Bacteriochlorophyll *a*: Influence of Axial Co-ordination on Reactivity and Stability. Design of an Improved Extraction Procedure

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It is shown that the dehydrogenation of bacteriochlorophyll *a* is dependent on the co-ordination state of the central Mg atom. It is proposed that this reaction goes *via* the radical cation which is reactive when five-co-ordinate but stable when six-co-ordinate. These observations help design an extraction procedure which gives reproducibly high yields of indefinitely stable microcrystalline bacteriochlorophyll.

In the preceding paper [1] we discussed the axial co-ordination states of bacteriochlorophyll *a* (1) (BChl) and their solvent dependence. Here we show that the number of co-ordinated ligands can have a profound effect on the chemistry and stability of BChl. We also describe an isolation procedure which exploits these effects to give microcrystalline material which is almost indefinitely stable in air and light at room temperature even though BChl is generally considered to be rather unstable. Major decomposition products include an allomer [2] and Δ-3, 4 BChl (2) [also known as bacterioviridin [3] (BVir) and 2-acetyl-2-desvinyl Chl *a* [4,5]]. Thus it is remarkable that although bacterial reaction centres can be isolated by readily available procedures, making the primary events of bacterial photosynthesis considerably more accessible to *in vivo* studies than those of plant photosynthesis, *in vitro* studies on bacteriochlorophyll *a* have been considerably less conspicuous than those on chlorophyll *a*, because of the reported instability and difficulties of extraction.

The work described here arose from the early observation in this laboratory that solutions of BChl in [$^2H_6$]acetone contain variable amounts of radical cation depending on the history of the solution.[6]

Results

The effect of illuminating solutions of BChl in various solvents was monitored by electronic absorption spectroscopy. Two quite different results were obtained. In acetone solutions exposed to the air, the spectrum changed cleanly, with good isosbestic behaviour (Figure 1a) to that of a yellow-green chlorin, $\lambda_{max.} = 679$ nm, $\alpha = 22$ l/g.cm in a reaction which was zero order in BChl. The product was deduced to be BVir (2) by comparison of its visible and n.m.r. spectra with those published by Lindsay-Smith and Calvin.[4] T.l.c. showed the presence of small amounts of additional, unidentified products. Similar results were obtained from solutions of BChl in THF and ether, but the reaction was slower. By contrast, illumination of methanol or pyridine solutions of BChl yielded spectra characteristic [7] of the radical cation (Figure 1b). One-electron oxidants such as iodine and tris(*p*-bromophenyl)ammonium hexachloroantimonate (TAH) give essentially identical results: formation of BVir in acetone was rapid and quantitative. In methanol, it was very slow and only found in the presence of an excess TAH: in pyridine no detectable dehydrogenation occurred.

The presence of radicals should also be reflected in the ^1H n.m.r. spectrum of BChl, hence we investigated the ^1H n.m.r. spectrum in [$^2H_6$]acetone. Dissolution of BChl in undegassed acetone gave a spectrum which was differentially broadened in a way which is characteristic [8] of rapid electron transfer between BChl and its radical cation (Figure 2):

$$BChl + BChl^{+\cdot} \rightleftharpoons BChl^{+\cdot} + BChl$$

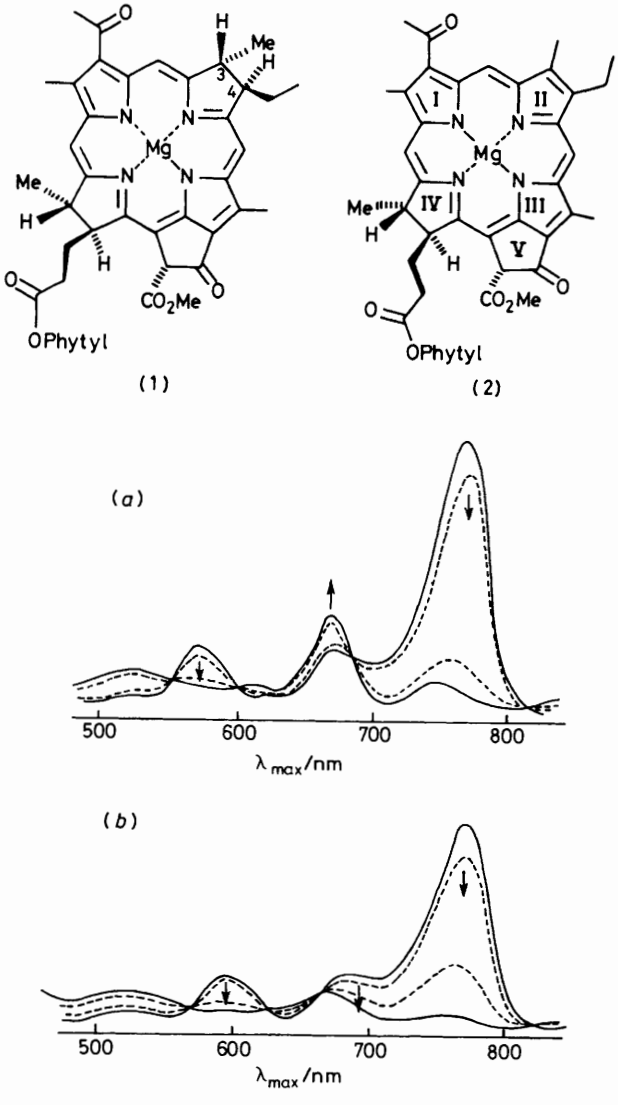


Figure 1. Changes in the electronic absorption spectrum of bacteriochlorophyll *a* (*a*) in acetone and (*b*) in methanol as solutions are illuminated in the air. The arrows show the direction of the change

Brief heating in the dark sharpened the spectrum. Brief illumination of the solution restored the differential broadening. Thorough degassing of the solution with N_2 also sharpened the spectrum, suggesting that the radical cation as expected, is destroyed.

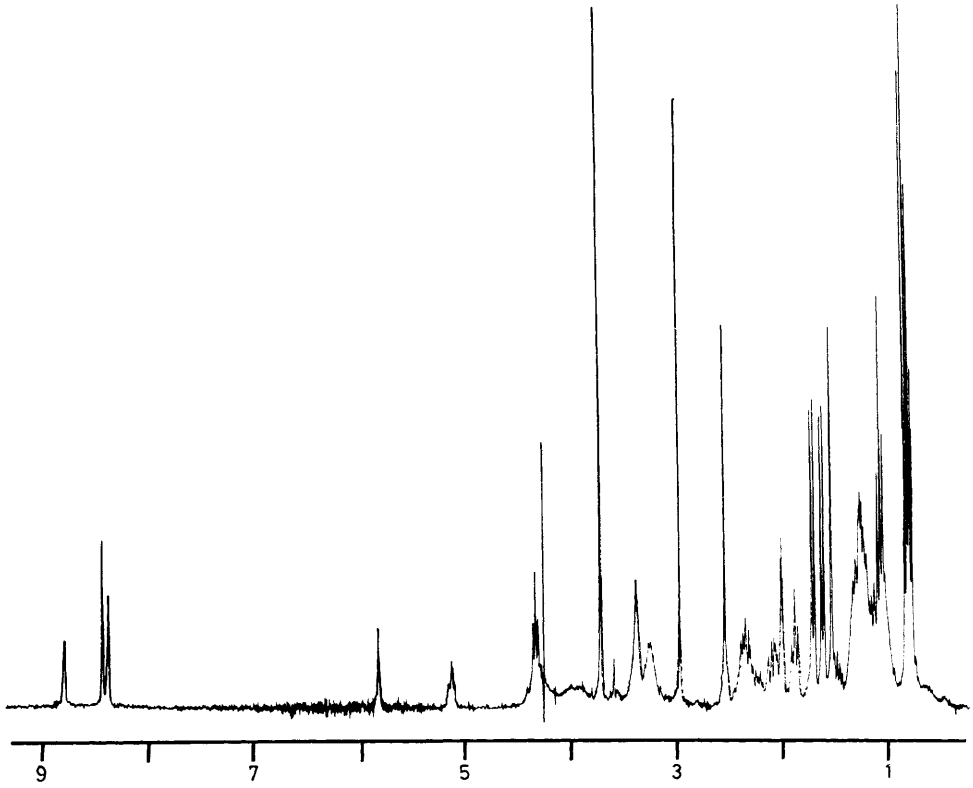


Figure 2. ^{1}H N.m.r. spectrum of bacteriochlorophyll *a* in un-degassed [$^2H_6$]acetone, indicating the presence of a small quantity of photochemically produced cation by differential line broadening particularly of 2 ring methyl singlets around δ 3.5

It is clear that both the chemical and photochemical oxidation of bacteriochlorophyll can be understood in terms of a single mechanism. We can furthermore rationalise the behaviour in terms of the co-ordination chemistry described in the preceding paper; [1] we know that the Mg atom of BChl is six-co-ordinate in methanol and pyridine, whereas it is five-co-ordinate in acetone, ether and THF. Correspondingly BChl can be readily converted *via* a mild process to BVir in five-co-ordinating solvents, but not in six-co-ordinating solvents. We suggest therefore that the first step on illumination or addition of a one electron oxidant is the formation of a radical cation: BChl ⟶ $BChl^{+\cdot}$. This is a slow rate limiting step photochemically, and the oxidant in the absence of an added reagent is probably O_2. Subsequent transformation to BVir ($BChl^{+\cdot}$ ⟶ BVir) formally requires loss of a proton and a hydrogen radical. This process occurs readily in five-co-ordinate BChl but is largely inhibited in six-co-ordinate BChl solutions, so that in the latter case the reaction stops at the radical cation.

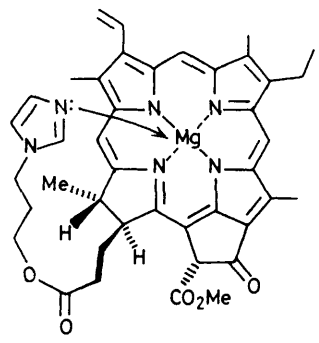
We do not know the fate of the lost H^+ and $H^\cdot$ but it seems reasonable to suppose that they may be transferred either to a co-ordinated group, perhaps water or O_2, or possibly two BChl molecules need to form a dimeric cation. There is no barrier to such processes in five-co-ordinate BChl which has a vacant site, but the six-co-ordinate species is ' protected '.

These results show that whatever the detailed nature of the processes involved, six-co-ordinate BChl is more stable to decomposition to BVir than five-co-ordinate BChl. Given this understanding of solvent influences on the stability of BChl, we designed an appropriate extraction procedure. BChl was extracted into ice-cold degassed methanol instead of the traditional acetone–water mixture. For chlorophyll *a* which is a dihydroporphyrin and so cannot be further oxidised in ring-II, these factors are not relevant, and acetone is the solvent of choice: this is because methanol causes the production of allomers,[2] and although allomers of BChl are also formed slowly, this is less serious than dehydrogenation. After initial filtration we selectively precipitated chlorophylls by sequential addition of dioxan and water.[9] The yield of BChl at this stage was 3—5 mg/g cell paste (*ca.* 60%). Chromatography on sucrose with light petroleum (b.p. 30—40 °C) containing 0.2—1.0% n-propanol gave a good separation allowing the BChl-containing section to be dug out and separately eluted. After evaporation, washing with light petroleum and drying, stable, analytically pure microcrystalline BChl, containing *ca.* 1.5 mol equiv. of water, was obtained in 30—50% yield based on crude extracts.

Discussion

These results confirm the observations of Lindsay-Smith and Calvin [4] on the dehydrogenation of what we now know to be five-co-ordinate BChl, and extend them in two directions: first, the mechanism appears to be radical-cation formation followed by loss of (formally) H^+ and $H^\cdot$. Second, the later steps in the dehydrogenation are effectively inhibited by six-co-ordination: the radical cation is protected from further reaction.

This protection idea is supported by other evidence as follows. (i) We previously took ' in beam ' EI mass spectra of bacteriochlorophyll and its allomer,[2] and found that spectra are only easy to obtain when solutions in pyridine or methanol were applied to the probe tip. Five-co-ordinate solutions in ether or oligomerised solutions in benzene reproducibly gave virtually no spectrum. This suggests that six-co-ordinate BChl is a single isolated species which can volatilise. Five-co-ordinate BChl, however, aggregates on heating on the probe, due presumably to mutual co-ordination, and hence does not readily yield a spectrum. Similar observations have been made on chlorophyll *a* (unpublished).



(3)

(ii) Electron transfer between neutral BChl and its radical anion in pyridine is slow on the n.m.r. time-scale at all accessible temperatures and concentrations.[10] This implies that electron transfer requires close proximity of two macrocycles perhaps in the form of a transient dimer, and that this process is inhibited by six-fold co-ordination.

(iii) The radical cation of the modified chlorophyll (3) which is protected by the intramolecularly bound imidazole is indefinitely stable, whereas the simple Chl-$a^{+\cdot}$ decomposes relatively rapidly.[11] Although the Chl-$a^{+\cdot}$ cannot decompose by the same route as $BChl^{+\cdot}$, it is clear that interaction with some other reactant is necessary.

In summary, we have powerful evidence that the co-ordination state of BChl critically affects its chemical reactivity, and have used this to our advantage in designing an extraction procedure.

Experimental

^1H N.m.r. spectra were recorded in the Fourier transform mode on a Bruker WH270 spectrometer as described in the preceding paper.[1] Electronic absorption spectra were recorded on a Perkin-Elmer SP8-100 spectrophotometer. Mass spectra were recorded on a Kratos MS50 mass spectrometer using a Fast Atom Bombardment (F.A.B.) source.[12] Sugar from British Sugar Corp.

Preparation of Bacteriochlorophyll a.—Chromatium D cell paste (100 g) was homogenised in a Waring blender with 700 ml of ice-cold methanol, in several fractions. The homogenate was filtered through a Buchner funnel, and the filtrate was centrifuged, at 3 000 rev min^{-1} and 10 °C in a Mistral 6L centrifuge for at least 15 min to remove cell debris. The supernatant liquid was diluted by 1/7th of its volume of sodium-distilled dioxan. Ice-cold distilled water was added dropwise to the solution until precipitation of BChl fractions was complete. Typically 50—150 ml of water was necessary. The resultant suspension was centrifuged at 3 000 rev/min^{-1} and 10 °C in a Mistral 6L centrifuge for at least 30 min. The supernatant liquid was poured off. If the supernatant liquid appeared dark in colour, the above procedure of precipitation with water, and centrifugation, was repeated. The precipitate was redissolved in diethyl ether (50—100 ml), transferred to a 500-ml round-bottom flask and the solvent was evaporated off on a rotary evaporator. If the precipitation had been repeated, all precipitates were combined. The green waxy solid left after evaporation was dried for 12 h at reduced pressure (0.1 mmHg). The crude extract was then dissolved in diethyl ether (*ca.* 20 ml) and loaded onto a column which had been packed with *ca.* 2 kg (B.S.C.) of dry icing sugar and pre-washed with several hundred ml of light petroleum (b.p. 30—40 °C). The column was run under reduced pressure from a water pump, and was first loaded with light petroleum (b.p. 30—40 °C) until the extract formed a discrete band at the top of the column. The eluant was pale yellow at this stage. The polarity of the solvent added to the column was gradually increased by adding n-propanol to the light petroleum. The initial proportion of n-propanol was 0.2% (v/v) and it was never necessary to increase the proportion to greater than 1.0%. As the polarity of the solvent increased, successive fractions were eluted, and the eluant disposed of. The column was stopped when the BChl fraction was a discrete blue/green layer in the middle of the column. At this stage the column appeared as follows (from top to bottom): (i) dark brown layer (proteins and cellular debris); (ii) discrete green layer (BChl oxidation products, *e.g.* BVir); (iii) blue/green layer (BChl); (iv) small blue/green layer (BChl epimer); and (v) red band (bacteriopheophytin).

The amounts of layers (i), (ii), (iv), and (v) were highly dependent on extracts. The eluant at this stage was light red. The central blue/green layer was dug out from the column with a long spoon, care being taken not to include the smaller band due to the epimer, if present. This layer was then packed into another column, and completely eluted with diethyl ether. The solution was evaporated on a rotary evaporator and dried using a trolley pump. Light petroleum (b.p. 30—40 °C) (50 ml) was added to the waxy precipitate and on scraping with a spatula, a microcrystalline suspension of BChl was formed. If the suspension was not easily formed then the light petroleum was evaporated off and the BChl dried further on a trolley pump before the operation was repeated. The suspension was collected on a Buchner funnel and washed several times with light petroleum (b.p. 30—40 °C) and distilled water. The precipitate was collected and dried in a desiccator over silica gel for several days. It was weighed, and stored in a refrigerator ready for use. It was unnecessary to distil or dry diethyl ether, light petroleum, or methanol for the extraction. Throughout the procedure light was excluded but rigorous precautions were unnecessary [Found: C, 70.1; H, 8.3; N, 5.7% ($BChl(H_2O)_2$ requires C, 69.7; H, 8.3; N, 5.9%; $BChl(H_2O)$ requires C, 71.2; H, 8.2; N, 6.0%], $\lambda_{max.}$ (MeOH) 769 ($\alpha_{max.}$ 47.4), 600 (14.3), and 364 nm (49.5 l/g.cm); *m/z* 911; h.p.l.c. 1 band on 2 Waters μ bondapak CN columns using hexane–toluene–acetonitrile (300 : 140 : 60) as eluant.

Photochemical Experiments.—Solutions were illuminated with two 13-W fluorescent lamps of length 15 cm. The n.m.r. tube and spectrophotometer cell were mounted between the lamps. The solutions were removed from the light and monitored at various intervals of time. Solutions for spectrophotometry were *ca.* 10^{-5}M, and for n.m.r. spectroscopy were *ca.* 10^{-2}M.

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References

1 R. G. Brereton and J. K. M. Sanders, preceding paper.
2 R. G. Brereton, V. Rajananda, T. J. Blake, J. K. M. Sanders, and D. H. Williams, *Tetrahedron Lett.*, 1980, **21**, 1671.
3 A. Holt and E. E. Jacobs, *Am. J. Bot.*, 1956, **41**, 718.

4 J. R. Lindsay-Smith and M. Calvin, *J. Am. Chem. Soc.*, 1966, **88**, 4500.
5 C. Weiss, *J. Mol. Spectrosc.*, 1972, **44**, 37.
6 J. C. Waterton, Ph.D., thesis, University of Cambridge, 1977.
7 D. C. Borg in 'Free Radicals in Biology,' ed. W. A. Pryor, Academic Press, New York, 1976, vol. I.
8 J. C. Waterton and J. K. M. Sanders, *J. Am. Chem. Soc.*, 1978, **100**, 4044.
9 K. Iriyama, N. Ogura, and A. Takamiya, *J. Biochem.* (*Tokyo*), 1974, **76**, 901.
10 R. G. Brereton and J. K. M. Sanders, *Org. Magn. Reson.*, 1982, **19**, 150.
11 I. S. Denniss and J. K. M. Sanders, *Tetrahedron Lett.*, 1978, 295.
12 M. B. Bazzaz, C. V. Bradley, and R. G. Brereton, *Tetrahedron Lett.*, 1982, **23**, 1211.

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