Co-ordination and Aggregation of Bacteriochlorophyll *a*: An N.M.R. and Electronic Absorption Study

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A detailed analysis is presented of the aggregation and co-ordination equilibria available to bacteriochlorophyll *a*, and n.m.r. spectroscopic titration experiments are proposed for their investigation. The results show that bacteriochlorophyll *a* is dimeric in wet benzene, five-co-ordinate monomeric in acetone or ether solution with one bound solvent molecule, and six-co-ordinate monomeric in pyridine or methanol solution with two bound solvent molecules. The second derivatives of the electronic absorption spectra show separate resolved peaks for five- and six-co-ordinate species near 575 and 600 nm respectively.

The solution equilibria of chlorophylls are dominated by the co-ordination demands of the central Mg^{2+} ion; it seems likely that co-ordination state also plays an important role in photosynthesis.[1,2] It is well established that Mg prefers to be five- or six-fold co-ordinated,[3-5] but only four sites are occupied by the pyrrole nitrogens in chlorophylls. The resulting co-ordinative unsaturation must be satisfied by a fifth and, in some circumstances, sixth basic axial ligand (Figure 1). In the absence of an external ligand the vacant co-ordination site is satisfied by a further chlorophyll molecule; this second molecule either co-ordinates directly using a carbonyl group or indirectly *via* a water molecule which hydrogen bonds to the carbonyl.[3-5] Hence chlorophylls exhibit a tendency to self aggregate and so possess correspondingly complicated solution equilibria.

Although the aggregation and co-ordination of chlorophyll *a* (Chl *a*) have been much studied by i.r. spectroscopy, solution osmometry and ^1H n.m.r. spectroscopy, they are still a matter of debate;[4] by contrast, because both the availability and stability of bacteriochlorophyll *a* (BChl) (1) are generally believed to be low, less work has been done on BChl and the effect on its chemistry is even less clear.

Here we report spectroscopic studies which elucidate BChl co-ordination properties in a variety of solvents. The following paper [6] reports evidence that the solution and solid-state stability of BChl are in part dependent on the co-ordination state and describes how we have used this evidence to design an improved high-yielding extraction procedure which gives stable microcrystalline BChl. In the third paper [7] we exploit this knowledge about the stability of BChl to provide concentrated but stable solutions suitable for natural abundance ^{13}C n.m.r. spectroscopy. A related paper will describe in detail the methods used for determining the equilibrium constants in this paper.[8]

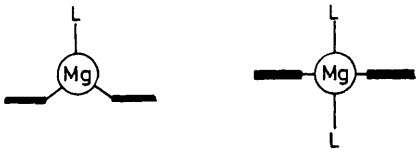
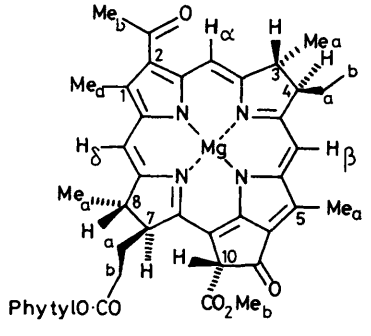


Figure 1. Schematic diagrams of bacteriochlorophyll *a* when five-co-ordinate (left) and six-co-ordinate (right)



(1)

There is a strong body of evidence to suggest that the molecules of BChl associate together in the photosynthetic reaction centre *in vivo* and that this complex is the primary electron donor of photosynthesis.[9,10] N.m.r. shift titrations on isolated BChl suggested that small ligands bind onto and dissociate BChl aggregates. Electronic absorption spectra indicate the monomeric BChl in solution can be either five- or six-fold co-ordinated: Evans and Katz suggest that the position of the Q_x band is strongly dependent on the co-ordination state, absorbing at *ca.* 580 nm for five-co-ordinate and 600 nm for six-co-ordinate material.[11] We extend these two observations to build a more detailed picture, but present first a description of the solution equilibria of BChl and a summary of the theory of the experimental design.

Principles.—The Scheme illustrates the principal BChl solution equilibria. We define the following constants:

$$K_0 = \frac{[BChl_2]^n_2}{[BChl_n]} \quad \{1\}$$

where K_0 is the constant for dissociation of $BChl_n$ aggregates to $BChl_2$ dimers,

$$K_1 = \frac{[BChl{\cdot}L]^2}{[BChl_2]{\cdot}[L]^2} \quad \{2\}$$

where K_1 is the disaggregation constant of a dimer induced by ligand L,

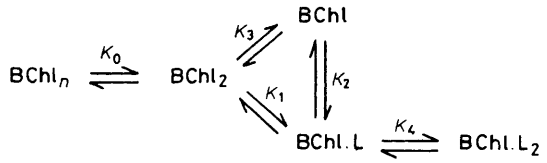
$$K_2 = \frac{[BChl]{\cdot}[L]}{[BChl{\cdot}L]} \quad \{3\}$$

where K_2 is the ligand binding constant to monomeric BChl,

$$K_3 = \frac{[BChl_2]}{[BChl]^2} \quad \{4\}$$

where K_3 is the dimerisation constant for BChl and

$$K_4 = \frac{[BChl{\cdot}L_2]}{[BChl{\cdot}L][L]} \quad \{5\}$$



Scheme

where K_4 is the second ligand binding constant. These are convenient definitions, although strictly K_0 to K_4 are not all independent variables.

We can induce the equilibria in the Scheme by changing the concentrations of L or BChl. The equilibria actually observed depend on the relative sizes of the various constants.

The total concentration of ligand needs to be considerably in excess of that of BChl for significant disaggregation shifts to be observable. Hence we monitored the BChl chemical shifts whilst varying the concentration of L, assuming fast exchange between species of different chemical shifts. For easy interpretation of such shifts it was necessary to ensure that the only effective process occurring during ligand titration was of the form $BChl_n + m \,.\, n \,.\, L \rightleftharpoons n(BChl \,.\, L_m)$. Conditions for this to be the case were established as follows.

(a) For the BChl to be initially more than 90% in the dimer form it was necessary for $(1 + 8K_3c)^{\frac{1}{2}} > 19$, where c is the total BChl concentration.[8] Assuming that for BChl, K_3 is similar to that for chlorophyll *a* (*ca.* 10^4 mol l^{-1}) then this imposes a lower limit on c of *ca.* 4.5 mM. Experimentally we confirmed that BChl shifts were concentration independent in the 5—15 mM range used in this work.

(b) For ease of analysis it was convenient to assume only a small proportion (<10%) of added ligand was bound so that L_{free} could be approximated by L_{total}. The condition [8] which needs to be satisfied is $(2/K_1c)^{\frac{1}{2}} \gg 9$. This imposes upper limits on c or K_1 which can give reliable measurements.

(c) In order that only five- or six-co-ordination in the final complex is observed it is necessary that *either* $K_4L \gg 1$, for six-co-ordination *or* $K_4L \ll 1$ for five-co-ordination.

In the event, suitable values of concentration and binding constants allowed conditions (a) and (b) to be satisfied. Had they not done so we would have needed either to employ other n.m.r. methods such as dilution shifts or ligand-binding shifts, or else choose another concentration range as may be conveniently studied by other methods of spectroscopy (such as i.r. or electronic absorption spectra). For most metalloporphyrins the tendency to self-aggregate is far less, K_1 is consequently small, and so dilution shifts and significant concentrations of monomeric metalloporphyrin make condition (a) hard to obtain experimentally; hence the experiments in this paper would not be possible.

In fitting experimental data to binding models we considered the following four equilibria

$$BChl_2 + 2L \rightleftharpoons 2BChl{\cdot}L \quad \{A\}$$
$$BChl_2 + 4L \rightleftharpoons 2BChl{\cdot}L_2 \quad \{B\}$$
$$BChl_3 + 3L \rightleftharpoons 3BChl{\cdot}L \quad \{C\}$$
$$BChl_3 + 6L \rightleftharpoons 3BChl{\cdot}L_2 \quad \{D\}$$

to allow for $n = 2$, 3 and $m = 1$, 2. Whereas it is often quite possible to fit poor experimental data to a variety of models we consider that we are only justified in making significant deductions about the co-ordination equilibria of BChl if the data fit one model very much more closely than others.

Equilibria {A}—{D} may be distinguished [8] by the 3 plots

(i) $c_1/(c - c_1)$ *vs.* L^2/c_1
(ii) $c_1/(c - c_1)$ *vs.* L^4/c_1
(iii) $\log \{c_1/[(c - c_1)L]\}$ *vs.* $\log (L/c_1)$

where c_1 is the concentration of monomeric BChl. Table 1 lists the behaviour of the data derived from equilibria {A}—{D} when graphs (i)—(iii) are plotted. It should be clear which stoicheiometry of disaggregation is in operation; appropriate constants can then be calculated from computational curve fitting.

Table 1. Behaviour of titration data arising from equilibria {A}—{D} when plotted in the manner of (i)—(iii) as discussed in the text

Equilibrium	(i)	(ii)	(iii)
{A}	*Linear*	*Non-linear*	*Linear*
$n = 1$, $m = 2$	Gradient $\propto \frac{1}{K}$	Asymptotic to *y* axis	Gradient = 1
{B}	*Non-linear*	*Linear*	*Non-linear*
$n = 2$, $m = 2$	Asymptotic to *x* axis	Gradient $\propto \frac{1}{K}$	Gradient probably negative
{C}	*Non-linear*	*Non-linear*	*Linear*
$n = 1$, $m = 3$	Asymptotic to *x* axis	Asymptotic to *y* axis	Gradient = 2
{D}	*Non-linear*	*Non-linear*	*Non-linear*
$n = 2$, $m = 3$	Asymptotic to *x* axis	Asymptotic to *x* axis	Gradient probably negative

Table 2. Chemical shifts (p.p.m.) of bacteriochlorophyll protons in different deuteriated solvents

Proton	Acetone	Pyridine	Benzene
α	8.81	9.19	9.00
β	8.43	8.45	8.46
δ	8.38	8.30	8.27
10	5.90	6.32	5.41
P2	5.14	5.29	5.29
P1	4.42, 4.36	4.58	4.08
8	4.40	4.33	4.17
4	4.32	3.87	3.96—3.91
3	4.10	4.09	
7	4.00	4.07	
10b	3.76	3.63	3.44
5a	3.30	3.40	3.39
1a	3.41	3.31	3.10
2b	3.02	2.98	2.68
4a, 7a	2.5—2.2	2.4—2.1	2.5—2.1
7b	2.20	1.82	2.05
3a	1.76	1.55	1.72
P3a	1.58	1.52	1.60
8a	1.67	1.41	1.55
4a	1.10	0.73	1.22

Results and Discussion

The ^1H n.m.r. spectra of BChl exhibit very different appearances according to solvent. We distinguish three principal types of spectra at room temperature: (a) in wet [$^2H_6$]benzene (Figure 2); (b) in [$^2H_6$]acetone (Figure 3), the spectra in [$^2H_4$]-methanol, [$^2H_8$]-THF, [$^2H_6$]-DMSO (100 °C) are of similar appearance, apart from some line-width differences; (c) in [$^2H_5$]pyridine (Figure 4). Chemical shifts are tabulated in Table 2.

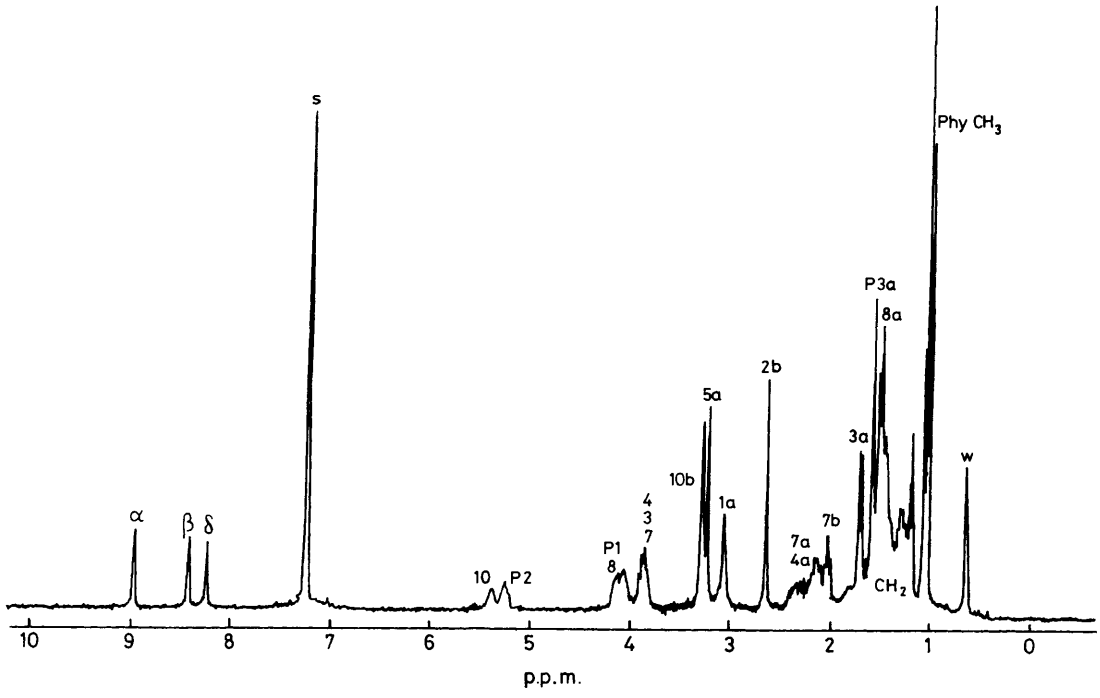


Figure 2. 270 MHz ^1H N.m.r. spectrum of BChl in wet [$^2H_6$]benzene; s = solvent, w = water

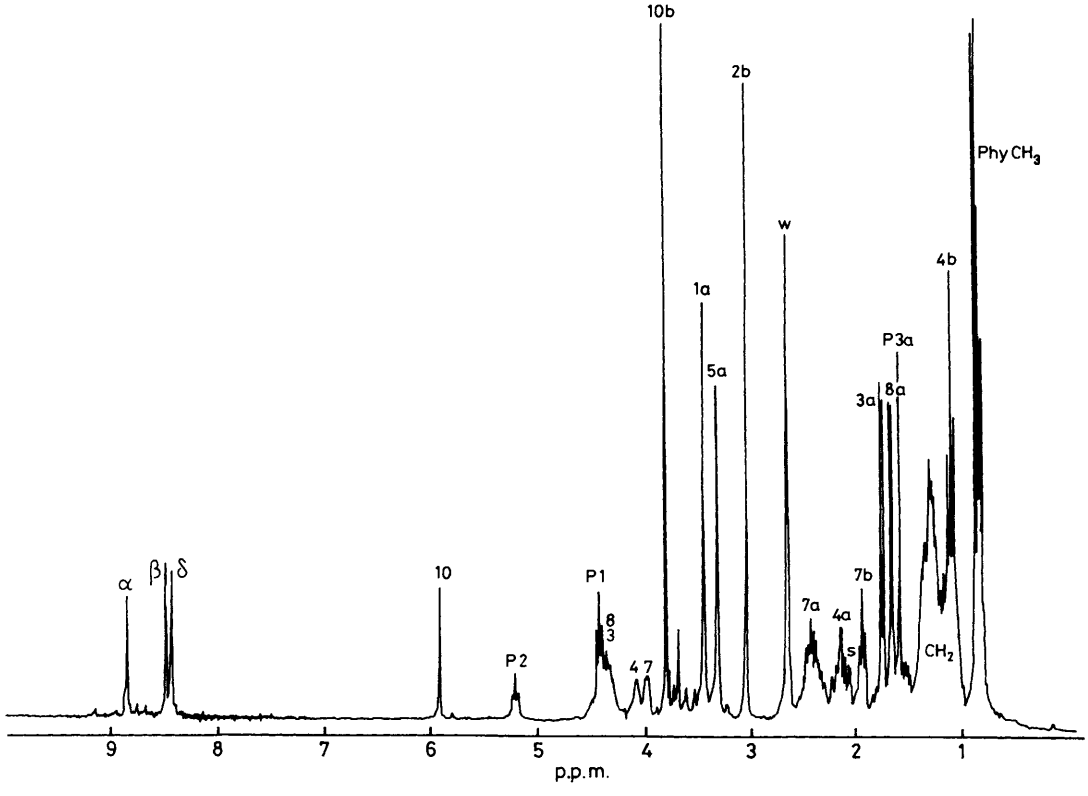


Figure 3. 270 MHz N.m.r. spectrum of BChl in [$^2H_6$]acetone solution

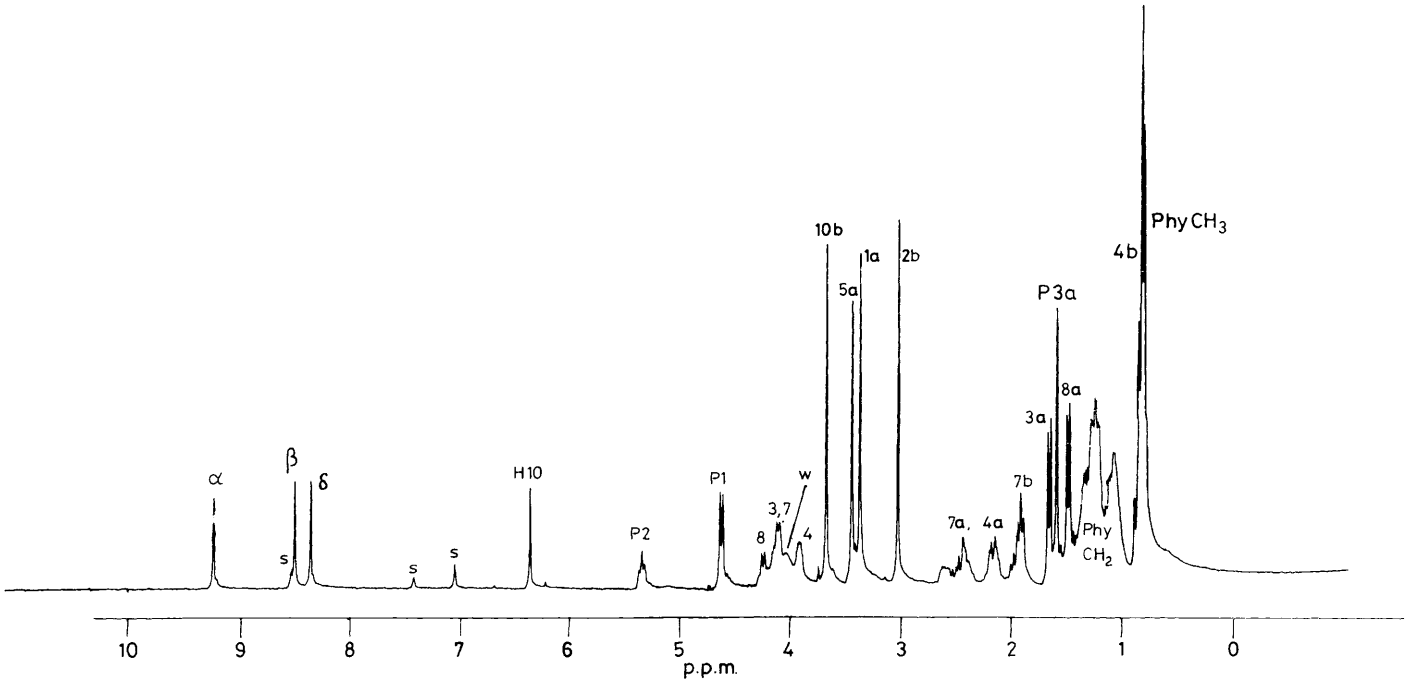


Figure 4. 270 MHz N.m.r. spectrum of BChl in [$^2H_5$]pyridine solution

Table 3. Chemical shifts of 10-H as [$^2H_6$]acetone is titrated into a solution of bacteriochlorophyll in [$^2H_6$]benzene [a]

μl acetone	δ (p.p.m.)
0.0	5.46
2.0	5.52
4.0	5.62
6.0	5.66
8.0	5.69
10.0	5.72
15.0	5.81
20.0	5.87
25.0	5.92
30.0	5.96
40.0	6.00
50.0	6.04
60.0	6.06
73.0	6.08
98.0	6.09
125.0	6.10

[a] 6.1 mg of BChl were dissolved in 0.4 ml of [$^2H_6$]benzene.

[$^2H_6$]Benzene cannot co-ordinate to the magnesium atom. Hence BChl will be aggregated, and the n.m.r. spectrum consequently broadened due to the rapid relaxation of protons in large aggregates. The spectra of BChl in hydrocarbon solvents are considerably broader than in benzene.[4] This is probably because benzene exhibits more specific association with the dipolar groups on the BChl periphery tending to favour smaller aggregates: we report elsewhere strong evidence for the existence of substantial solvent shifts of BChl in benzene.[8] A second factor determining the size of aggregates in benzene appears to be the presence of small quantities of water. Spectra in very dry benzene are broad, and we routinely added small quantities of H_2O or D_2O to obtain the limiting spectrum as in Figure 2.

We therefore suggest that BChl is predominantly associated as small oligomers in benzene under the conditions studied. The spectrum in [$^2H_5$]pyridine was assigned by the same techniques previously employed for the spectrum in [$^2H_6$]-acetone.[12] The spectrum in [$^2H_6$]benzene was assigned by chemical-shift titration into [$^2H_6$]acetone; in the latter solvent the spectrum has previously been rigorously assigned.[12]

The resonance that shifts the most between different solvents in 10-H. Its chemical shift changes as [$^2H_6$]acetone is titrated into a solution of 10 mM BChl in [$^2H_6$]benzene are given in Table 3.* The graphs (i)—(iii) (Figure 5) show that the best fit is obtained for $n = 2$ and $m = 1$, *i.e.* a dimer in benzene is dissociated into monomers with one solvent molecule attached. We find at 25 °C that $K_1 = 0.1$ l/mol. At a concentration of 10 mM we expect from condition (b) to be able to measure values of $K_1 < 2.5$ l/mol so the value is well within measurable limits. Figure 6 shows curves for $K = 0.1K_1$, $0.5K_1$, $2K_1$, and $10K_1$; all experimental points fit well within the confidence limits $K_1 = 0.05$—0.2 l/mol.

A different situation is encountered when pyridine is titrated in a solution of BChl in [$^2H_6$]benzene. The titration curve (Figure 7a) is steeper and fits the same model less well. A value of $K = 15$ l/mol is considerably above the range in which we have confidence. Since we know BChl is dimeric in benzene the experiment suggests that BChl is not predominantly five-fold co-ordinated in pyridine.

Further evidence comes from the electronic absorption spectra. In acetone (and wet benzene) the Q_x band absorbs at 580 nm whereas in pyridine it absorbs at 600 nm. Evans and Katz have suggested that five-fold co-ordinated BChl whether in monomeric or oligomeric form absorbs at *ca.* 580 nm whereas six-fold co-ordinated BChl absorbs at *ca.* 600 nm, suggesting that BChl is predominantly six-co-ordinated in pyridine.[11]

* The chemical shift discrepancy for 10-H between Table 2 and 3 arises from the benzene-induced solvent shift experienced even by acetone-solvated monomers.[8]

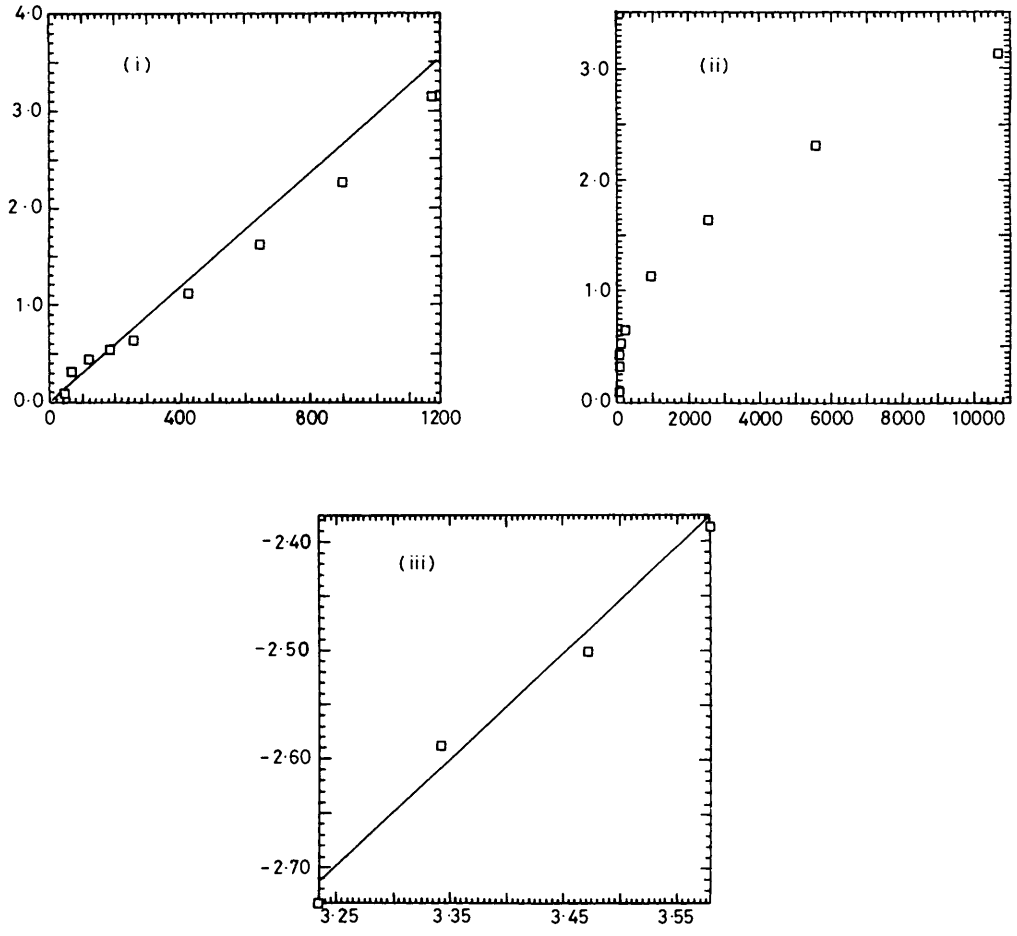


Figure 5. Plots of (i) $c_1/(c - c_1)$ *vs.* L^2/c_1; (ii) $c_1/(c - c_1)$ *vs.* L^4/c_1;(iii) log $\{(c_1/[(c - c_1)L]\}$ *vs.* log (L/c_1) as [$^2H_6$]acetone is titrated into a [$^2H_6$]benzene solution of BChl

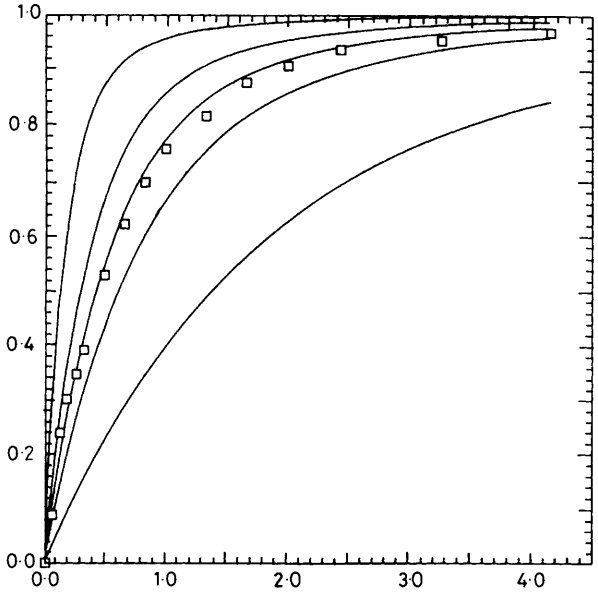


Figure 6. Experimental chemical shifts of 10-H for the acetone titration together with theoretical titration curves for K_1 = 0.01, 0.05, 0.1, 0.2, and 1.0 l mol^{-1}. *x* axis is conc. of acetone in mol l^{-1}

To investigate this further we recorded the electronic absorption spectra in a mixture of solvents to monitor the change of co-ordination state. Pyridine binds very strongly to BChl, so only small amounts are necessary to change the position of the absorption maximum from 580 nm in acetone to 600 nm in a mixture of acetone and pyridine. However, methanol, although it exhibits the same behaviour as pyridine in the n.m.r. titration (Figure 7b) appears to bind less strongly. The position of the maximum in a 9 : 1 (v/v) solution of acetone–methanol is intermediate between that in the pure solvents.

To analyse the gradual change of the absorption maximum as methanol is titrated under a solution of BChl in acetone, we need to be able to distinguish a non-specific solvent shift from a net shift resulting from a band consisting of two different forms (five- and six-co-ordinated BChl). We expect the latter to be the correct interpretation. The conventional method is deconvolution but so many adjustable parameters are required for curve fitting that the result is often of limited validity.[13]

Derivative spectra are frequently used to enhance resolution; 4th derivatives of chloroplast absorption spectra have been employed to study small changes in absorption.[14] We transferred digitised spectroscopic data to an IBM 370/165 mainframe computer and calculated the first and second derivatives. Because higher derivatives increased noise un-

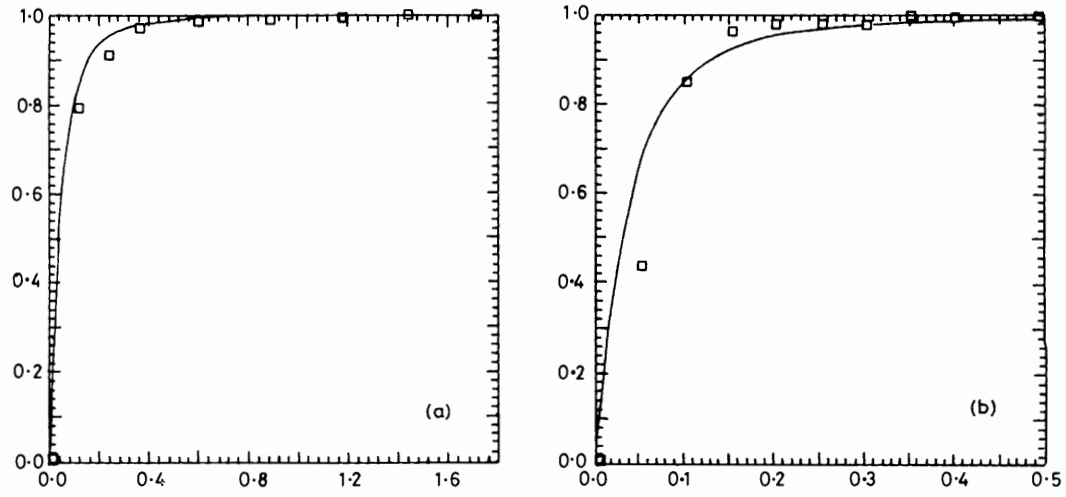


Figure 7. 10-H Titration curves for the addition of (a) [$^2H_5$]pyridine and (b) [$^2H_4$]methanol to a [$^2H_6$]benzene solution of BChl. Lines are calculated best fits to the five-co-ordination model

acceptably some smoothing was necessary. As in n.m.r. spectroscopy,[15] optimal enhancement should be a compromise between increasing resolution and preserving signal intensity, and we found that the method of Savitsky and Golay [16] of smoothed higher derivatives was very effective.

The smoothed first- and second-derivative spectra are illustrated in Figure 8. The spectrum in pure methanol exhibits a single band at 600 nm; in the 9 : 1 mixture the major band is at 600 nm with a smaller band at 575 nm. This is good evidence for the existence of two discrete forms of BChl in solution.

From recording the electronic absorption spectra of BChl in a variety of solvents we find it is predominantly five-co-ordinate in acetone, diethyl ether, tetrahydrofuran, and dioxan, and predominantly six-co-ordinate in methanol and pyridine.

In the cases of methanol and pyridine this evidence is further confirmed by plots of (i)—(iii) (not shown) which clearly favour six-co-ordination. The K values were too large to be safely estimated by our techniques. An interesting observation is that the n.m.r. spectrum of BChl does not appear to be directly dependent on co-ordination state: the spectrum in methanol more closely resembles that in acetone than in pyridine. The reasons for this are unclear but may reflect ring current effects in the pyridine case.

The following papers [6,7] describe some chemical consequences of the results presented here. Similar observations on five- *vs.* six-co-ordination in quinone-capped magnesium-containing porphyrins have also been reported by one of us.[17]

Experimental

Bacteriochlorophyll *a* was extracted from chromatium D cell paste by the procedure described in detail in the following paper.

^1H N.m.r. spectra were recorded at 270 MHz, 25 °C on a Bruker WH270 spectrometer. Pulse widths of between 45° and 90° were used, spectral widths of 4 000 Hz, data-lengths of 8 K and no relaxation delay were employed. Typically spectra were average over 5—10 min at the concentrations studied.

Chemical-shift titrations were performed as follows. A pre-weighed amount of BChl (*ca.* mono-co-ordinate with H_2O) was dissolved in a deuteriated solvent in an n.m.r. tube. Internal $SiMe_4$ was added. The height of solvent in the n.m.r. tube was measured and solution volumes calculated assuming a cylindrical diameter of 0 42 cm (Wilmad PP 528). BChl concentrations were *ca.* 10 mM.

The concentration of added ligand was calculated from the volume added in μl. All concentrations were adjusted to allow for volume changes in solution as more ligand was added.

The curves were analysed as follows. If the shift of BChl in wet [$^2H_6$]benzene is S_1 Hz and the final shift is S_2 Hz, then the observed shift $\delta = S_1(c_1/c) + S_2(c - c_1)/c$ where c_1 and c are defined as in the text. Hence $c_1/c = (\delta\text{-}S_1)/(S_2 - S_2)$ giving the proportion of monomeric BChl.

The curves were analysed as described elsewhere.[8] To obtain equilibrium constants we used routines for constrained minimisation [18,19] of the residuals Σ (obs. − pred.)^2 and employed software locally available from the Numerical Algorithm Library.

Electronic absorption spectra were recorded on a Perkin-Elmer SP8-100 spectrophotometer, at 25 °C, and the digital readings at 0.2 nm intervals were manually transferred to an IBM 370/165 mainframe computer for subsequent processing as described in the text. The spectrum of BChl in a 9 : 1 v/v mixture of acetone and methanol was obtained by volumetrically adding methanol to a solution of BChl in acetone. Solvents were AnalaR grade.

Acknowledgements

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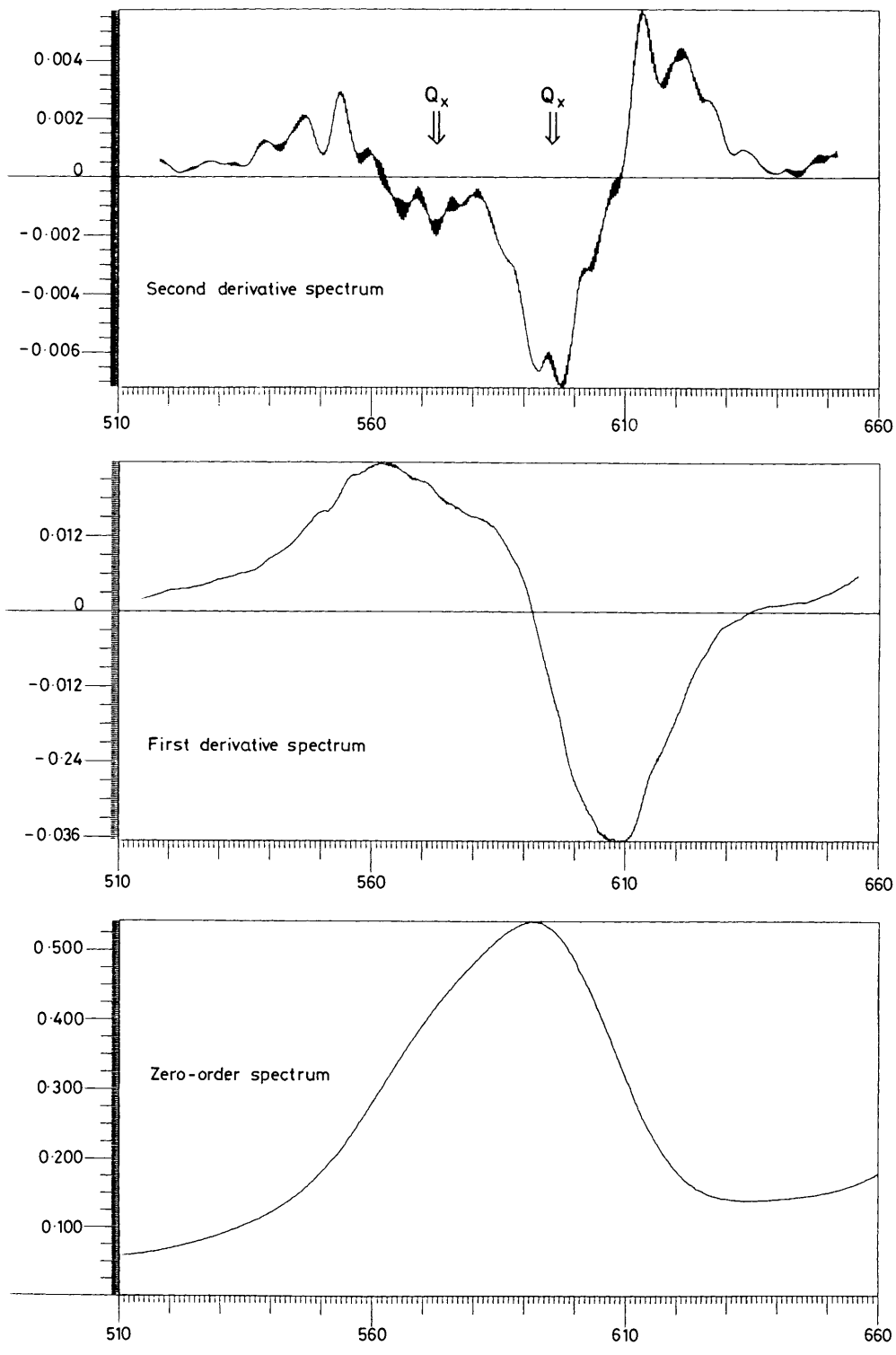

Figure 8. Portions of the electronic spectrum of bacteriochlorophyll *a* in acetone–methanol (9 : 1), and the smoothed first and second derivatives. The *x* axis is wavelength in nm, and the *y* axis is intensity of appropriate derivative

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