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Organization of Antenna and Photo-Reaction Centre Chlorophylls on the Molecular Level [and Discussion]

J. J. Katz, W. Oettmeier, J. R. Norris and S. I. Beale

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Organization of antenna and photo-reaction centre chlorophylls on the molecular level

By J. J. Katz, W. Oettmeier† and J. R. Norris
Chemistry Division, Argonne National Laboratory, Argonne, Illinois, 60439, U.S.A.

Recent investigation, mainly by nuclear magnetic (n.m.r.) and electron spin (e.s.r.) resonance spectroscopy, provide experimental support for the view that both antenna or light-harvesting chlorophyll and photo-reaction centre chlorophyll are aggregated species, but are aggregated in quite different ways. The spectral properties of antenna chlorophyll appear to be best explained in terms of chlorophyll oligomers, $(Chl_2)_n$, in which the chlorophyll molecules are bound to each other via keto C=O···Mg coordination interactions, whereas light conversion occurs in special pairs of chlorophyll molecules probably held in the necessary configuration by a water molecule between them: [Chl H_2O Chl]. Photo-reactive bacteriochlorophyll *a* appears to have a very similar special pair structure, but antenna bacteriochlorophyll *a* appears to be a much more complex entity than is the antenna in green plants, and it is possible that a variety of bacteriochlorophyll *a*–water adducts are involved in antenna behaviour. Considerations of photosynthetic membrane structure suggest that antenna chlorophyll and the special pair photo-active chlorophyll may occupy the annular region of the thylakoid lipid bilayer.

Introduction

A fairly coherent picture of the role of chlorophyll in photosynthesis has emerged over the past several decades. Universally regarded as the primary photo-acceptor, chlorophyll was shown by Emerson & Arnold (1932) to act cooperatively in the absorption of light. The light energy absorbed by the so-called 'light-harvesting' or 'antenna' chlorophyll molecules is funnelled to a small number of 'special' chlorophyll molecules that act as a photo-reaction centre where conversion to oxidation-reduction potential occurs. The combination of antenna and photo-reaction centre chlorophyll constitutes a photosynthetic unit. From information derived largely from spectroscopic studies of various kinds on chlorophyll *in vitro* and *in vivo*, it is now possible to delineate (or at least to impose quite rigorous boundary conditions on) the structures of the antenna (Cotton, Trifunac, Ballschmiter & Katz 1974) and photo-reaction centre (Norris, Scheer, Druyan & Katz 1974) chlorophyll. Both of these entities, which we will describe below, are themselves composed of chlorophyll aggregated in different ways. As far as we can see at present, monomeric chlorophyll molecules, other than providing the basic building blocks of which antenna and reaction centre chlorophyll are constituted, have no functional significance in photosynthesis.

The main interest in the past in the biosynthesis of the photosynthetic apparatus has focused on the biogenesis of chlorophyll on the one hand, and on the morphology of the chloroplast in green plants, the chromatophore in photosynthetic bacteria, and the photosynthetic membrane in blue-green algae on the other. In this communication we propose to examine the biosynthetic problems posed by the postulated structures for antenna and photo-reaction centre chlorophylls. These questions occupy the ground between the chemical and biochemical studies directed to the elucidation of the biosynthetic pathways leading to the chlorophylls, and the morphological

† Resident Research Associate.

investigations at the light microscope and especially the electron microscope levels that aim at a description of organelle structure. It will become evident that much more information on the morphology of the photosynthetic apparatus at the molecular level will be needed, perhaps by such new methods as neutron diffraction on organelles of suitably adjusted isotopic composition, for anything like a detailed answer to the questions we raise here. Nevertheless, we consider it opportune to begin the process of fitting our postulated organized chlorophyll entities into currently accepted structures of the photosynthetic apparatus, and to explore possible ways in which dissonances can be resolved.

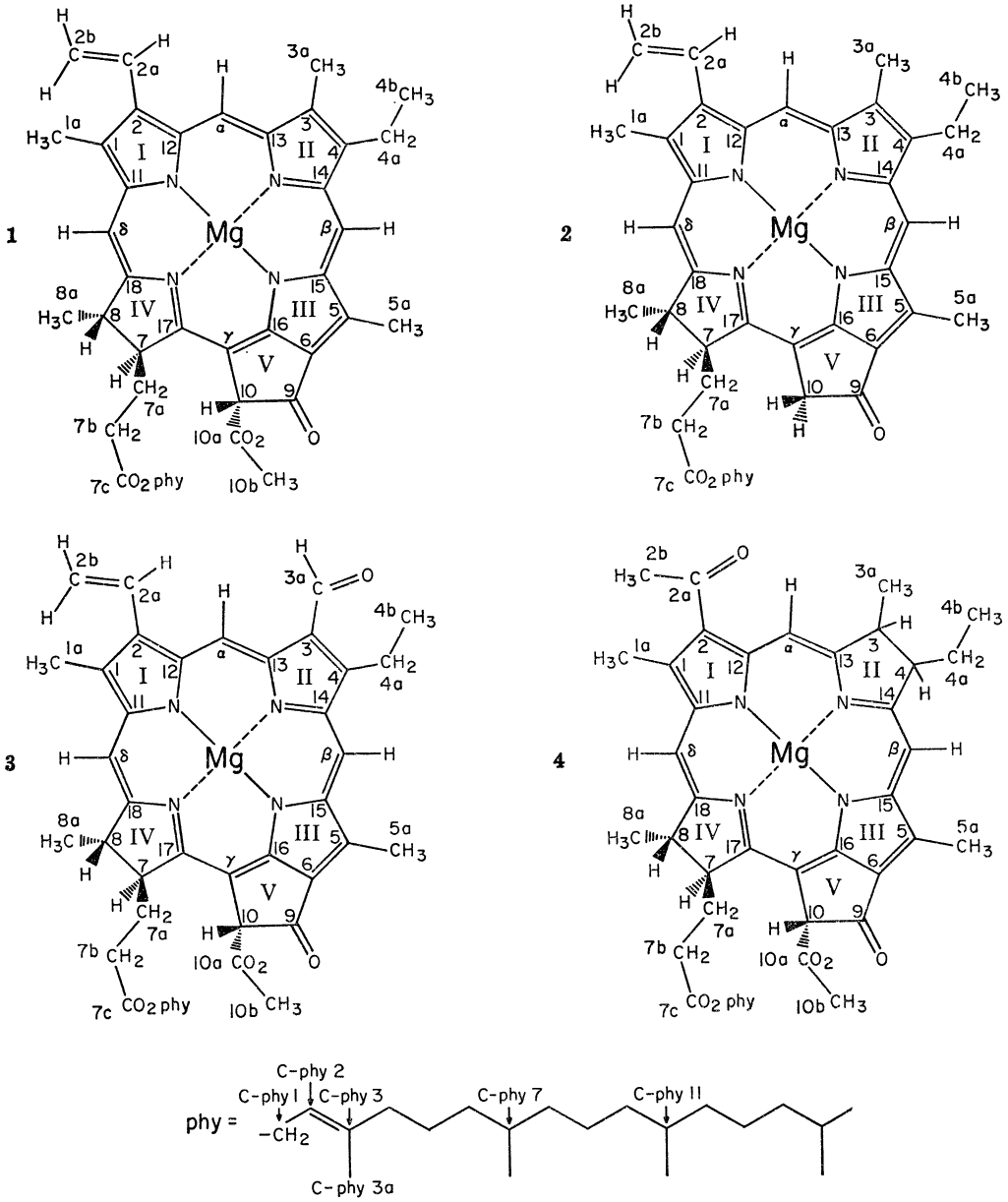

FIGURE 1. Structure of the chlorophylls: **1**, Chlorophyll *a* (Chl *a*); **2**, Pyrochlorophyll *a* (Pyrochl *a*); **3**, Chlorophyll *b* (Chl *b*); **4**, Bacteriochlorophyll *a* (Bchl *a*). All of the carbon atoms are numbered in **1**.

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Antenna chlorophyll

The most obvious experimental feature that indicates organization within the antenna chlorophyll system is the unusual position of the red optical transition. Whereas chlorophyll *a* (Chl *a*) (figure 1) dissolved in solvents such as diethylether, acetone, pyridine and the like absorbs light in the red portion of the spectrum near 665 nm, antenna chlorophyll in green plants absorbs near 680 nm, a red shift of the red maximum of some 15–20 nm. It has long been recognized, particularly by the Brody's (1961) and by Krasnovskii *et al.* (1952, 1955), that some type of aggregation of the chlorophylls that brings the ring macrocycles close together could account for the red shift, at least in a qualitative fashion. As an experimental basis for relating the electronic transition spectra to the structure of a particular chlorophyll species was lacking, little could be said about the nature of the aggregated antenna chlorophyll species. Modern spectroscopic techniques, proton (Closs *et al.* 1963; Katz, Dougherty & Boucher 1966) and carbon-13 (Boxer, Closs & Katz 1974) nuclear magnetic resonance (^{1}H m.r., ^{13}C m.r.), electron spin resonance (e.s.r.) (Warden & Bolton 1974) used in conjunction with infrared (i.r.) (Katz *et al.* 1963, 1966) and computer-assisted electronic transition (e.t., visible absorption) spectroscopy (Cotton *et al.* 1974) have provided a firm experimental basis for an understanding of the nature of self- or endogamous interactions of chlorophyll, and for the (exogamous) interactions of chlorophyll with electron-donor (nucleophilic) molecules generally (Katz 1973).

Coordination properties of chlorophyll

All of the chlorophylls are molecules with an unusual combination of electron donor-acceptor properties (Katz 1968). I.r. and n.m.r. studies (summarized by Katz 1973) strongly suggest that the central magnesium atom of Chl *a* and bacteriochlorophyll *a* (Bchl *a*) are coordinatively unsaturated when written with the coordination number 4 as in the conventional structural formulas of figure 1. Rather, the magnesium atom, in order to allievate its coordination unsaturation, must have an electron donor group in one or both of the Mg axial positions, so that the coordination number of the central Mg atom is formally always 5 or 6. The recognition of an absolute requirement for the central Mg atom to assume a coordination number of 5 or 6 makes possible the rationalization in terms of a variety of chlorophyll species of different structures of a large amount of data on the solvent dependence of i.r., ^{1}H m.r. and ^{13}C m.r. spectra.

Investigations by ^{1}H m.r. that take advantage of the ring current effect on the chemical shifts of the protons of a ligand bound to the central Mg atom (Katz *et al.* 1968) show that the coordination number 5 is by far the preferred Mg coordination number, but that both axial positions may be filled under forcing conditions, as in solution in a neat strong base such as a primary amine. Predilection for a coordination number of 5 is also observed for the Mg in Bchl *a* (Evans & Katz 1975). Thus, Chl *a* dissolved in a polar solvent occurs as chlorophyll monomer, with one or both of the Mg axial positions occupied by a molecule of solvent, e.g. Chl·L_1 and Chl·L_2. In such solutions, the chlorophylls exhibit n.m.r. and i.r. spectra directly interpretable in terms of the conventional structural formulas of figure 1.

In the absence of extraneous nucleophiles, Chl *a* can function as its own electron donor by virtue of the several donor (carbonyl) groups at its disposal. In a non-polar solvent carefully freed from nucleophilic impurities, a Mg coordination number of 5 (or 6) in one chlorophyll molecule can be achieved by electron donation from the C=O function of another chlorophyll.

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In non-polar media dimers will be generated by such an interaction, and in dimers the i.r., n.m.r. and e.t. spectra will all differ, not surprisingly, from those of monomeric chlorophyll. In the case of the e.t. spectra the changes observed in the dimer are in the direction of the changes observed in antenna chlorophyll. The coordination behaviour of the central Mg atom is thus central to the state assumed by the chlorophyll in different environments.

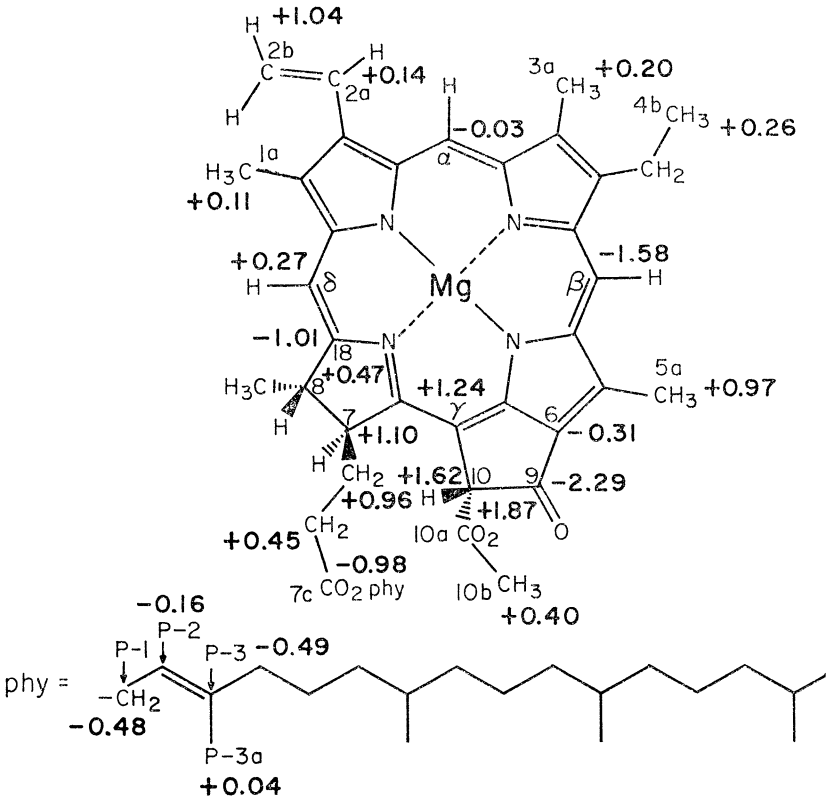

FIGURE 2. Chlorophyll *a* ^{13}C incremental shifts (Δ (ppm) = $\delta_{monomer} - \delta_{dimer}$) for the indicated carbon atoms (Shipman, Janson, Katz & Ray 1975).

Chlorophyll dimers, trimers and oligomers

Molecular mass measurements by vapour phase osmometry (Ballschmiter, Truesdell & Katz 1969) and by ultracentrifugation establish the state of Chl *a* in carbon tetrachloride or benzene to be the dimer, Chl_2. The ring V keto C=O function was earlier shown by ^{1}H m.r. (Closs *et al.* 1963) and i.r. (Katz *et al.* 1963) to be the chief donor function in the dimerization. While the ^{1}H m.r. evidence for ring keto C=O donor participation is quite unequivocal, it is nevertheless indirect, and the possibility of a donor contribution from either or both of the ester C=O functions at 10*a* and 7*c* (figure 1) could not be completely excluded. ^{13}C m.r. now provides direct evidence for ring V keto C=O as the paramount donor (Katz *et al.* 1972; Katz & Janson 1973). When dimers are formed the resonance(s) of the carbon atom(s) of the donor C=O group(s) are expected to show a strong downfield shift from the deshielding resulting from the coordination interaction. The resonances of the carbon atoms brought into the close vicinity of another ring macrocycle are to the contrary expected to show a shift to either higher or lower field, with those carbon atoms near the coordination centre necessarily showing an upfield shift. The ^{13}C chemical shifts in all 55 carbon atoms in monomer Chl *a* have now been assigned (Boxer

et al. 1974) and thus it is possible to assign the chemical shifts in the dimer by a titration procedure similar to that used for the ^{1}H m.r. spectra (Closs *et al.* 1963). The results of such an experiment are summarized in figure 2 in the form of incremental chemical shifts (for the indicated carbon atoms) of the carbon atoms in the dimer relative to the monomer (Shipman *et al.* 1975). To qualify as a donor group, a C=O function must show a large (negative) downfield shift in the dimer, and the carbon atoms in the immediate vicinity of the donor carbonyl groups that are not a part of the conjugated system must experience a (positive) upfield shift from the ring current effect. It is apparent that the C_9=O is the only carbonyl function that exhibits donor properties. The C_9=O carbon atom is strongly deshielded, and shows the largest negative incremental shift of any carbon atom in the dimer. The sp^3 carbon atoms in the immediate vicinity of C_9 all show a positive incremental shift, including the C_{10a}=O in the ring V carbomethoxy group. The C_{7c}=O also fails to exhibit donor behaviour; although C_{7c} shows a downfield shift, the carbon atoms in the attached phytyl chain also are deshielded, thus arguing for a ring current origin for all of these negative incremental shifts. The ^{13}C m.r. data thus lead to the same conclusion as do the ^{1}H m.r. results, i.e. the ring V keto C=O function is the donor group in Chl_2 formation. Additional support for this view also is contributed by the aggregation behaviour of pyrochlorophyll *a* (Pyrochl *a*) (figure 1). Pyrochl *a* lacks a carbomethoxy group at position 10 in ring V. Both i.r. and ^{1}H m.r. data on Pyrochl *a* dimers in benzene or carbon tetrachloride agree that (Pyrochl *a*)$_2$ is a more stable entity than is (Chl *a*)$_2$, and argue for a destabilizing contribution from the carbomethoxy group to Chl *a* dimer formation.

Direct molecular mass measurements show the process of chlorophyll aggregation via keto C=O···Mg interactions proceeds far past the dimer stage in hostile solvent such as aliphatic, or cycloaliphatic hydrocarbons, and that in these media, Chl *a* oligomers, $(Chl_2)_n$, with $n > 10$, are formed in concentrated solutions (0.1 M). Because of the favourable relaxation properties of ^{13}C, ^{13}C m.r. (unlike ^{1}H m.r.) spectra of Chl *a* hexamers are still well defined, and the results of Boxer *et al.* (1974) provide strong evidence that the geometry of the dimer is repeated in the oligomer, i.e. no radically different chlorophyll–chlorophyll interactions are used to form the oligomer. Thus, the preponderance of the experimental evidence favours the ring V keto C=O···Mg as the principal coordination interaction involved in the genesis of the oligomer.

The self-aggregation behaviour of Chl *b* and Bchl *a* is considerably more complicated and is only incompletely understood. Chl *b* and Bchl *a* both possess additional donor functions: an aldehyde-CHO group in position 3 in Chl *b* (figure 1), and an acetyl CH_3CO function at position 2 in Bchl *a*. Both of these groups appear to be considerably stronger donors than are the ester C=O functions, but weaker than the ring V keto C=O. I.r., which serves very well to provide structural information on Chl *a* self-aggregates, is far more difficult to apply to Chl *b* and Bchl *a*. In Bchl *a* self-aggregates, i.r. evidence indicates donor participation by both the ring V keto C=O and the ring II acetyl C=O groups, with the keto C=O as the stronger donor. A more quantitative assessment of the relative donor strengths of the carbonyl functions will have to be deferred until ^{13}C m.r. data for these two chlorophylls become available.

There can be little doubt that both Chl *b* and Bchl *a* have a stronger tendency to self-aggregation than does Chl *a*. This is already evident in the limited solubility of Chl *b* and Bchl *a* in nonpolar solvents. Whereas Chl *a* readily forms 0.2 M solutions in *n*-octane, Chl *b* solutions more concentrated than 10^{-4} M cannot be prepared in aliphatic hydrocarbon solvents, and even in cyclohexane, in which Chl *a* is freely and highly soluble, Chl *b* solutions more concentrated

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than about 5×10^{-2} M cannot be formed. The aggregation behaviour of Chl *b* in CCl_4 appears to be best described by a trimer $\rightleftharpoons$ oligomer equilibrium at 25 °C. Xylene is a more disaggregating solvent, and direct molecular mass measurements show that Chl *b* in xylene at 65 °C is less aggregated than in benzene at 37 °C. In xylene solutions, Chl *b* aggregation is best rationalized in terms of a dimer $\rightleftharpoons$ oligomer equilibrium. In a mildly aggregating solvent such as cyclohexane at 37 °C, a still rather dilute solution of Chl *b* shows an aggregation number near 30, indicative of a far greater degree of self-aggregation than for Chl *a*. In benzene solution at 37 °C the smallest unit of Chl *b* extrapolates to trimer in very dilute solution, and to dimer in methylcyclohexane solution at 65 °C (figure 3). A solution of Chl *b* in a non-polar solvent thus contains a complex mixture of Chl *b* dimers, trimers, and oligomers. Small variations in concentration, temperature, and solvent composition can be expected to have significant effects on the relative donor strengths of the —CHO and keto C=O groups and thus on the mix of Chl *b* species present. As Chl *b* always occurs in green plants only when larger amounts of Chl *a* are present, the nature and consequences of Chl *a*–Chl *b* interactions becomes of interest. Addition of Chl *b* to a benzene solution of Chl *a* does not appear to cause any marked changes in the state of the Chl *a*, as judged from the observed molecular mass average (figure 4). In cycloaliphatic, and particularly in aliphatic hydrocarbon solvents, Chl *b* is a cross-linking agent for Chl *a* oligomers. When concentrated solutions of Chl *a* in aliphatic hydrocarbon solvents are titrated with Chl *b*, the size of the aggregates increases rapidly. Even though the solubility of Chl *b* in *n*-hexane is less than 10^{-4} M, solutions of Chl *b* at least as concentrated as 2×10^{-2} M can be prepared when twice the molar concentration of Chl *a* is present. The ratio of Chl *a*/Chl *b* in green plants approximates 2:1, and thus it is natural to speculate that a 2:1 mixture of Chl *a* and *b* will tend to be present as *aba*, and that these may form oligomers with the sequence *–abaabaaba–*. The visible absorption spectrum in the red of such a mixed oligomer is somewhat different from that of a pure Chl *a* oligomer, but not remarkably so. It is possible that green plant spectral maxima near 673 nm reported in the literature may originate in antenna chlorophyll containing *aba* components.

I.r. data on Bchl *a* aggregates indicate donor participation by both the ring V keto=O and the ring II acetyl C=O. Because of overlapping absorption maxima in the carbonyl stretch region, a detailed analysis has as yet not been possible, and the structural aspects of Bchl *a* oligomers remain obscure. It is evident from the limited molecular mass data available that Bchl *a* resembles Chl *b* more closely than Chl *a* in its aggregation behaviour, and that Bchl *a* solutions in cycloaliphatic or aliphatic hydrocarbon solvents contain entities of much larger molecular mass than is the case for Chl *a* at comparable concentrations. As in the case of Chl *a* *v.* Pyrochl *a*, pyrobacteriochlorophyll *a* (Pyrobchl *a*) forms more strongly bound aggregates than does Bchl *a*, as judged from the relative intensity of the respective keto C=O i.r. absorption maxima. Again, the carbomethoxy ester C=O appears to cause steric problems in the formation of aggregates, and the replacement of the $—CO_2CH_3$ group by H permits a more strongly interacting aggregate to form.

Deconvolution of visible absorption spectra

Even casual examination indicates a resemblance between the visible absorption spectrum of Chl *a* dissolved in benzene or carbon tetrachloride and antenna chlorophyll. It has long been noted that the red absorption peak of Chl *a* in dilute solution in benzene or CCl_4 develops a red-shifted shoulder near 680 nm. Likewise, the resemblance in spectral properties of Chl *a*

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films, monolayers and colloidal dispersions and those of antenna chloropyll have long been recognized. For the most part, investigators were content to attribute the shift to long wavelength in the red absorption band to 'aggregation', and therefore to characterize antenna chlorophyll as 'aggregated' chlorophyll. Greater precision in interpretation proved elusive for a variety of reasons. Chlorophylls are intense absorbers of light with extraordinarily high

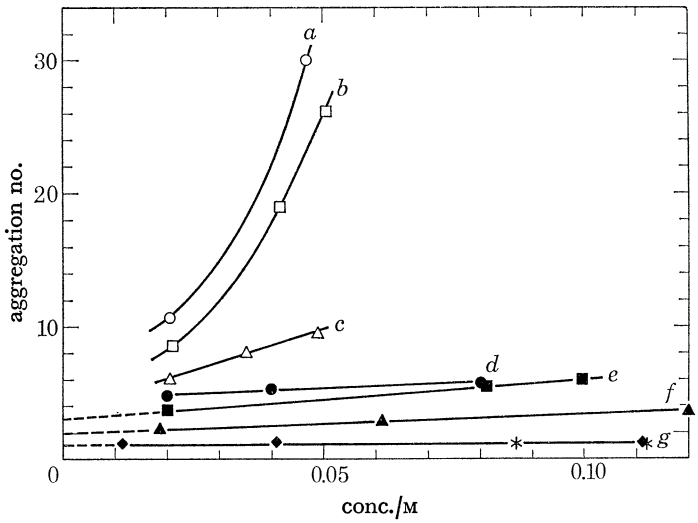

FIGURE 3. Aggregation of chlorophyll *b* from vapour phase osmometry. The aggregation number is defined as the ratio of the molarity calculated as monomer to the observed molarity. (*a*) cyclohexane, 25 °C; (*b*) cyclohexane, 37 °C; (*c*) methylcyclohexane, 65 °C; (*d*) benzene, 25 °C; (*e*) benzene, 37 °C; (*f*) xylene, 65 °C; (*g*) pheophytins *a* and *b* in benzene, 37 °C.

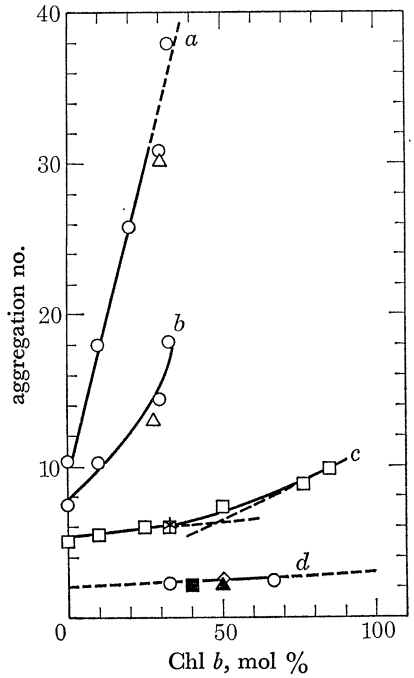

FIGURE 4. Aggregation behaviour of chlorophyll *a*–chlorophyll *b* mixtures as a function of *a*/*b* ratio. The aggregation number is defined as in figure 3. (*a*) *n*-hexane, total chlorophyll conc. 6×10^{-2} M; (*b*) *n*-hexane, total chlorophyll conc. 4×10^{-2} M; (*c*) cyclohexane, [Chl], $6\text{–}8 \times 10^{-2}$ M; (*d*) benzene, [Chl], 6.2×10^{-2} M–1.2×10^{-1} M. All at 25 °C.

extinction coefficients, with the result that chlorophyll visible absorption spectra are almost always recorded on very dilute solutions (10^{-6} to 10^{-4} M) when conventional absorption cells are used. In the chloroplast, however, chlorophyll is in a highly concentrated form, at a concentration estimated to be in the vicinity of 0.2 M. Consequently, spectral comparison between the very dilute solutions used in the laboratory and antenna chlorophyll in the plant were not entirely convincing. As the effects of nucleophiles on the aggregation state of chlorophyll was not recognized, experimenters generally failed to remove nucleophiles such as water from their systems. It is easily possible to prepare 10^{-2} M solutions of water in benzene or carbon tetrachloride, and thus to have present a thousandfold excess of water when a 10^{-5} M solution of Chl *a* is examined. Under such conditions, the Chl *a* will be fully disaggregated to its monomeric form, Chl·L_1, and no red-shift in the spectrum will occur. Thus, experiments on dilute solutions were poorly reproducible and thus added to the confusion. With films or monolayers, which are essentially highly concentrated solutions of Chl *a* in an aliphatic hydrocarbon solvent (the phytyl chain moiety), the spectral resemblance to antenna chlorophyll is unmistakable, but in these systems there was no easy way to characterize the state of aggregation of the chlorophyll, and no other structural alternatives to the dimer were seriously entertained. In any event, the red bands in both antenna chlorophyll and a Chl *a* film are so featureless as to prohibit a detailed comparison. In retrospect it can be seen that visible absorption spectra, by far the most widely applied spectroscopic technique used for chlorophyll and photosynthesis studies, are the easiest to use and the most difficult from which to draw structural information. Only in conjunction with structural information derived from i.r. and magnetic resonance studies can visible absorption spectroscopy yield the structure of the chlorophyll species in which the electronic transitions occur. It is by an examination of chlorophyll solutions in which the chlorophyll species present have been characterized by other means that a convincing argument can be made for the identification of antenna chlorophyll with the Chl *a* oligomer present in concentrated solutions in aliphatic hydrocarbon solvents.

The red envelopes in the absorption spectra of antenna chlorophyll or Chl *a* oligomers are broad and show practically no structure at room temperature. Comparisons on the basis of estimated wavelength maxima carry little conviction. To make a comparison of *in vitro* and *in vivo* absorption spectra less subjective, resort has been had to computer-assisted deconvolution techniques. In this procedure, visible absorption spectra are digitized and a computer fits the experimental curve with Gaussian components. A Gaussian line shape for the deconvolution is chosen because the red envelope in the visible absorption spectrum of monomeric Chl *a* is very close to a Gaussian in line shape, and an analysis of the physical situation in terms of the statistics of aggregates of a large number of objects also suggests that the criteria for Gaussian line shapes are met in chlorophyll self-aggregates. The deconvolution is carried out by a computer program that fits the component peaks by a process of successive iterations to any degree of precision desired. The red envelopes of both Chl *a* oligomers and antenna chlorophyll are already so close to Gaussian that a unique deconvolution cannot be obtained. Consequently, a boundary condition is applied to the deconvolution of the *in vitro* Chl *a* solution spectra. Dilute solutions (10^{-5} to 10^{-6} M) of Chl *a*·L_1 have a red envelope that has sufficient structure to assure a unique deconvolution. Deconvolution is required to be self-consistent over the entire concentration range, i.e. the envelopes over the range 10^{-6} to 10^{-1} M must be fitted with Gaussian components that have the same wavelength maxima, and only the width at half-height and the intensity or peak height allowed to vary. In effect, the assumption is made that changes in the Chl *a* species

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that occur in going from the most dilute solutions where dimers predominate, to the most concentrated, where oligomers are the principal species present, are gradual and progressive. By this procedure the deconvolutions of the oligomers are related to the dimer. Figure 5 shows the results of deconvolution of the visible absorption spectra of a series of Chl *a* solutions in hexane. The most surprising result is that the entire series of solutions can be deconvoluted with the same set of Gaussian components. The wavelength of the Gaussians are essentially the same

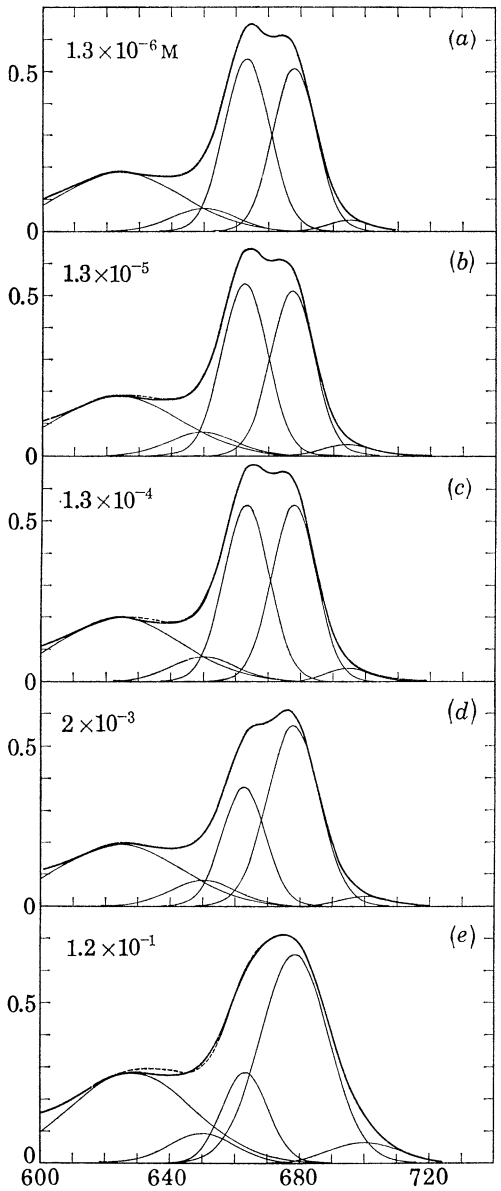

FIGURE 5

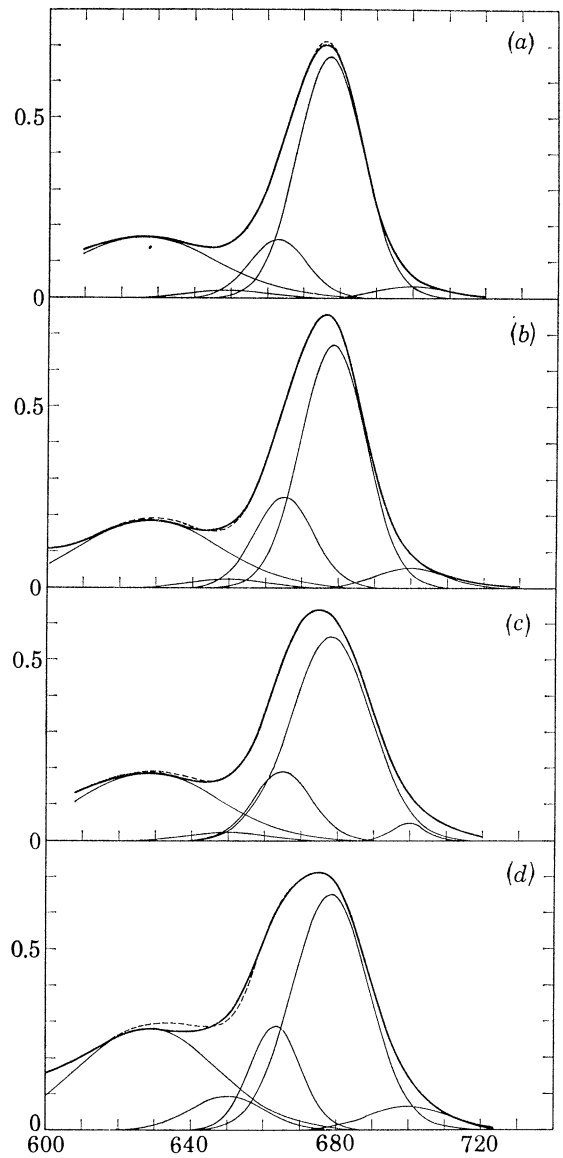

FIGURE 6

wavelength/nm

FIGURE 5. Deconvolution of the spectra of chlorophyll *a* solutions in *n*-hexane as a function of concentration (Cotton *et al.* 1974).

FIGURE 6. A comparison of the deconvolution of the *in vivo* and *in vitro* chlorophyll *a* red envelopes in (*a*) *Tribonema aequale* sonicate; (*b*) an active centre preparation containing antenna chlorophyll prepared from the blue-green alga *Synechococcus lividus*; (*c*) a similar preparation from the blue-green alga *Anabaena variabilis*; and (*d*) a 1.2×10^{-1} M solution of chlorophyll *a* in *n*-hexane. No chlorophyll *b* is present in any of the *in situ* preparations.

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over the entire concentration range, and only the relative areas of the main blue and red Gaussians change with aggregation. A similar experiment carried out over a similar concentration range in benzene, where the Chl *a* occurs largely as dimer in the concentration range 10^{-6} to 10^{-1} M, results in deconvolution components that have essentially the same area in all of the solutions. Thus, a (rough) relationship between the size of the aggregate and the areas of the red and blue Gaussian is established. The relationship is not exact because the change in size is also accompanied by changes in the geometry of the chlorophyll molecules relative to each other. It must be emphasized that in Chl *a* oligomers increase in size is not necessarily accompanied by additional red shifts in the red maxima. The red shift from 665 to 680 nm described for these solutions results from the increase in the area of the red Gaussian at the expense of the blue, and this has been interpreted by visual examination as a red shift. The red shift in the dimer e.t. spectra relative to monomer Chl *a* arises from the juxtaposition of the two dimer chlorophyll macrocycles. When oligomers are formed from the dimer, no new orientations result, i.e. the red shift in the oligomers is largely due to interactions between nearest neighbours, and thus the red shift is relatively insensitive to oligomer size. Comparison of deconvoluted Chl *a* spectra in aliphatic hydrocarbon solutions to antenna chlorophyll in a variety of organisms is remarkably similar. (The organisms selected for comparison do not contain Chl *b*, thus simplifying the comparison.) A concentrated solution of Chl *a* and antenna chlorophyll in a variety of organisms have red absorption maxima that can be deconvoluted with the same Gaussian components. The relative areas of the blue and red Gaussians in the deconvoluted spectra are such as to suggest that Chl *a* in the antenna is even more highly aggregated than it is in a 0.1 M solution in *n*-hexane (figure 6).

Identification of antenna chlorophyll with Chl *a* oligomers, $(Chl_2)_n$, places some important restrictions on chloroplast structure. The oligomer is stable only in a highly non-polar hydrophobic environment from which extraneous nucleophiles, especially water, are rigidly excluded. If water is, in fact, present in the vicinity of the oligomer the water activity must be reduced by some mechanism so that it cannot compete with another Chl *a* molecule for coordination at Mg. Further, it is evident in the *in vitro* system that the spectral red shift results from chlorophyll–chlorophyll interactions. The widely accepted view that the red-shift in antenna and photoreaction centre chlorophylls arises from some sort of chlorophyll–protein interaction finds no support in laboratory experiments, in which red-shifted Chl *a* species are generated by chlorophyll–chlorophyll interactions, or by water in an aliphatic hydrocarbon solvent. The entire envelope and the red-shift are an intrinsic property of the oligomer. The red and blue Gaussian components can be associated with specific chlorophyll–chlorophyll orientations. It is evident that whatever else may be said about the structure of $(Chl_2)_n$, there is no way in which an entity formed by keto C=O···Mg interactions can have macrocycle planes oriented in a parallel fashion. The macrocycles must be much closer to an orthogonal configuration than to a parallel one. These requirements thus impose some rather severe restrictions on models for antenna chlorophyll in green plant photosynthetic membranes.

Bchl *a* oligomers have also received study, but need not be considered further here, as the evidence is quite strong that antenna Bchl *a* in photosynthetic bacteria is not an anhydrous but a hydrated species. Antenna chlorophyll in photosynthetic bacteria thus appears to have a basically different structure than does green plant antenna chlorophyll. The differences are discussed below.

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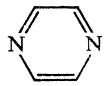
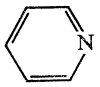
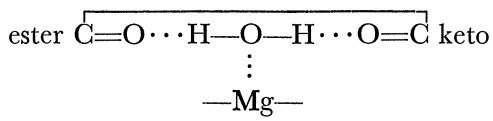
Photo-reaction centre chlorophyll

We now turn to a class of chlorophyll-ligand adducts that has a special significance for discussions of the nature of *in vivo* photo-reactive chlorophyll. When a chlorophyll–chlorophyll self-aggregate is titrated with a mono-functional base such as acetone or pyridine, the existing keto C=O···Mg interactions are disrupted and the species, Chl·L_1 and Chl·L_2, are formed. Unlike aggregated chlorophyll species, whose red-maxima are red-shifted and which are non-fluorescent, the monomer chlorophyll species absorb in the red but at shorter wavelengths and are intensely fluorescent. It is clear that a bifunctional ligand such as pyrazine,  (cf. pyridine, ), has the possibility to cross-link chlorophyll molecules by coordination to the central Mg atoms of two Chl *a* molecules, bringing the chlorophyll molecules into more or less close proximity to an extent that depends on the dimensions of the bifunctional ligand. Numerous examples of such bifunctional ligands are known and include diols, diamines, di-nitriles and the like. The most interesting bifunctional ligand is water, H_2O. Water acts as a bi-functional ligand because it acts as an electron donor at oxygen to the Mg of one Chl *a* molecule, and can form one or two hydrogen bonds to the oxygen functions of another, thus cross-linking them. Water-adducts of Chl *a* and Bchl *a* have remarkable altered spectral and photochemical properties, and provide the best model yet of photo-reactive chlorophyll in both green plants and photosynthetic bacteria.

The chlorophyll a*–water adduct*

The introduction of water into a solution of Chl *a* oligomers in an aliphatic hydrocarbon solvent causes the colour to change from blue (λ_{max} = 680 nm) to yellow-green ($\lambda_{max} \approx$ 740 nm). The red-shift is about 80 nm, the largest red-shift known in any Chl *a* species. Removal of water as by pumping in vacuum causes reversal of the spectral change. Accompanying the change in the visible absorption spectrum is an equally drastic change in the infrared spectrum in both the C=O and OH stretch regions. The ir spectra are compatible with a structure in which water is coordinated to the Mg atom of one Chl *a* molecule and hydrogen-bonded to both carbonyl functions of ring V of another. The free keto C=O absorption in (Chl_2) and $(Chl_2)_n$ vanishes in the Chl *a*–H_2O adduct, and the ester C=O absorption maxima are split, indicative of nonequivalence of the ester C=O functions in the propionic acid side chain at position 7c and the carbomethoxy C=O group of ring V at position 10a. Consequently, the basic interaction that cross-links the two Chl *a* molecules has been formulated:



Strouse (1974) has deduced a structure by X-ray diffraction for ethyl chlorophyllide *a*·$2H_2O$ (ethyl chlorophyllide is Chl *a* (**1**) in which the phytyl residue is replaced by ethyl). The Strouse structure differs in some respects from that advanced for the Chl *a*–H_2O adduct from i.r. It should be noted that ethyl chlorophyllide·$2H_2O$ in a crystal is not in the same situation as a Chl *a*–H_2O adduct in an hydrocarbon solvent; the latter can assume configurations of minimum energy not accessible in a crystal. The effect of the phytyl chain on the formation of

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a Chl *a*–H_2O adduct in a hydrocarbon solvent must also be taken into account; for example, ethyl chlorophyllide is essentially insoluble in aliphatic hydrocarbons, whereas Chl *a* is highly soluble, suggesting that solvation effects will be very different for the two compounds. Solvation of the phytyl chain in aliphatic hydrocarbon solvents may make a significant contribution to the energy state of the Chl *a*–H_2O adduct and lead to structures different from those of ethyl chlorophyllide·$2H_2O$ in the crystal. Both structures have much in common, however, and therefore we prefer the structure originally advanced for the Chl *a*–H_2O (P740) adduct pending further experimental evidence.

Given the basic unit structure of the Chl *a*–H_2O adduct (–Chl H_2O Chl–) it is readily seen that the process of cross-linking the (–Chl H_2O Chl–) sandwiches by H_2O can continue until a large micelle with the structure (–Chl H_2O Chl H_2O Chl H_2O Chl–) results. Analysis indicates that the P740 species can have a stoichiometry close to 1 Chl:1 H_2O. The adduct is clearly of colloidal dimensions, as it can be readily sedimented in a few minutes in an ultracentrifuge. X-rays, which yield no diffraction pattern with $(Chl_2)_n$, show a 0.75 nm periodicity in $(Chl \cdot H_2O)_n$, which can be assigned to the Mg–Mg distance between the two cross-linked Chl *a* molecules of the repeating unit.

The $(Chl \cdot H_2O)_n$ adduct we have described here is the Chl *a* species referred to in the chlorophyll literature as 'crystalline' chlorophyll (Jacobs, Vatter & Holt 1954). It is implicated in such physical phenomena as chlorophyll photoconductivity (Bromberg, Tang & Albrecht 1974) and in a number of anomalous or unexplained spectral properties (Tomita 1968; Sherman & Linschitz 1967; Sherman & Fujimori 1968).

Of the Chl *a* species prepared in the laboratory, $(Chl \cdot H_2O)_n$ is the only one to show photoactivity. Irradiation with red light produces a reversible photo-e.s.r. signal in the adduct, but not in $Chl \cdot L_1$, $Chl \cdot L_2$, (Chl_2), or $(Chl_2)_n$. The photo-e.s.r. signal is extraordinarily narrow with a linewidth of the order of 10^{-4} T (1 G) (peak-to-peak). The free radical signals that can be generated in $Chl \cdot L_1$, (Chl_2) or $(Chl_2)_n$ by chemical oxidation with iodine, ferric chloride or potassium ferricyanide have linewidths $\approx 9.5 \times 10^{-4}$ T (9.5 G). The unusually narrow (for an entity composed of such large molecules) linewidth of the photo-e.s.r. signal in $(Chl.H_2O)_n$ suggests that spin delocalization must occur over the entire $(Chl \cdot H_2O)_n$ micelles, and that the process of spin migration is very efficient and proceeds at such a high rate as to average out all of the hyperfine electron-nuclear interactions, causing the collapse of the e.s.r. signal to the very narrow one observed.

Norris, Uphaus, Crespi & Katz (1971) have developed a simple but rigorous treatment that correlates the linewidth with the number of chlorophyll molecules over which the unpaired spin is delocalized. Given a linewidth ΔH_m for the monomer free radical, then the linewidth ΔH_N for a free radical signal delocalized over N chlorophyll molecules is given by

$$\Delta H_N = \Delta H_m / \sqrt{N}.$$

The linewidth, ΔH_m, for monomer chlorophyll free radicals is *ca.* 10^{-3} T (10 G); thus, delocalization of the unpaired spin over a micelle composed of 100 chlorophyll molecules would narrow the linewidth to $\sim 10^{-4}$ (1 G). By the same analysis the hyperfine protein–electron coupling constants follow the relation

$$A_N = A_m / N$$

and the $1/N$ factor is a more stringent criterion than the $1/\sqrt{N}$ factor that applies to the linewidths.

The $(Chl \cdot H_2O)_n$ adduct does not appear to occur *in vivo*, at least to any significant extent, although chlorophyll species absorbing near 740 nm have been reported to be present in green plants, and efforts have even been made to isolate them. Although the 740 nm species does not have physiological significance, its photo-activity in terms of a reversible photo-e.s.r. signal makes it a highly suitable model for photo-reactive chlorophyll in both green plants and photosynthetic bacteria. How the $(Chl \cdot H_2O)_n$ adduct can be used for this purpose is discussed below after the hydration behaviour of bacteriochlorophyll is described.

Bacteriochlorophyll a–*water adducts*

The interactions of Bchl *a* with water are very much more complex than those of Chl *a*, as befits a molecule with a larger number of carbonyl functions that can participate in hydrogen bonding. Consequently, a description of these hydrates must at this time be largely phenomenonological. Evans & Katz (1975) have shown that the coordination number of the Mg atom in Bchl *a* can be deduced from the wavelength maximum in the yellow region of the spectrum: 5-coordinate species absorb near 580 nm, 6-coordinated near 603 nm. Infrared spectra are distinctive for the various Bchl *a*–H_2O species, but the spectra are so complex that it is difficult to draw more than general structural information from them.

To add to the complexities of the situation, the visible absorption spectra are recorded on Bchl–H_2O adducts that are of colloidal dimensions. Hence, the spectra must be corrected for scattering; and if the spectra of the adducts are to be compared to those of photosynthetic bacteria, these must also be corrected. All of the visible absorption spectra reported here, both for laboratory-prepared Bchl *a*–H_2O adducts and for intact photosynthetic bacteria, are corrected for light scattering. We follow the custom here of referring to the various pigment species by their absorption maxima, i.e. P740 for the $(Chl \cdot H_2O)_n$ species absorbing at 740 nm, etc.

Unlike the case for Chl *a*, Bchl *a* forms a whole series of hydrated species. A very dry solution of Bchl oligomer (P775) in methylcyclohexane is the starting point. Introduction of water into this system immediately forms the first hydrate, P850. Further hydration (by continued sonication) forms a second and third hydrate:

$$\text{P775} \rightleftharpoons \text{P850} \rightleftharpoons \text{P825}.$$
$$\updownarrow$$
$$\text{P800}$$

Whether P850 is converted by additional hydration to P825 or to P800 depends on the environment. In a cycloaliphatic hydrocarbon solvent, additional hydration of P850 forms P825, but if the P850 that forms initially is collected by centrifugation and resuspended in water (along with a little of the methylcyclohexane solvent), then P800 is obtained. Whether P825 and P800 are hydrated to the same extent but have structures in which the Bchl molecules are ordered differently, e.g. in P800 in an aqueous environment, the phytyl chains may be on the same side, whereas in P825 in an hydrocarbon solvent, the phytyl chains may extend on both sides of the micelle (cf. Katz *et al.* 1968). It seems quite clear that both P825 and probably P800 are both more extensively hydrated than P850 as both are produced from P850 by additional exposure to water.

Further evidence for an essential identity between P825 and P800 follows from the coordination number of the Mg in these species. P850, the least hydrated Bchl–H_2O adduct contains 6-coordinated Mg. Both P825 and P800 contain 5-coordinated Mg (as does the Chl $a \cdot H_2O)_n$, P740),

but anhydrous P775 also has a Mg coordination number of 5. Visible absorption spectra for the various Bchl–H_2O adducts are shown in figures 7 and 8.

The displacement of the red absorption maximum in the Bchl *a*–H_2O adducts to *shorter* wavelengths with increasing hydration is surprising. In the Chl *a*–H_2O system, the maximally hydrated species is the most red-shifted, but in the Bchl *a*–H_2O system the reverse appears to be true, with the most highly hydrated species absorbing at shorter wavelengths. The e.s.r. properties of the Bchl–H_2O described support this conclusion. It is also worthy of note that at least one of the Bchl–H_2O adducts, P800, requires an aqueous environment for existence; a water–oil interface (provided by residual methylcyclohexane) has the ability to stablize this Bchl–H_2O adduct. P740 formed from Chl *a*, to the contrary, is stable in either an hydrocarbon

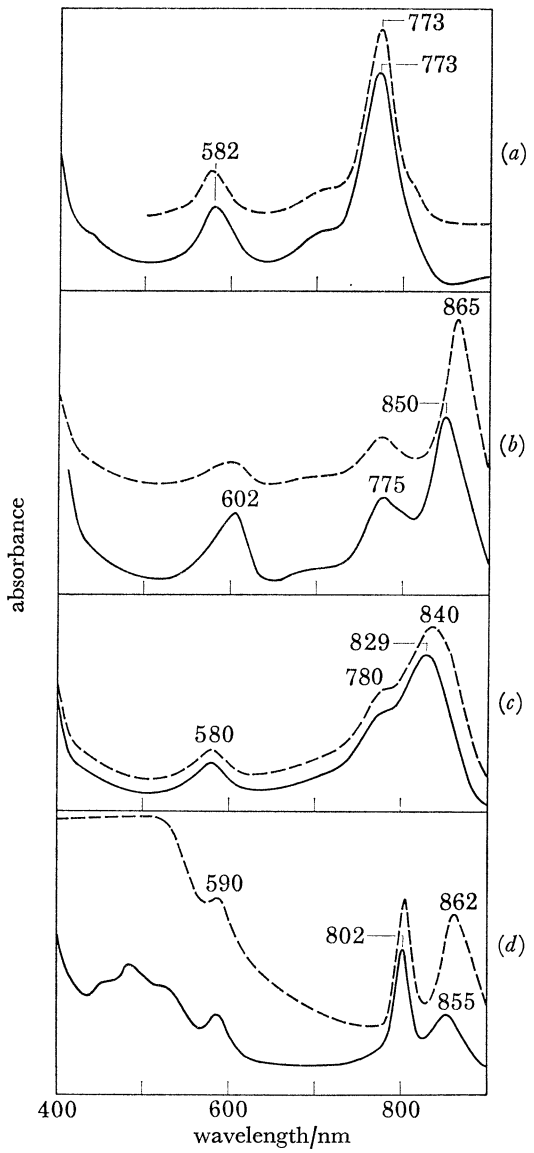

FIGURE 7. A comparison of the visible absorption spectra of various bacteriochlorophyll species corrected (——) and uncorrected (– – –) for light scattering. (*a*) very dry bacteriochlorophyll oligomers in methylcyclohexane; (*b*) P850 hydrate in methylcyclohexane; (*c*) P825 hydrate in methylcyclohexane; (*d*) *R. spheroides*.

or water environment. The P800 species has another anomalous feature. Unlike the P850 and P825 species, which require large corrections for scattering (P850 in an ordinary Cary absorbs at 865 nm), the P800 species shows no light scattering effect on the spectrum and absorbs at the same wavelength in both an ordinary Cary spectrophotometer as in a Cary fitted with an integrating sphere attachment. This would suggest that the anisotropy and size of the P800 species are intrinsically much smaller than those of the other Bchl–H_2O *in vitro* adducts.

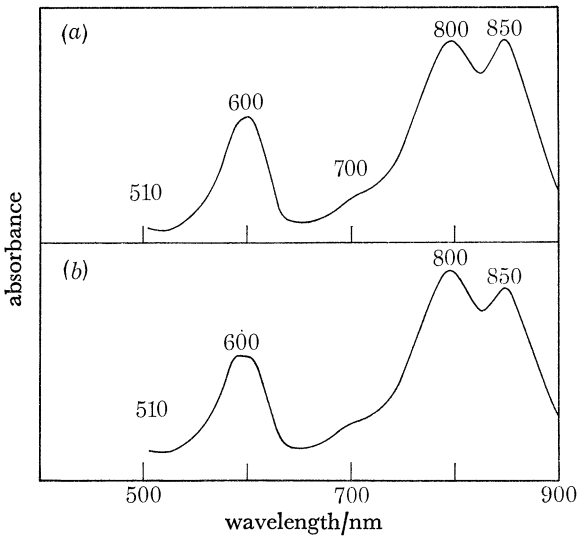

FIGURE 8. Visible absorption spectra of (*a*) an *in vitro* Bchl *a*–water P800 preparation; (*b*) a preparation with an increased proportion of P800. Note the increase in absorption at 580 nm associated with P800.

E.s.r. activity of bacteriochlorophyll a*–water adducts*

As is the case for monomeric Chl *a* species, a broad free radical signal (*ca.* 13 G; 13×10^{-4} T) can be induced in monomeric Bchl *a* only by chemical oxidation. However, both P850 and P825 are weakly photo-active in red light, and yield a narrower, reversible photo-induced e.s.r. signal. In spectra uncorrected for scattering, reversible photo-bleaching is observed at 865 nm. The photo-e.s.r. signal and the bleaching are observed even in the absence of added electron donors or acceptors. We attribute the weakness of the photo-signal to the low concentration of acceptors present in these systems and the reversibility of the electron transfer when the light is turned off. These Bchl *a*–water adducts are extraordinarily sensitive to light. Ambient room light is sufficient to prevent the e.s.r. signal from decaying while the sample is still in the e.s.r. cavity, and to demonstrate photo-reversiblity total darkness is essential. P800 characteristically shows a very narrow e.s.r. signal, quite reminiscent of that of P740, in the dark, i.e. with no additional illumination. We suspect, but have not proved, that this is actually a light-signal induced by low levels of ambient light. P825 appears to have a somewhat narrower signal than P850. Bacteriopheophytin acts as a chain-breaker in these systems because it functions only as acceptor for hydrogen bond formation, and the stacking by water cannot be propagated past this point. The smaller P850 particles produced by added bacteriopheophytin have a narrower absorption peak at 850 nm in the visible spectra, a more intense photo-e.s.r. signal, and are photo-bleached to a greater extent than is ordinary P850. Table 1 summarizes the e.s.r. of the aggregate derived from the narrowing of the linewidth in the aggregate relative to the monomer. From the exceptionally narrow linewidth of P800 we

believe it is a highly ordered structure and probably corresponds more closely to the P740 of Chl *a* than the other Bchl–water adducts.

Although a detailed understanding of the structure and photochemistry of the Bchl *a*–water adducts is still lacking, it is clear that these aggregated species between them exhibit the necessary properties of narrowed photo-e.s.r. linewidth, photo-activity, and the large optical red-shifts required of paradigms for bacterial photosynthesis.

TABLE 1. E.S.R. PROPERTIES OF MONOMERIC BACTERIOCHLOROPHYLL *a* AND BACTERIOCHLOROPHYLL *a*–WATER ADDUCTS

system	ΔH_{PP}† G; 10^{-4} T	e.s.r. agg. number N‡
P770§	12.8 ± 0.2	1
P850	8.6–9.5	1.8–2.2
P825	6–9.5	1.8–4.5
P800‖	~ 1.5	~ 73

† All signals have a g value of 2.0027 ± 0.0002.

‡ $N = \left(\frac{\Delta H_{PP,\ \text{monomer}}}{\Delta H_{PP,\ \text{aggregate}}}\right)^2$.

§ This is monomer Bchl·L_1. It is not photo-active. The free radical signal is produced by oxidation of Bchl in CH_2Cl_2/CH_3OH (50:50) by I_2.

‖ This is a signal observed in the absence of additional light irradiation.

Photo-reaction centre chlorophyll: the special pair

Commoner, Heise & Townsend (1956) were the first to discover that irradiation of intact photosynthetic organisms or chloroplast preparations with red light produces a relatively intense, reversible, e.s.r. signal with a Gaussian lineshape, a linewidth of about 7.5×10^{-4} T (7.5 G), and a g-value very near that of the free electron. Current thinking holds that this e.s.r. signal originates in chlorophyll cation free radicals formed in the primary light conversion act in photosynthesis. Extensive and detailed researches (summarized by Warden & Bolton 1974) associate the free radical signal with the oxidation of the so-called photo-reaction centre chlorophyll, which in green plants is strongly red-shifted to 700 nm (P700). Although the e.s.r. signal is conventionally assigned to $P700^+$ and written Chl^+, it is apparent that the signal cannot originate in a monomeric Chl *a* species. Monomer Chl^+ is readily prepared in the laboratory, and characteristically yields signals with a linewidth $\approx 9 \times 10^{-4}$ T (9 G), which is considerably broader than the *in vivo* $P700^+$ signal. To explain the narrowing of the *in vivo* photo-e.s.r. signal, we resort to the same process invoked to account for the line narrowing in P740. If the *in vivo* linewidth is narrowed by a process of spin delocalization, how many Chl *a* molecules does delocalization involve? The equation $\Delta H_N = \Delta H_m/\sqrt{N}$ can be solved for the value of N, as both the monomer and the *in vivo* linewidths are known, and for green plants, blue-green algae, and photosynthetic bacteria the value of N closely approximates 2. The unpaired spin in $P700^+$ is thus delocalized over 2 Chl *a* molecules and over 2 Bchl *a* molecules in photosynthetic bacteria. Because the $(Chl \cdot H_2O)_n$ adduct P740 possessed the property of spin delocalization, we make the assumption that a molecule of water orients the two chlorophyll molecules in the photo-reaction centre in the same configuration as occurs in P740; in effect, one (–Chl H_2O Chl–) unit of P740 is used as the model for P700. No direct evidence is available that bears on the question of whether the two chlorophyll molecules present in *in vivo* P700 actually are positioned by water, but for a variety of reasons we consider this a reasonable hypothesis even though other

orienting mechanisms (such as a protein matrix or some other bifunctional ligand) cannot be excluded at this time.

The formulation of photo-reactive chlorophyll as (Chl H_2O Chl) accounts very well for the observed e.s.r. line-shape in all photosynthetic organisms or preparations in which a comparison can be made. We refer to the two chlorophyll molecules that share the unpaired spin as a 'special pair' rather than as a dimer. The term dimer should be used only for (Chl_2), a well-defined chlorophyll *a* species that is not photo-active. As there is good reason to believe that green plant antenna chlorophyll is comprised of (Chl_2) dimer units, the use of the term dimer for the two special chlorophylls in the reaction centre only serves to perpetuate the confusion so prevalent in the literature.

Because of the importance of the special pair concept we have sought for additional experimental verification. As pointed out above, the hyperfine (h.f.) coupling constants of *in vivo* and *in vitro* free radicals should be reduced by a factor of 1/2 in a special pair relative to the h.f. splittings in the monomer. This is a more rigorous experimental test than the linewidth relation, which relates the special pair to the monomer by the factor of $1/\sqrt{2}$. However, chlorophyll free radical e.s.r. signals, especially *in vivo*, do not show sufficient structure to permit extraction of h.f. coupling constants. Recourse must therefore be had to indirect techniques such as electron-nuclear double resonance (endor) spectroscopy, a high-resolution extension of e.s.r. first introduced by Feher (1956), which in conjunction with selectively deuterated photosynthetic organisms and chlorophylls allows the assignment of the endor spectra and thus establishes the values of the needed h.f. coupling constants (Norris *et al.* 1974). Hyperfine couplings extracted by this procedure for monomeric Chl *a* and Bchl *a* are compared to the *in vivo* h.f. coupling constants in the photosynthetic bacterium *Rhodospirillum rubrum* and the blue-green alga *Synechococcus lividus* in table 2. These results provide strong support for the special pair concept. Corresponding h.f. coupling constants in the monomer chlorophyll free radicals are very nearly twice those in the *in vivo* reaction centre, i.e. the 1/2 relation expected for the special pair holds.

TABLE 2. COMPARISON OF *IN VITRO* AND *IN VIVO* ENDOR DATA

	hyperfine coupling constants/MHz		aggregation number†	
protons‡	Bchl†	Chl *a*†	*R. rubrum*§	*S. lividus*‖, ¶
(α, β, δ, 10)	1.4††	0.67‡‡	1.7	—
1a	5.32	—	2.4	—
(1a, 3a, 4a)	—	3.19§§	—	1.9
	—	3.72§§	—	2.2
5a	9.8	7.45	2.1	2.0
7, 8	14.0‖‖	11.8	2.0	2.2
		av.	2.0	2.1

† Defined as the ratio of h.f. coupling constants *in vitro*/h.f. coupling constants *in vivo*.
‡ Proton numbering from structure 1. Parentheses indicate the h.f. coupling constant arise from all of the indicated protons.
§ Endor recorded at 15 K.
‖ Endor reported at 100 K.
¶ Endor from *C. vulgaris* and *S. lividus* are indistinguishable.
†† Includes contributions from 7a, 8a, 3a and 4a protons.
‡‡ Includes contributions from 7a and 8a protons.
§§ These peaks are resolved but are not assigned.
‖‖ Includes contributions from 3 and 4 protons.

All of the evidence we know of now available that bears on the question supports the view that the primary act in light conversion in photosynthesis takes place in a special pair of chlorophyll molecules, probably related to each as are the chlorophyll molecules in the P740 Chl *a*–H_2O adduct.

Models for green plants and bacterial photosynthesis

Our suggested structures for antenna and photo-reactive chlorophylls can now be fitted together to provide models for the light conversion step in photosynthesis. Although photo-reaction chlorophyll appears to have the same basic structure in both green plants and photo-synthetic bacteria, the antenna chlorophylls in these organisms are different either in structure, or environment, or both. It is therefore convenient to discuss models for these two classes of photosynthetic organisms separately.

Model for light-conversion in green plants

The chlorophyll oligomer that we take to represent antenna chlorophyll can easily be linked to the photo-reactive [Chl H_2O Chl] sandwich by a keto C=O···Mg to the Chl *a* in the special pair that is the acceptor for hydrogen bonding, or by a keto C=O···Mg interaction from the other Chl *a* in the special pair to the Mg atom of a terminal Chl *a* molecule of the antenna oligomer $(Chl_2)_n$. Associated with the special pair will be a conduit or electron transport chain to conduct the electron created in the light conversion step away to be used for chemical reduction. There must also be an electron transport chain to transfer an electron from water to restore the positive hole in the special pair to its original state.

We can summarize the operation of this model by the following scheme:

$$\begin{aligned}
&\rightarrow (Chl_2)_n\,[\overset{x^-}{Chl}\ HOH\ Chl]\ Y \xrightarrow{h\nu} && 1\\
&\quad (Chl_2)_n^*\,[\overset{x^-}{Chl}\ HOH\ Chl]\ Y \longrightarrow && 2\\
&\quad (Chl_2)_n\,[\overset{x^-}{Chl}\ HOH\ Chl]^*\ Y \longrightarrow && 3\\
&\quad (Chl_2)_n\,[\overset{x^-}{Chl}\ {}^+(OH)^-\ (H)^+\ Chl^-]\ Y \longrightarrow && 4\\
&\quad (Chl_2)_n\,[\overset{x^-}{Chl}\ HOH\ Chl]^+\ Y^- \longrightarrow && 5\\
&\quad (Chl_2)_n\,[\overset{x}{Chl}\ HOH\ Chl]\ Y
\end{aligned}$$

Step 1 describes the absorption of light by the antenna chlorophyll. The excitation energy is transferred in step 2 to the photo-reaction centre special pair, where charge separation occurs as indicated in step 3. There are several ways in which charge separation can occur, but at this time we prefer a mechanism in which one Chl *a* molecule acts as donor to the other Chl *a* molecule of the special pair. The two Chl *a* molecules are not equivalent; one of them is co-ordinated to a water molecule, whereas the other is hydrogen bonded, and although both Chl *a* molecules can be attached to the antenna, the mode of attachment cannot be the same, so that the two Chl *a* molecules have a different relationship to the antenna. Chl *a* is recognized as a compound that can be both easily oxidized or reduced. The instrinsic asymmetry of the special pair can be expected to make one of the Chl *a* molecules a slightly stronger electron donor, and the other a slightly stronger electron acceptor, thus effecting charge separation by internal oxidation-reduction. In essence, the excitation results in an internal redox reaction in the special

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pair, probably from the singlet state. By transfer of a proton from the coordinated water molecule, the excited special pair is an electrically neutral species, thus avoiding the energetic requirements for charge separation. At this stage, the special pair could be described as a triplet state with a charge transfer contribution. An electron is then transferred in step 4 to an electron acceptor, Y, forming Y^-. Electron transfer from the donor X^- returns the special pair radical cation to its initial state in step 5. What we have described in this model is an electron pump energized by light.

This model accounts for the optical properties of both the antenna and photo-reaction chlorophyll, and for the e.s.r. signal observed in photosynthesis. As the special pair [Chl H_2O Chl] is red-shifted relative to the antenna chlorophyll, it becomes a sink for the excitation energy trapped by the antenna. The special pair radical cation [Chl H_2O Chl]$^+$ is unable to oxidize antenna chlorophyll, for we have shown that P740$^+$ cannot oxidize $(Chl_2)_n$. The arrangement we postulate effectively localizes the oxidized special pair until it can be reduced by the normal electron donor, hence the mechanism that prevents chemical or photo-chemical damage to chlorophyll is highly efficient. Two Chl *a* molecules in the photo-reaction centre makes it possible to conduct electrons in and out of the special pair simultaneously.

The entity [Chl$^+$ $(OH)^-$ $(H)^+$ Chl$^-$] is expected to have the e.s.r. spectrum of a triplet. A triplet signal has recently been observed by Dutton, Leigh & Seibert (1972) in photosynthetic bacteria. Triplet states we have been able to observe by e.s.r. in green plants as well as bacteria are polarized, i.e. have anomalous intensities indicating both emissions and absorptions that are a consequence of a non-equilibrium (non-Boltzmann) population of the three energy levels of the triplet. The polarization exhibited by the *in vivo* triplet spectra of photosynthetic bacteria are consistent with the special pair excited as we have indicated. The *in vivo* triplet spectra also suggest a charge-transfer contribution to the rather unusual triplet state formed in the two Bchl *a* molecules that constitute the special pair; this may also apply to Chl *a* special pairs. The triplet studies are in an early stage, but they offer a real possibility of deducing the geometry of the special pair and its relation to the electron transport chain.

The case of photosynthetic bacteria

Constructing a model for bacterial photosynthesis is both simpler and more complex than for green plants (yellow-green algae, blue-green algae). For Chl *a* organisms, the identification of antenna chlorophyll with $(Chl_2)_n$ oligomers is persuasive. However, we have only been successful in constructing P700 special pairs in the laboratory in dilute form as in a film or solid matrix, where the prospects of concentrating them are not promising. In the case of photosynthetic bacteria, we have been able to produce laboratory systems that mimic the optical and e.s.r. properties of intact bacteria remarkably closely, but we know only the general features of the structure of the entities responsible.

Figure 7*d* shows the visible absorption spectrum of the photosynthetic bacterium *Rhodopseudomonas spheroides* as recorded in an ordinary spectrophotometer and the spectrum corrected for light scattering. Figure 9 illustrates the dependence of the (scattering-corrected) visible spectrum of *R. spheroides* on the age of the culture; it is evident that *in vivo* P850 and P800 are two different species, as they evidently can occur in quite different concentrations. Figure 10 compares the spectra of *R. spheroides* and an *in vitro* preparation of P800 and P850. The resemblance is striking. Certain differences, however, must be pointed out. The linewidths of the *in vitro* preparation are distinctly broader than those of intact *R. spheroides*. From the 587 nm

maximum in the bacteria, we judge the coordination number of the Mg to be 5, whereas in the laboratory preparation it is evident from the yellow absorption maximum that a considerable amount of Mg with coordination number 6 is present. The P848 (bacteria) and P850 linewidths are both broad enough to include a sizeable contribution from a true P865 species.

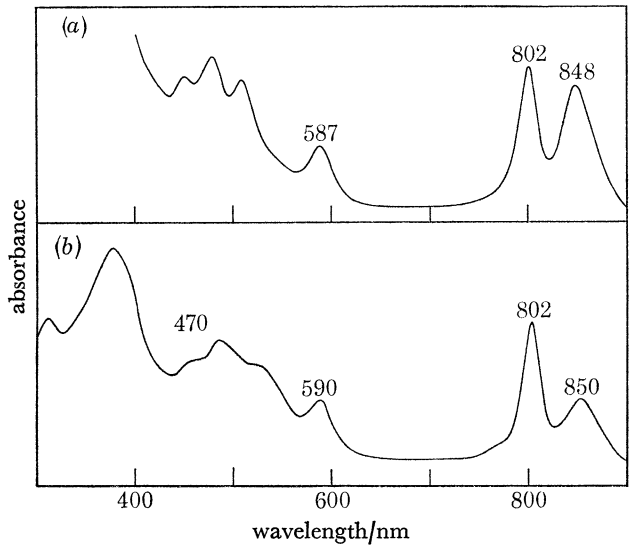

FIGURE 9. *In vivo* visible absorption spectra of *R. spheroides* showing P800 and P850. (*a*) A fresh culture; (*b*) spectrum of the same culture many weeks later. These results show that *in vivo* P800 and P850 are two distinct species.

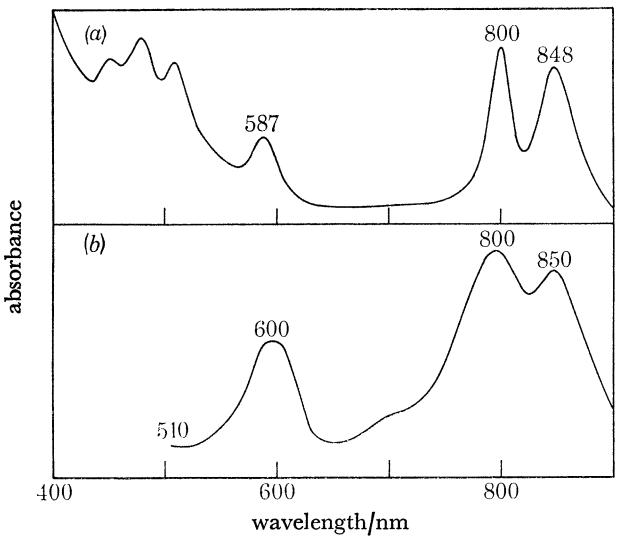

FIGURE 10. A comparison of an *in vivo* absorption spectrum of a photosynthetic bacterium and an *in vitro* bacteriochlorophyll–water adduct. (*a*) *In vivo R. spheroides*; and (*b*) an *in vitro* P800 and P850 preparation.

The *in vitro* preparation of figure 10*b* shows weak intrinsic e.s.r. activity and a narrowed e.s.r. linewidth, consistent with the presence of bacteriochlorophyll special pairs in the preparation. We judge that our *in vitro* P850–P800 operation contains a certain amount of P865, but as yet we have not been successful in the preparation of a pure P865 species, reminiscent of the situation with P700 in green plants. It appears possible that *in vivo* P865 represents (–Bchl H_2O Bchl–)

remote from other such pairs, or (–Bchl H_2O Bchl–) at a lipid–water interface or some other special environment where the hydrophilic/hydrophobic balance is different from the environment occupied by the bulk of the chlorophyll in the photosynthetic membrane. Although we cannot exclude the possibility that *in vivo* P865 is a less hydrated species than P850, our experiments with *in vitro* P800 seem also to indicate that differences in spectra may result not only from extent of hydration but also from environmental effects on the configuration. P800 *in vitro* can be formed only in the presence of an organic–water interface. In our preparations, hydration in methylcyclohexane results only in the formation of P850 (initially) and then P825. Only if P850 prepared in methylcyclohexane is collected and resuspended in water, along with a little methylcyclohexane, is P800 observed to form; in the absence of at least a small amount of the organic solvent, no P800 is obtained. We can, therefore, suppose that P825 and P800 are similar in composition but contain Bchl in different orientation, and that both of these *in vitro* species are more extensively hydrated than P850. Our *in vitro* P850 preparation may then contain a small amount of even less extensively hydrated Bchl, in the sense that the water molecules inserted into the Bchl oligomer are more distant from each other than in the bulk of the *in vitro* P850. In any event, it appears likely that all of the Bchl *a* in *R. spheroides* is hydrated, and that a large fraction of it occurs at an oil–water interface. This is a quite different picture from that of green plant chlorophyll, where the evidence requires at least the antenna chlorophyll to occur in a rigidly hydrophobic and water-free environment, or at least, if water is in fact present, to be in an environment in which the water activity is greatly reduced. The experiments described here suggest fundamental differences between green plant and bacterial antenna chlorophyll, but a basic identity in the special pair used for light conversion.

A principal uncertainty at the present is the structure of *in vitro* P800. It is possible that P825 and P800 are hydrated to about the same extent, or that P800 is the most extensively hydrated Bchl that can be prepared, making it the Bchl equivalent of P740. A more remote possibility is that P800 is a largely anhydrous species, this latter because of its comparatively small red-shift. We have been severely limited in the application of i.r. spectroscopy because of the large amounts of water present in P800 and the relatively low concentrations of Bchl *a* in our P800 preparations. Fourier transform i.r. spectroscopy may greatly improve the acquisition of i.r. data, and resonance enhanced Raman spectroscopy and circular dichroism measurements are likely to provide information from which structural conclusions about *in vitro* P800 can be drawn.

All of the species responsible for the major absorption peaks in the visible spectrum of *R. spheroides* are hydrated. Consequently, a clear distinction between antenna and photo-active chlorophyll in photosynthesis on a structural basis is not as satisfying as in the case of green plants. The existence of special pairs, presumably [Bchl *a*–H_2O–Bchl *a*] in bacteria is strongly supported by e.s.r. and endor data. Just what differentiates the photo-active special pairs from the other hydrated species is puzzling. It may be that the distinguishing feature of a special pair is the isolation of the water molecule, i.e. it occurs in a sequence –Bchl Bchl H_2O Bchl Bchl–. Additional hydration to form –Bchl H_2O Bchl H_2O Bchl Bchl– may be the P850 species, and full hydration forms P800 which is the analog of P740. The reason why the charge separation occurs in the least hydrated form rather than in P800, for intact photosynthetic bacteria show a *ca.* 9×10^{-4} T (9 G) e.s.r. signal, must relate to the light absorption properties of the Bchl *a*-hydrates. The least hydrated P865 is the energy trap, and additional hydration causes the formation of species absorbing at *shorter* wavelengths that are unable to trap the excitation. Or,

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as discussed below, no suitable electron acceptors are available in the vicinity of P800 because of its location in the photosynthetic membrane.

The availability of electron acceptors may be crucial to the decision of where a special pair occurs. No matter the number of special pairs, no special pair cation free radical can be formed if an electron acceptor is not present in close proximity. The difference between antenna and special pair chlorophyll in photosynthetic bacteria may be as much a function of the presence or absence of a suitable electron acceptor as a question of Bchl *a* geometry.

We believe the simple *in vitro* Bchl–water systems we describe here come astonishingly close to reproducing the important optical and e.s.r. properties of *R. spheroides*. These systems contain no protein, and thus whatever the protein functions in the photosynthetic apparatus of bacteria, and these must of course be many and important, organization of chlorophyll appears not to be one of them. Protein–Bchl interactions need not be invoked to account for the spectral properties of *in vivo* Bchl *a*. Lipids, however, may be heavily involved in the genesis of the red-shifts, either by providing an hydrophobic medium (necessary for the survival of some Bchl–H_2O species) or a water–oil interface that imposes specific orientations on the Bchl *a* molecules in the Bchl *a*–water adducts.

Biosynthesis considerations

We have described models for plant and bacterial photosynthesis that specify the state of organization of antenna and photo-reactive chlorophyll in quite specific terms. Ignoring for the moment some of the uncertainties in the details of the structure of some of the important Bchl *a* species in bacteria, it is clear that monomeric chlorophyll must play a very restricted role if any in photosynthesis. Antenna and photo-reactive chlorophyll (in all photosynthetic organisms) are large organized entities that function cooperatively. How are such entities assembled from their component units, and what sort of milieu must be provided to ensure their stability? To view chlorophyll as merely another component of a lipid bilayer membrane would be a great over-simplification. Chlorophyll 'antenna' is not achieved by the intercession of covalent bonds, and is thus basically different from the formation of proteins from amino acids for example. The chlorophyll interactions described here seem without precedent or analogy in other biological systems. The biogenesis of the organized chlorophyll species involved in photosynthesis thus raises a category of new questions. To attempt a detailed answer to these in the present state of knowledge would not be fruitful. However, it does appear appropriate to consider how the organized chlorophyll structures we have described here can be fitted into current views on the nature of the photosynthetic membrane.

Current views on the photosynthetic apparatus

The light dependent reactions of photosynthesis are considered to occur in a specialized membrane system, the thylakoid. In electron micrographs the thylakoid systems appear to consist of large stacks of thylakoids (granum, grana lamellae), interconnected by single thylakoids (stroma lamellae). In comparison to contemporary understanding of the biochemistry of photosynthetic electron transport, in which knowledge is extensive but still far from complete, much less information is available about the fine structures of the lamellar system. The techniques of low-angle X-ray diffraction and freeze- and deep-etching procedures in electron microscopy provide the larger portion of evidence for the actual fine structure of the photosynthetic membrane.

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From his X-ray data, Kreutz (1972) proposed a model for the photosynthetic membrane of higher plants, which consists of a bilayer system (8 nm thick), built up by structural protein, porphyrin rings, and lipids, with the phytol chains of the chlorophylls anchored in the protein layer.

The other technique, freeze- and deep-etching, including recovery techniques (Park & Sane 1971; Kirk 1971), does not only allow a view of the outside and inside of the thylakoid membrane, but reveals the interior of the bilayer membrane by fracturing along the hydrophobic region within the membrane. Whereas the outer faces of the membrane do not exhibit any particular fine structure, the two inner complementary fracture faces reveal a high population of distinct protein entities. At least two different protein species are observed, small particles about 11 nm and large particles about 17.5 nm in diameter. The latter occur exclusively in the partition region of the thylakoid, i.e. where thylakoids are stacked together. According to more recent evidence, they may even protrude through two adjacent bilayers of the thylakoid system (Goodenough & Staehelin 1971; Pendland & Aldrich 1973).

It has been pointed out that the small particles probably are associated with photosystem I activity and the large ones with photosystem II activity. As only the partition region possesses the large particles, photosystem II activity may be restricted to the partition region. This interpretation, however, has been questioned by Goodenough & Staehelin (1971) based on the results that a *Chlamydomonas* mutant lacking grana regions is nevertheless capable of performing the entire photosynthetic electron transport process.

The view of the thylakoid membrane as a lipid bilayer with protein particles of different size embedded into it but reaching to the surface (and accessible to soluble electron transport carriers and antibodies as well) appears to be inconsistent with the earlier model of Kreutz. In an attempt to reconcilate the electron microscopy and X-ray data, Kirk (1971, 1972) has pointed out that Kreutz' electron density data may fit into this later model, by assuming an asymmetric distribution of the proteins in the lipid bilayer.

Whereas the Kreutz model accounts for the location of chlorophyll in the photosynthetic membrane but is inconsistent with other evidence, the model derived from the electron microscopy leaves the important question of the localization of the chlorophyll completely open.

Weier & Benson (1967) have associated chlorophyll mainly with the partition region of the thylakoid membrane, which they consider to be highly hydrophobic. This opinion, however, is difficult to reconcile with the fact that changes in the aggregation of the thylakoids (unstacking and stacking) can be easily induced by changes of the ionic strength of the suspension medium.

The present view of the localization of the chlorophyll in the membrane system favours incorporation or at least close association of the chlorophylls with the proteins (Kirk 1971). This conclusion is mostly derived from the observation that upon detergent treatment of chloroplasts and subsequent separation of the smaller species, a large amount of the chlorophyll is found in the protein fractions. However, it cannot be excluded that detergent treatment brings together initially separated protein and chlorophyll in one micelle, thus facilitating the binding of one to another (Kirk 1971).

Thus, we conclude that the location of chlorophyll in the membrane system, the nature of its interactions with protein and/or lipid and its relationship to the various electron transport carriers remains relatively open.

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Biosynthesis of Chl a *special pairs*

It will be useful to review the limited amount of information that exists in the origin of *in vivo* Chl *a* special pairs. The e.s.r. signal from $P700^{+}$ varies greatly in linewidth as a function of isotopic composition. Algae in which ^{12}C is replaced by ^{13}C have the broadest signal (*ca.* 13 G; 13×10^{-4} T), whereas organisms in which ^{1}H has been replaced by ^{2}H (deuterium) have the narrowest signal (*ca.* 2.9 G; 2.9×10^{-4} T). An organism composed of ^{13}C and ^{2}H compounds has a $P700^{+}$ linewidth of about 12×10^{-4} T (12 G). We have found it possible to follow the biosynthesis of P700 by observing the time-dependence of the e.s.r. signal of $P700^{+}$ when algae growing in $^{2}H_2O$ are switched from $^{12}CO_2$ to $^{13}CO_2$ (95 % ^{13}C). In such an experiment, a gradual increase in the linewidth is not observed. Rather, a broad signal (about 12×10^{-4} T) superimposed on the existing narrow e.s.r. signal, appears very early. It would appear that the pool of ^{12}C precursors for P700 Chl *a* production must be very small, or that it never mixes with the pool freshly formed from $^{13}CO_2$, otherwise the change in linewidth would be gradual rather than abrupt.

Antenna Chl *a* is in great abundance relative to P700 special pairs. If special pairs were formed from one molecule of ^{13}C-Chl *a* and one of ^{12}C-Chl *a*, the linewidth would be a slowly changing average intermediate value as a function of the extent of growth on $^{13}CO_2$. Thus, the evidence available at this time suggests that special pairs are not produced from pre-existing antenna chlorophyll. It is important to note that ^{14}C methods cannot be used in a similar way, as ^{14}C does not distinguish antenna from reaction centre chlorophyll. Stable isotope effects on e.s.r. lineshapes *in vitro* and *in vivo*, however, would appear to have distinct utility in the study of the biosynthesis of reaction centre and antenna chlorophylls.

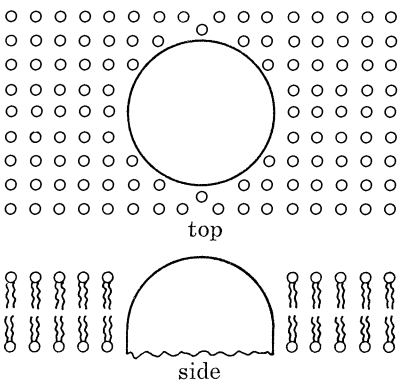

FIGURE 11. A schematic representation of the photosynthetic membrane as a lipid bilayer. A top and a side view are shown. The circular object represents a protein immersed in a digalactosyl lipid bilayer.

Chlorophyll and the thylakoid

Our reading of the literature indicates that none of the models proposed for the thylakoid are very specific about the site of the chlorophyll. The lipid bilayer is at least 6 nm thick. Embedded in the bilayer are at least two kinds of globular proteins, the smaller about 11 nm, the larger 17 nm in diameter (figure 11). The lipid, which we will assume to be predominantly digalactosyl dilinolenin (digalactolipid), is about 3.9 nm in length. A Chl *a* molecule is about 1.3 nm across the porphyrin ring and about 3.5 nm in overall length. Three ways of incorporating the Chl *a* antenna into the bilayer appear possible. First, the antenna Chl *a* molecules can be immersed in

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one layer of the lipid bilayer. From the relative dimensions of the Chl *a* molecule and the lipid it is possible to immerse completely a Chl *a* molecule in one-half of the digalactolipid bilayer. The simplest assumption about the arrangement of the Chl *a* molecules in the antenna relative to each other is that they are orthogonal, with the donor Chl *a* molecule keto C=O···Mg bond at right angles to the macrocycle plane of the acceptor Chl *a*. As the phytyl chain in Chl *a* is quite flexible, it is not much of a problem as judged from models to insert the phytyl chains of the chlorophyll so that they are parallel to the fatty acid residues in the digalactolipids. The digalactolipids are perpendicular to the plane of the lamellae, but the arrangement of the planes of the Chl *a* molecules in the antenna is not well established to the surface of the lamellae (figure 12*a*). It is obvious that adjoining Chl *a* molecules in the antenna are not parallel to each other, nor can they be arranged in such a way if the keto C=O···Mg interactions are to be maintained. The antenna Chl *a* orientation we postulate here is consistent with the orientation for antenna Chl *a* deduced from magnetic studies (Becker, Geacintov, Van Nostrand & Van Metter 1973), which show the Chl *a* to be perpendicular to the surface of the lamellae.

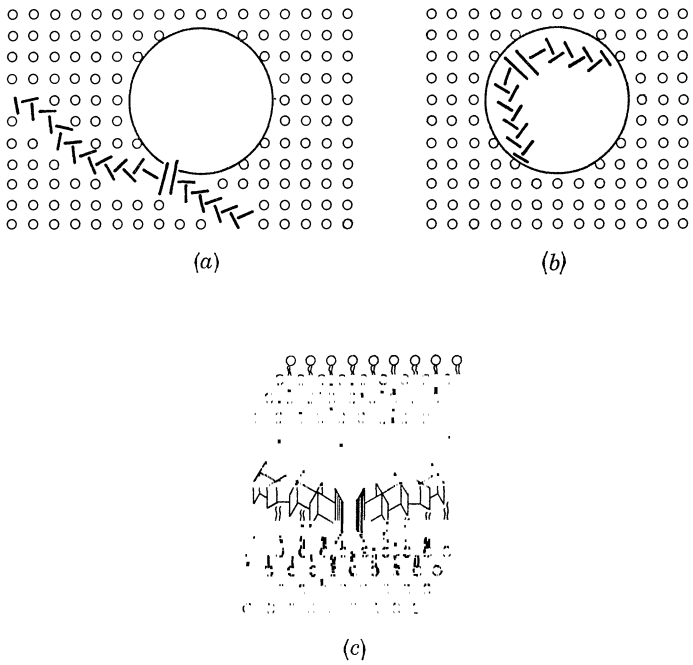

FIGURE 12. Schematic representations of alternative ways of incorporating antenna and special pair photo-reaction centre chlorophylls in the thylakoid membrane. The phytyl chains are not shown. The special pairs are indicated by two parallel, unconnected bars or squares. (*a*) Insertion into one layer of the lipid bilayer; (*b*) insertion within the interior of a protein component of the bilayer, presumably in an hydrophobic region of the protein; the phytyl chains may be inserted into the lipid; and (*c*) the antenna and the special pair are placed in the annular region of the lipid bilayer. In all representations, the special pair is placed very near the protein for access to the electron transport chains.

A second alternative placement of the antenna is inside the protein units (figure 12*b*). Antenna with at least as many as 20 Chl *a* molecules can be inserted inside the proteins, presumably in an hydrophobic region (figure 12*b*). Placing the Chl *a* special pair in the protein also assures access to the electron transport chains.

The third and perhaps the best place to insert the antenna Chl *a* is in the annular region between the lipid bilayers (figure 12*c*). It is generally accepted that this region is free of water,

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and if the ends of the lipid chains are some 1.5 nm apart, then insertion of the Chl *a* macrocycles is feasible. As the protein units protrude through this region, the special pair may be conceived to consist of Chl *a* molecules of the antenna in close proximity to the protein, where the water molecules associated with the protein can be used for special pair formation. If only the Chl *a* in the antenna immediately adjacent to or embedded in the protein is convertible to special pairs, then the troublesome problem of how the admission of very limited amounts of water to this region of the thylakoid is regulated is largely avoided. The Chl *a* phytyl chains find a natural location immersed in a parallel fashion in the hydrocarbon moieties of the digalactosyl lipid. This arrangement resembles very closely the milieu of Chl *a* oligomers in an hydrocarbon solvent such as hexadecane. In the annular arrangement the macrocycle planes can be made perpendicular to the surface of the thylakoid lamellae.

Placing the antenna in the annular space between the lipid bilayer avoids some serious problems that arise when the antenna is placed in the upper layer of the bilayer (figure 12*a*). Some years ago, Lippincott *et al.* (1962, 1964) observed that treatment of an aqueous suspension of tobacco chloroplast fragments with methanol or other water-miscible solvents causes a change in the visible absorption spectrum from 670 to 740 nm, and a concurrent change in the photo-e.s.r. signal from about 7 to 1×10^{-4} T (7 to 1 G). This is an exact description of the consequences of the introduction of water into a Chl *a* oligomer solution in an aliphatic hydrocarbon solvent. Indeed, the Lippincott *et al.* experiment is one of the best arguments for the existence of Chl *a* oligomers in the plant. Now, if the antenna is located in the top portion of the lipid layer (figure 12*a*) very near the aqueous region of the chloroplast, then it is difficult to see why extensive hydration of the antenna does not occur. Antenna in the annular region of the bilayer, however, is in the aliphatic hydrocarbon environment essential for maintenance of the integrity of this Chl *a* species, and well shielded from hydration. In the arrangement proposed here, the Chl *a* special pairs are considered to be generated very near or even in the protein that extends through the annular region of the bilayer. Thus, we can find a reasonable location for both antenna and special pair Chl *a* in the currently accepted photosynthetic membrane structure.

The case for photosynthetic bacteria is much more complex than for green plants, and not much can be said at this time about the distribution of Bchl *a* in the bacterial photosynthetic membrane. A large fraction of the Bchl *a* in a bacteria appears to be located at a water–lipid interface, unlike the case for green plants. Either the location of the Bchl *a* antenna and special pairs are basically different in bacteria, or the bacterial photosynthetic membrane has a significantly different structure, or perhaps both. More data on both the photosynthetic membrane in bacteria and on the structure of the Bchl *a*–water adducts will be required to delineate the fine structure of the bacterial photosynthetic apparatus.

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Discussion

S. I. Beale (*The Rockefeller University, New York, N.Y.* 10021, *U.S.A.*). The linear oligomer model appears to imply that each trap can have only one or two antenna chains associated with it, and each antenna only one or two traps. Could you please comment on this?

J. J. Katz. The model described here allows one or two antenna chains to be structurally connected to a special pair of traps.

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