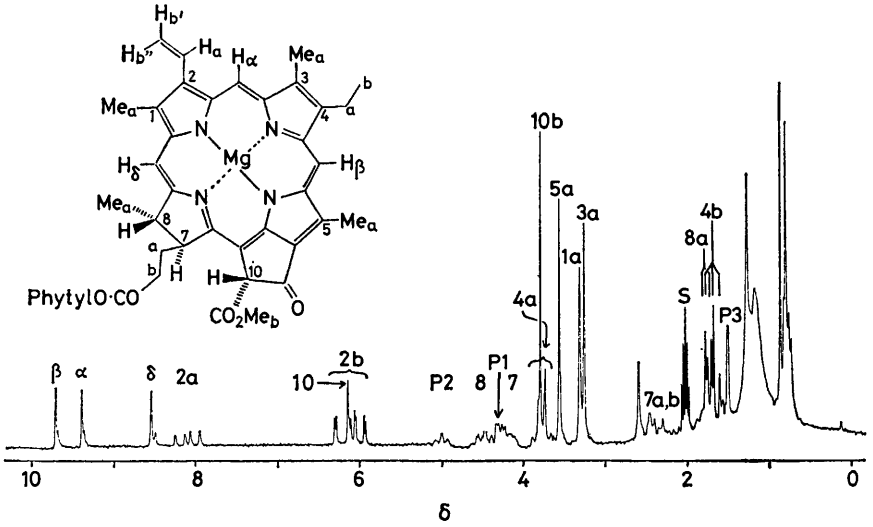


FIGURE 1 100 MHz ^{1}H N.m.r. spectrum of chlorophyll-a, (5); 20mM in [$^2H_6$]acetone

be separated chromatographically.* Esterification with diazomethane before or after separation gives (7) and (8) in good yield. Previous preparations of these compounds required chlorophyllases from the leaves of deciduous (and, in Britain, rare) plants,[10] whereas spinach beet is available throughout the year.

methyl (and methylene) proton T_1 values increase gradually with distance from the macrocycle, so that, for example, in (2) the propionate methyls (T_1 *ca.* 1.1 s) are readily distinguished from ring methyls (T_1 *ca.* 0.5 s).[1,2] This has been confirmed by deuteriation of the ester methyl group.

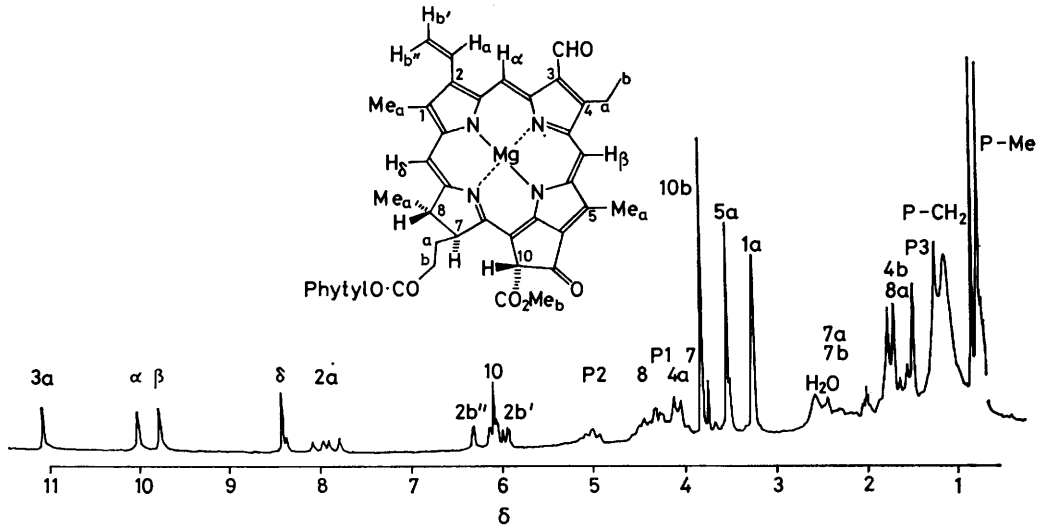


FIGURE 2 100 MHz ^{1}H N.m.r. spectrum of chlorophyll-b, (6); 30mM in [$^2H_6$]acetone

Proton n.m.r. spectra of (5), (6), and (9) are shown in Figures 1—3, together with the assignments determined in this work and the conventional numbering scheme. The spectrum of (5) demonstrates the presence of an

The *meso*-protons of (1) and γ-H of (3) and (4) have $T_1 = 0.8$ s whilst α-H, β-H, and δ-H of (3) and (4) have $T_1 = 1.0$ s.† Whether the shorter values are due to enforced proximity of the adjacent protons or other

* These acids are useful synthetic intermediates.[2]

† Partial *meso*-signal overlap in (2) prevents accurate measurement.

[10] F. C. Pennington, H. H. Strain, W. A. Svec, and J. J. Katz, *J. Amer. Chem. Soc.*, 1964, **86**, 1418.

reasons is not clear, but they are reproducible. They are also manifested by a consistently greater linewidth for the γ-H signal as predicted by equation (5) (see section on coupling and linewidths).

TABLE 1

T_1 Values for metalloporphyrins

Substituent	H	T_1/s CH_2	Me
H	0.8—1.0		
Me			0.5—0.6
CH_2Me		0.25—0.35	0.7—0.8
$CH_2CH_2CO_2Me$		α0.25—0.35	1.1
α β		β0.4—0.6	

Table 3 gives T_1 values for methyl groups which should obey equation (4) and simply reflect mobility. The methyls on the aromatic pyrrole rings all have T_1 *ca.* 0.6 s, but 8a-H [and in (9), 3a-H] have T_1 *ca.* 0.4 s, reflect interproton distances: this effect is seen clearly in (9) for α-H which is flanked on one side by a carbonyl group. The longer distance to proton neighbours gives α-H a much longer T_1 value than β-H or δ-H. Similarly, 10-H relaxes slowly in all the chlorophylls; in (5′), 10-H is in a sterically even less crowded environment and T_1 is longer still.* The methine protons on the reduced pyrrole rings have very short T_1 values which reflect their crowded environment.

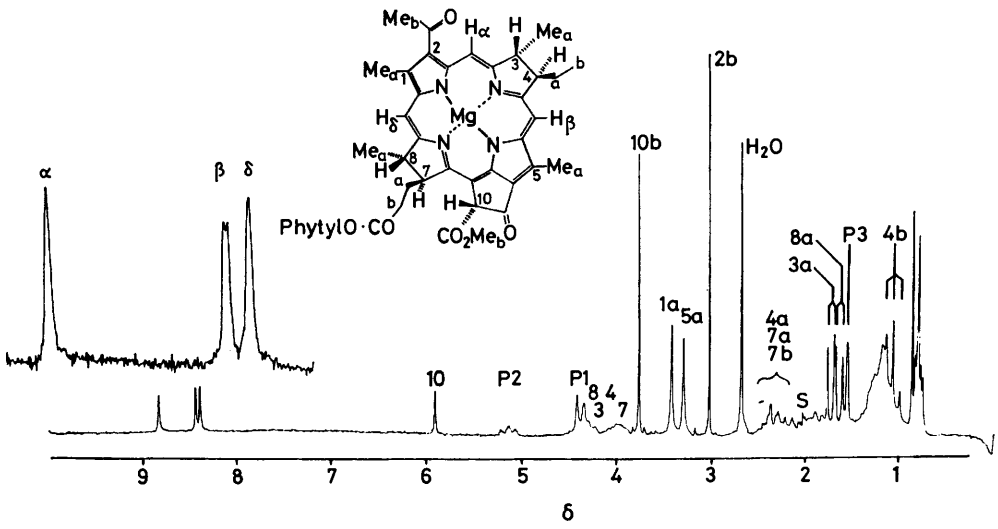


FIGURE 3 100 MHz ^{1}H N.m.r. spectrum of bacteriochlorophyll-a (9); 25mM in [$^2H_6$]acetone under N_2 atmosphere. *Inset*: Expansion of δ 8—9 region using 0.12 Hz/data point

Results for chlorophylls (5)—(9) are given in Tables 2—4, the assignments anticipating the n.o.e. results.

TABLE 2

T_1 Values in seconds for fixed protons in chlorophylls

Proton	(5)	(6)	(7)	(8)	(9)
α	1.0	0.8	1.3	1.5	1.0
β	0.9	0.6	1.1	1.1	0.7
δ	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]

[a] For (5′) T_1 = 2.0 s. [b] For (7′), T_1 = 2.0 s. [c] Estimate by null point due to signal overlap.

TABLE 3

T_1 Values in seconds for methyl groups in chlorophylls

Proton	(5)	(6)	(7)	(8)	(9)
1a	0.7	0.6	0.9	0.9	0.5
2b					1.3
3a	0.6		0.9		0.3
4b	0.7	0.6	0.9	0.8	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
7-CO_2Me			1.8 [a]	1.8 [a]	

[a] 3.47 δ

Table 2 presents T_1 values for protons directly attached to the macrocycle. These should follow equation (3) and presumably due to greater steric hindrance to internal rotation. This effect is particularly clear in (7) and (8). The 4b methyl protons in (5)—(8) relax at the same rate as the 'aromatic' ring methyls, probably indicating limited mobility of the ethyl group. In contrast the ethyl group of (9) is out of the plane of the macrocycle and might be expected to be more mobile: this is reflected in the observed longer T_1 (relative to the ring methyls). In all the chlorophylls the 10b-methyl ester group relaxes slowly, as does the 2-acetyl group in (9). The 7-Me groups of (7) and (8) have very characteristically long T_1 values allowing their assignments to be made easily.

TABLE 4

T_1 Values in seconds for phytyl protons

Signal	(5)	(6)	(9)	(12)	(13)
1-P	0.7	0.7	0.5	2.7	1.4
2-P	1.7	1.3	1.2	3.0	2.8
3a-P	1.3	1.4	1.1	3.0	2.2
CH_2-P	1.05	1.4	1.1	1.5	1.6
7a-P, 11a-P	1.2 [a]	1.4 [a]	1.2 [a]	1.8	1.8
15a-P, 16-P	2.0 [a]	1.9 [a]	2.2 [a]		

[a] Estimated by null point.

* Other T_1 values which can be measured in (5′) are not significantly different from (5).

The remaining protons of the macrocycle, are neither fixed nor in methyl groups but they can be interpreted. The diastereotopic methylene protons 7a-H,7b-H [and in (9), 4a-H] have $0.2 \leqslant T_1 \leqslant 0.4$ s, probably reflecting both large r^{-6} and short τ. 2b-H and 2a-H in (5)—(8) have T_1 *ca.* 1 and 1.5 s respectively; the slower relaxation in the latter case reflects lack of a geminal partner.

The partially resolved signals due to the methyl groups of the phytyl chains in chlorophylls relax at markedly non-uniform rates in [$^2H_6$]acetone (Table 4). The 6 H doublet at δ 0.85 relaxes slowly, with T_1 *ca.* 2 s, and is tentatively assigned to 15a-P and 16-P. The

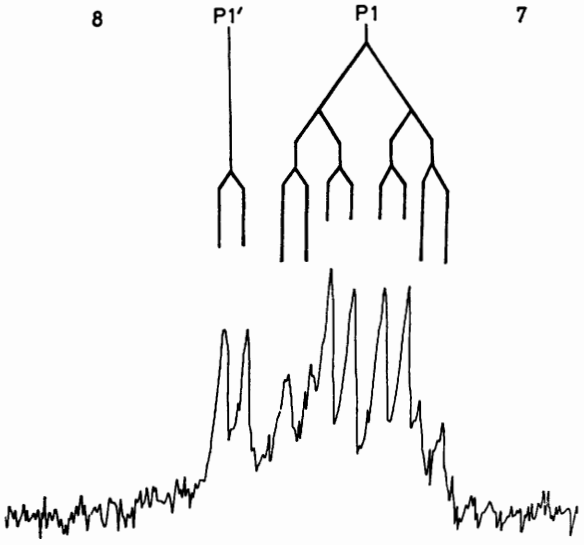


FIGURE 4 Portion of 270 MHz ^{1}H n.m.r. spectrum of (5) in the presence of sufficient (5)$^{+\cdot}$ to broaden 7-H and 8-H

3 H doublets (7a-P, 11a-P) at δ 0.82 and 0.80 are conformationally more restricted, giving T_1 *ca.* 1.3 s. 1-P, 2-P, and 3a-P are also conformationally restricted by more than is characteristic for esterification of the phytyl group [*cf.* (13)]. In (12) and (13), 7a-, 11a-, 15a-, and 16-P are equivalent at 100 MHz (δ 0.87) and apparently relax with the same T_1.* We conclude, therefore, that when the chlorophylls are dissolved in [$^2H_6$]acetone, the phytyl group takes up a preferred conformation. This idea is supported by observation of *ca.* 0.1 p.p.m. non-equivalence of the 1-P protons ($J = 11$ Hz) in the chlorophylls but not in the C-10 epimers, (12) or (13) (Figure 4).

Metal-free porphyrins and metalloporphyrins in non-polar solvents are aggregated.[3] We find (J. K. M. S., unpublished results) that this leads to shorter *absolute* T_1 values but within a molecule relative T_1 values remain unchanged.

In summary, relaxation times provide a method for sorting methyl and methylene groups according to distance from the macrocycle, and in favourable cases can help assign *meso*-protons.

Nuclear Overhauser Enhancements.—Irradiation of the methylene resonance of (1) causes an increase of *ca.* 40% in both the peak height and integral intensity of the *meso* resonance. This is close to the theoretical maximum effect. The correspondence of integral and peak height increases rules out decoupling effects.

Table 5 lists the n.o.e. values observed in (3) when high power irradiation was used to saturate all the methyl resonances simultaneously. The assignments of γ-H and δ-H follow directly from the results; those of α-H and β-H are found as follows. Irradiation of the lowfield (δ 3.81 in [$^2H_6$]acetone) 6 H singlet with a field too weak to saturate those at δ 3.66 and 3.68 gives a small n.o.e. (*ca.* 10%) on the δ 10.40 and 10.27 signals and simultaneously sharpens the vinyl methine signals at δ 8.5 by removing long-range coupling (see next section). The δ 3.81 methyls must therefore be 1a-H and 3a-H and hence the δ 10.40 proton is α-H. Similar results are obtained in $CDCl_3$–pyridine (Tables 5 and 6) for (3) and

TABLE 5

Assignments and n.o.e.s for *meso*-protons of (3) and (4)

	α-H	β-H	γ-H	δ-H
δ ($CDCl_3$–pyridine) (4)	10.24 [a]	10.15 [a]	9.96	10.07
δ ($CDCl_3$–pyridine) (3)	10.31 [a]	10.22 [a]	9.97	10.14
δ ([$^2H_6$]acetone) (3)	10.40	10.32	10.15	10.27
(signal irradiated)		(% increase)		
vinyl-CH	15	15		
-CH_2			30	
-CH_3	20	20		30

[a] The α-β distinctions are made by analogy with acetone results for (3).

TABLE 6

Assignment of methyl groups in (3)

	1a,3a	5a,8a	Ester [a]
δ ($CDCl_3$–pyridine)	3.77	3.65, 3.63	3.60
δ ([$^2H_6$]acetone)	3.81	3.68, 3.66	3.65

[a] Assigned by long T_1.

(4). These results agree with the limited assignments made by other methods.[3]

Analogous results are obtained for chlorophyll-a in [$^2H_6$]acetone. Irradiation of the vinyl-CH signal (δ 8.08) gives a 15% increase in the δ 9.34 signal which must, therefore, be α-H. α-H also experiences a 20% n.o.e. from high power irradiation at *ca.* δ 3.3 which is due to the 3a-H methyl group. Low power irradiation (see next section) pinpoints this resonance at δ 3.26. Similarly δ-H (δ 8.51) experience 20% n.o.e. from 1a-H (δ 3.32), 15% from 8a-H (δ 1.75, d, $J = 7$), whilst β (δ 9.67) is enhanced 25% by 4a-H (δ 3.74, q, $J = 7.5$). These assignments are indicated in Figure 1. Assignments for (6) (Figure 2) follow closely those for (5).

For bacteriochlorophyll-a (9) there are no unambiguous starting points since the recognisable signals for the macrocyclic periphery consist entirely of apparent singlets and a *pair* of doublets due to 3a-H and 8a-H. N.o.e.s at *meso* protons are observed as follows: 8a-H, 1a-H ⟶ δ-H; 5a-H ⟶ β-H; 3a-H ⟶ α-H (Figure 5). These assignments are the only ones consistent with the structure and are confirmed by long-range coupling constants.

* The decay is a good exponential for $> 2T_1$.

Long-range Coupling and Linewidths.—In the previous section we noted the presence of a small, *ca.* 0.3 Hz,

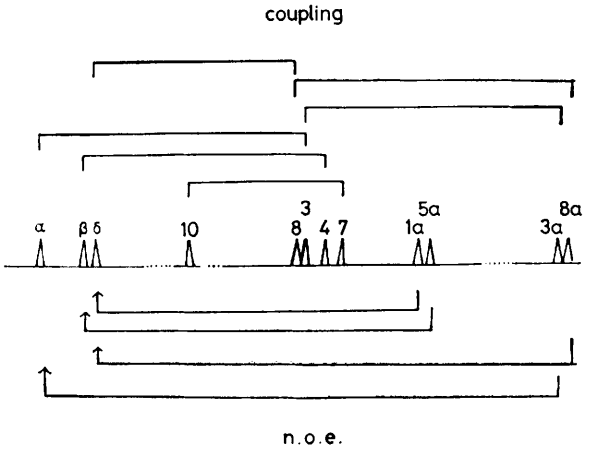


FIGURE 5 Schematic representation of coupling and n.o.e.s in (9)

unresolved five-bond coupling between methyl and vinyl methine protons attached to the same pyrrole ring.*

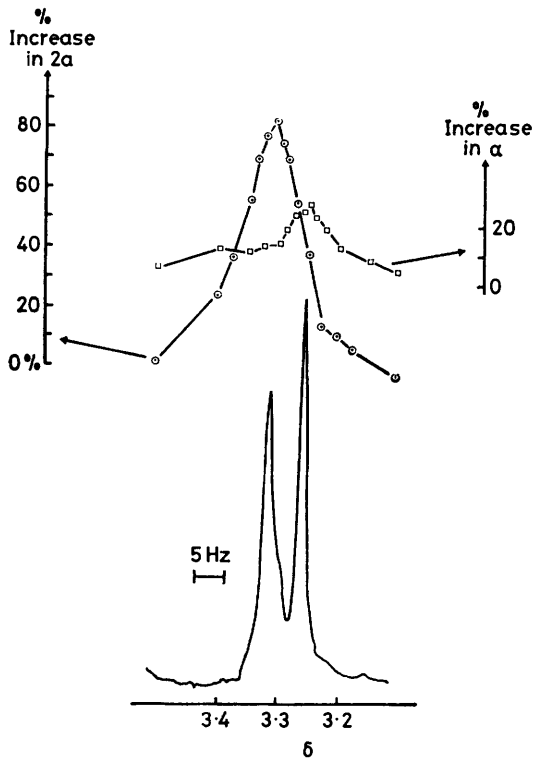


FIGURE 6 Assignment of 3a-H (δ 3.26) and 1a-H (δ 3.32) in (5) by long-range coupling (—○—) and n.o.e. (—□—)

This is illustrated for (5) in Figure 6, which plots peak-heights for 2a-H and α-H as a function of irradiation frequency. Integration shows that the 2a-H,1a-H effect is due solely to coupling, whilst the α-H,3a-H effect is a n.o.e. Irradiation of 2a-H sharpens 1a-H as expected.

In addition, irradiation of the 8-H methine resonance affects δ-H; the linewidth decreases by *ca.* 0.2 Hz, whilst there is no significant n.o.e. This 4-bond allylic coupling is most apparent in (9): when a large number of data points is used to define the spectrum, a 0.8 Hz coupling to 4-H can be directly observed on β-H (see inset, Figure 3). Smaller couplings of *ca.* 0.2 Hz are inferred for 3-H ⟶ α-H and 8-H ⟶ δ-H from irradiation experiments which simultaneously assign the 3-H,-3a-H and 8-H,8a-H pairs (Figure 5). An unresolved long-range 7-H,10-H coupling of *ca.* 0.4 Hz is also inferred in all the chlorophylls but not in the C-10 epimers.

These allylic couplings and associated linewidth effects are most useful, and it is perhaps surprising that they have not been reported previously. In all our spectra the linewidth variations between different methyl groups is very striking. This is in part due to long-range coupling (*e.g.* 1a-H *vs.* 3a-H) and in part due to inherent differences imposed by equation (5). Thus in (5), 10b-H is always the sharpest singlet methyl; 2b-H in (9) is even sharper as it has a longer T_1 value; and the added methyls in (7) and (8) are sharp for the same reason. Similarly, in (3) and (4), γ-H and the propionate methylene nearer the ring are always broader † because of their short T_1 value.

Solutions of (9) in [$^2H_6$]acetone often contain low (*ca.* 10^{-8}M) steady-state concentrations of the radical-cation $(9)^{+\cdot}$ even when thoroughly degassed. This leads to electron transfer broadening of the hyperfine-coupled resonances and accounts for the exceptional broadness of 1a-H and 5a-H in Figure 3. Knowledge of the relevant coupling enables ready identification of this source of broadening.[1,2]

Observation of the above described coupling and linewidth phenomena requires relatively dilute solutions and therefore Fourier transform techniques. High-field studies at ⩾200 MHz are unlikely to reveal these effects as computer word length usually limits digitisation to *ca.* 0.5 Hz/data point. In contrast, Figures 1—3 were obtained at 100 MHz with 0.25 Hz/point and the inset to Figure 3 with 0.12 Hz/point.

The four-bond allylic coupling is clearly dependent on very subtle geometrical factors. In (9) the relationship 4-H,β-H is apparently the same as 3-H,α-H and 8-H,-δ-H but the coupling constant, 0.8 Hz, is *ca.* four times larger. An analogous 0.9 Hz coupling has been used in the structure elucidation of sirhydrochlorin monolactone.[11]

Conclusions.—These results taken together provide an overdetermined set of assignments for (5)—(9) without resort to chemical modification or biosynthetic labelling. Assignments are in agreement with those most recently

* There is similar six bond coupling to the terminal vinyl portons.

† This is not due to long-range coupling between these protons. It is also not due to non-equivalence of the 6-CH_2 and 7-CH_2 protons as we observe the same effect in the symmetrical uroporphyrin-II-octamethyl ester (J. K. M. S., unpublished).

[11] A. R. Battersby, K. Jones, E. McDonald, J. A. Robinson, and H. R. Morris, *Tetrahedron Letters*, 1977, 2213.

published.[12] Detailed relaxation behaviour in these compounds remains to be elucidated but T_1 measurements provide a useful tool in natural product structure elucidation.[11]

EXPERIMENTAL

Proton n.m.r. spectra were obtained at *ca.* 310 K at 80 MHz (Varian CFT 20), 100 MHz (Varian XL-100), or 270 MHz (Bruker WH270) in the Fourier transform mode. Five to one hundred free induction decays following 90° pulses were collected and transformed without exponential weighting. Chemical shifts are in δ units (p.p.m.) from $SiMe_4$ and were measured from internal $SiMe_4$ or calculated *via* the solvent deuterium field-frequency lock.

For relaxation and intensity measurements intervals between pulses were *ca.* four times the longest T_1 measured. Integral intensities were determined from these fully relaxed spectra by cutting and weighing spectral peaks. Spin-lattice relaxation times were determined at 100 MHz using the inversion-recovery (180°-t-90°) method,[5] only the initial exponential recovery being used in the calculation. Nuclear Overhauser enhancements were measured by comparing integral intensities of the *s* resonance when the *i* resonance and a blank spectral region were being separately irradiated. Quoted n.o.e.s are the average of several experiments and are ±5%.

Porphyrin spectra were obtained from dilute (10—25 mM) solutions in deuteriochloroform containing sufficient pyridine to disaggregate the porphyrin,[3] or in [$^2H_6$]acetone. The chlorophyll spectra were all obtained in [$^2H_6$]acetone. Solutions of (9) were degassed with dry N_2 to minimise production of $(9)^{+\cdot}$. Preliminary experiments on the other compounds revealed no dependence of T_1 on degassing and it was discontinued.

Field desorption mass spectra of all the chlorophylls were kindly obtained for us by Dr. H. R. Morris of Imperial College. Each showed only the expected molecular ion. Compounds (1), (3), and (4) were gifts from Dr. E. McDonald of this Department, (12) was obtained from Sigma, (13) was a gift from Professors E. H. Ahrens and A. R. Battersby, and (2) was synthesised as described elsewhere.[13]

Sucrose Chromatography: General Procedure.—The chlorophylls were isolated, separated, and purified by chromatography in the dark on powdered sucrose (British Sugar Corporation CP icing sugar containing 1½% calcium phosphate). The absorbent was dry-packed into 7 cm (1 kg) or 9 cm (2 kg) diameter columns using a steel tamper. The chlorophylls were applied to the dry column dissolved in a suitable solvent and the chromatography was run with water pump suction at the foot of the column. Once the pigments were well separated, the column was sucked dry and the required bands were dug out with a long-handled spoon. The chlorophylls were eluted from sucrose with an appropriate solvent.

Chlorophylls-a and -b (5), (6).—Chlorophylls-a and -b were prepared from leaf beet perpetual spinach (*Beta vulgaris* var. *cicla*) by an improved procedure based on the methods of Strain and Svec,[14] and Iriyama, Ogura, and Takamiya.[9] Chlorophyll solutions and precipitates were kept in the dark as much as possible. The spinach was grown in the University Botanic Garden.

Solvents were laboratory reagent grade and were used as received, except that dioxan and diethyl ether were redistilled from, and stored over, sodium.

Spinach was freshly picked and the leaf midribs were removed, only the soft green parts of growing leaves being used; 200 g (fresh weight) of this preparation were washed first with water and then with methanol. The leaves were then homogenised for 2 min in a Waring blender using 1 000 ml methanol previously cooled to −25 °C, and filtered through a Büchner funnel. The precipitate was washed until it was pink with about 500 ml cold methanol. The supernatant was combined with ⅐ its own volume of dioxan and stirred magnetically, while ice-cold water was added dropwise. On addition of a little water the red fluorescence of the solution disappeared and on addition of more water a green precipitate was observed. The addition of water was continued until the supernatant liquid was yellow-green in colour. About 200 ml water was required, although this varied considerably between preparations. The precipitate was collected by centrifugation at 0 °C. It was redissolved in 7 : 1 methanol–dioxan (300 ml) and reprecipitated with water (50 ml), again added dropwise with stirring. The second precipitate was collected by centrifugation, redissolved in ether (100 ml), and washed with ice-cold water (3 × 300 ml). It is important to use cold water for washing chlorophyll solutions in ether as this reduces the formation of stable emulsions. The ether solution was evaporated and stored at 0 °C in the dark.

The chlorophylls were separated from each other, from their pheophytins (formed in small amounts during the preparation) and from other lipid pigments by column chromatography. The extract was dissolved in ether (25 ml), diluted with light petroleum (b.p. 30—40 °C; 75 ml) and applied to a sucrose column with a loading ratio of 10 000 : 1. The yellow pigments were eluted with 9 : 1 light petroleum : ether. Chlorophylls were then separated by elution with light petroleum containing 0.2%, increasing to 0.6% of n-propanol. The chlorophylls were separately eluted from the isolated sucrose with ether which was then concentrated to 100 ml, washed with 3 × 300 ml cold water, and evaporated. Each chlorophyll was then rechromatographed in the same way using a loading ratio of 7 500 : 1. The solids obtained were dried *in vacuo* to yield *ca.* 120 mg of chlorophyll-a and 40 mg of chlorophyll-b. The u.v./visible spectra [$\lambda_{max.}$ ($\log_{10}$ ε) in ether] were as follows.

Chlorophyll-a. Found: 410(4.95), 430(5.12), 535(3.68), 579(3.94), 619(4.21) and 664(4.94) [lit.,[15] 410(4.88), 430(5.07), 534(3.58), 578(3.92), 615(4.16), and 662(4.95)]. *Chlorophyll-b.* Found: 429(4.73), 453(5.18), 548(3.76), 595(4.01), 645(4.67) [lit.,[15] 430(4.76), 455(5.20), 549(3.81), 595(4.06), and 644(4.75)].

Methyl chlorophyllides (7), (8). Healthy leaves of perpetual spinach were stripped of large stalks and blended for 1 min with 3 volumes/g leaves of acetone–water (4 : 1). The mixture was degassed on a water pump and set aside in the dark for 3—4 h with occasional swirling. Chlorophyll hydrolysis can be followed by extracting aliquots of clear supernatant liquid with equal volumes of light petroleum: the chlorophyllide acids are soluble in the acetone layer whilst the chlorophylls and yellow pigments dissolve in the upper petroleum layer. When the reaction was completed,

[12] H. Scheer and J. J. Katz in 'Porphyrins and Metalloporphyrins', ed. K. M. Smith, Elsevier, Amsterdam 1975, p. 599.
[13] J. K. M. Sanders, C. G. Newton, and J. C. Waterton, *J. Magnetic Resonance*, in the press.
[14] H. H. Strain and W. A. Svec in 'The Chlorophylls' ed. L. P. Vernon and G. R. Seely, Academic Press, London, 1966, ch. 2.
[15] Ref. 12, p. 880.

the mixture was filtered under vacuum through a large Büchner funnel and extracted with petroleum (0.7 vol) to remove yellow pigments. The aqueous acetone was then extracted with ether (0.7 vol).* The ethereal extract was concentrated and treated with an excess of ethereal diazomethane until methylation was complete. On further concentration of the solution, wet (7) and (8) were precipitated; they were collected by centrifugation and dried *in vacuo*.

Chromatography was performed as described for the chlorophylls but using similar conditions to those given by Chow *et. al.*[16] Mixed product (100 mg) was dissolved in pyridine (20 ml), diluted to 700 ml with light petroleum, applied to the column (1 kg), and then washed with 0.5% pyridine in petroleum (200 ml). The column was eluted with 0.5% pyridine and 0.5% n-propanol in petroleum and then washed with petroleum when the pigments had separated. The bands were dug out and (7) and (8) eluted separately from the sucrose with dry distilled acetone. These solutions were evaporated and each pigment rechromatographed separately as above. Finally the solid residues were triturated with dry, distilled light petroleum, filtered, dried *in vacuo*, and stored at 0 °C in the dark. U.v./visible spectra were in agreement with structures (7) and (8).

Bacteriochlorophyll-a. Bacteriochlorophyll was obtained as a by-product from a corrin extraction[17] from *Chromatium D.*

From 500 g of bacteria a semisolid precipitate from aqueous ethanol at pH 4 was obtained. This was resuspended in water buffered at pH 7, filtered through a Büchner funnel and washed with water. The lipid precipitate was dissolved in methanol–dioxan (7 : 1; 3 l) and centrifuged at 0 °C to remove any insoluble debris. It was then precipitated with water (*ca.* 800 ml) and collected by centrifugation at 0 °C. The precipitate was resuspended in water and recentrifuged. The yield was *ca.* 15—20 g.

This extract was three times chromatographed on sucrose as described for chlorophylls-a and -b. The first column had a loading ratio of *ca.* 2 000 : 1 and served simply to remove the most polar and non-polar material. Uv./visible spectrum [$\lambda_{max.}$ ($\log_{10}$ ε) in ether] 359(4.81), 394(4.58), 540sh(3.52), 572(4.26), 705sh(3.96), 768(4.89) (lit.,[15] 359-(4.87), 392(4.62), 530sh(3.44), 577(4.32), 697sh(3.96), and 773(4.96).

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* Evaporation of this wet solution yields a precipitate of almost pure mixed chlorophyllides (0.05% yield based on net weight of leaves).

[16] H. C. Chow, R. Serlin, and C. E. Strouse, *J. Amer. Chem. Soc.*, 1975, **97**, 7230.

[17] V. B. Koppenhagen, F. Wagner, and J. J. Pfiffner, *J. Biol. Chem.*, 1973, **248**, 7999.