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ELECTRONIC ORIGINS OF MONOMERIC AND DIMERIC
CHLOROPHYLL a AS DETERMINED FROM
WAVELENGTH SELECTIVE FLUORESCENCE
AND RAMAN EXCITATION PROFILES

by

Nancy Elaine Binnie, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in the partial fulfilment of
the requirements for the degree of
Master of Science
Department of Chemistry

Carleton University
Ottawa, Ontario
Canada

22 December, 1988

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Nancy Elaine Binnie, B.Sc.
in partial fulfilment of the requirements
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ABSTRACT

The energy level diagrams of monomeric and a dimeric species of Chlorophyll a (Chl a) in hexane solution derived from optical absorbance, wavelength-selective fluorescence and Raman spectroscopic measurements are reported here. It was found that the excitation profile for the resonance enhanced Raman intensities of normal modes of the dimer is of great importance in finding the location of the (0,0) transitions between the ground state and the electronic origins in the Soret region. Present information points to the identification of an approximately T-shaped structure for this dimer where bonding occurs from a carbonyl group on the macrocycle periphery of one chlorophyll molecule (donor) to the magnesium atom centred in the macrocycle of another Chl a molecule (acceptor). In the Soret region, the electronic origins of this dimer are at 448.0 nm and 435.8 nm for the donor and acceptor molecules respectively.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
$(\text{Chl } \underline{a} \cdot \text{H}_2\text{O})_n$	Chl <u>a</u> ligated with n molecules
BChl <u>a</u>	bacteriochlorophyll <u>a</u>
BChl <u>a</u> .L ₁	monoligated BChl <u>a</u> , ligand on the magnesium
BChl <u>a</u> .L ₂	biligated BChl <u>a</u> , ligand on the magnesium
B _x , B _y	absorption transitions in the blue region of the chlorophyll spectra (Soret region)
Chl <u>a</u>	chlorophyll <u>a</u>
C--C, C--N	vibrational or stretching modes of the bond
CD	circular dichroism
CW	continuous wave
δ	delta
DPE	deoxophylloerythrinmethylester
EtChl <u>a</u>	ethylchlorophyllide <u>a</u>
ESR	electron spin resonance
FEMO	free electron molecular orbital
FWHH	full width at half height
IR	infra-red
ISC	intersystem crossing
LHC	light harvesting complex
L _n	n = number of ligands, L
MeChl <u>a</u>	methylchlorophyllide <u>a</u>
MePPb	methylpheophorbide
Mg	magnesium
MgOEP	magnesium octaethylporphyrin
MgPC	magnesium phthalocyanin
NiDPE	nickel deoxophylloerythrinmethylester
NMR	nuclear magnetic resonance
OEP	octaethylporphyrin
Pheo	pheophytin
Pheo <u>a</u>	pheophytin <u>a</u>
π	pi
PMT	photomultiplier tube
Q _x , Q _y	absorption transitions in the red region of the chlorophyll spectra
r _p -HPLC	reverse phase high pressure liquid chromatography
σ	sigma
S _n	nth excited singlet state
TLC	thin layer chromatography
TPrP	tetraprophylporphyrin
TPP	tetraphenylporphyrin
VODPER	vanadyl deoxophylloerythroetiporphyrin
Xe	xenon

CHAPTER I

A. Introduction

1A.1 Chlorophyll a and the Biological System

The interaction of plant pigment molecules with their environment is fundamentally important in determining specific properties necessary for light capture (absorption), energy transfer and electron transfer since these characteristic properties are moderated by the chemical environment. In the plant, aggregation with proteins or other molecules provides the necessary structure for the antenna and reaction centre chlorophylls¹. Studies of *in vivo* light harvesting complexes have been made^{2,3} as well as light harvesting complexes isolated from chloroplasts^{3,4}. From studies of the electronic properties^{2,5,6}, it has been proposed that the bulk of the chlorophyll pigments in the LHC are present as monomeric units bound in the protein matrix via the magnesium and through hydrogen bonding to the ring V keto carbonyl group to amino acids^{4,7,8}. In these chlorophyll units, the magnesium appears to be primarily a penta-coordinated species^{4,7,8}. However, bacteriochlorophyll a (BChl a) appears to be a penta-coordinated species only in the alga R. Spheroides and a mixture of penta- and hexa-coordinated in other purple bacteria⁸.

In the photosynthetic unit, the chlorophyll molecules act cooperatively as primary and accessory pigments harvesting light and undergoing excitation into the singlet electronic state⁹. The

excitation energy is transferred to a few 'special pair' chlorophylls which compose the reaction centre or 'trap'. Here the electronic excitation energy is used for chemical oxidation and reduction.

Studies of the structure and function of photosynthetic organisms have followed a number of different directions. Investigations have been made of intact in situ systems, whole cells and partially intact organelles. By extensive degradation of the original plant material, the individual components may be isolated and studied. Models of simple in vivo systems can be assembled by combining isolated pigments either in solution, solid, film or aggregate (i.e. colloidal assembly). Unfortunately, this wide area of research has not always resulted in unified theories. Any introduction in the field of Chlorophyll research can be based on hundreds of references. A complete literature survey is not presented here, however literature relevant to the experimental work in this thesis is discussed in the following sections.

1A.2 Spectroscopic Techniques and Species Differentiation

Ideally, the simplest environmental system is a dilute solution of one pigment type in an 'inert' solvent where the isolated molecules are unperturbed. This ideal situation is very difficult to achieve¹⁰. The main difficulty lies in the chemical properties of the chlorophyll, in particular the coordination unsaturation of the central magnesium in the planar porphyrin structure. Consequently, the metal is always ligated to some nucleophile and will always be penta- or hexa-coordinated¹¹. Coordination with external ligands produces very specific and sometimes sizable effects on the electronic properties of the molecule. An

unambiguous assignment of the energy level diagram of chlorophyll a (Chl a) is complicated by the presence of various species with different degrees of ligation, association (i.e. hydrogen bonding) and solvation. Each of these 'species' may exhibit different or degenerate absorption and emission properties causing difficulties in the interpretation of observations.

Chl a is the molecule considered to be the most important pigment in green plants because it is generally present in the largest quantity and is observed in all photoactive organelles of the plant. An extensive literature on the electronic absorption properties of Chl a exists (for instance see reviews in ref. 9) and includes attempts in resolving the contributions from individual species in convoluted absorption bands⁹. These analyses provide a great deal of information about the positions of the electronic levels of the individual species components, but are considered by some to be of limited validity due to the large number of adjustable parameters required for curve fitting¹². As a consequence, there is a need for methods of analysis which rely more heavily upon empirical observations than 'fitted' data, and a need for complete studies of each species. Other reliable and sensitive methods to monitor these properties are available, and are able to resolve minor or less active species from the bulk of the solution. One seemingly practical method for analysing different species is through the measurement of emission spectra. Emission spectra can be selectively enhanced by tuning to regions in the absorption spectrum where the absorption cross-section for a particular species is larger than for other species. Fluorescence excitation profiles can map out

individual absorption bands which may not be resolved in the absorption spectra, permitting the direct correlation of absorption and emission bands. An excited state lifetime of the corresponding species can be measured using laser excitation at a wavelength where the fluorescence produced is attributed to one species alone. These data may be used to assess the state of ligation and/or association of the molecules under study.

Fluorescence methods may be limited in their usefulness, because they do not provide direct information about positions of the electronic (0,0) origins (a manifestation of selection rules, i.e. Stokes shift). Origins may be found from Raman excitation profiles of the normal modes of vibration, as long as the observed Raman scattering can be correlated with the desired species, in the same way absorption and fluorescence can be correlated to one species. Interferences arise from the low quantum yields of some species and the non-linear behavior caused by population saturation effects with high photon fluxes under some conditions of laser excitation. It is the purpose of this work to gain a better understanding of the physical properties of individual species of Chl a, in particular the monomeric forms. The need for this basic knowledge becomes apparent when observations of solutions containing higher aggregates composed of these basic sub-units must be interpreted.

Raman spectroscopy complements infra-red (IR) absorption measurements for the investigation of vibrational modes, however Raman spectra have a distinct advantage over IR measurements due to the monochromatic sources used for electronic excitation of Raman measurements. Like all absorption techniques, IR spectra reveal almost

exclusively the bulk constituents of the solution. Through the use of the phenomena of resonance excitation, Raman spectroscopy is able to probe the individual components of a solution, similar to resonance fluorescence excitation. By varying the excitation frequency, a selective amplification of each species in the solution may be found. The non-coincidence of the electronic absorption spectrum for different species allows a zone of resonance excitation to be found for each species. These properties have been well exploited in the investigation of chlorophyll species in solution, in particular the identification of vibrational bands arising from or changing due to aggregation. Conversely, resonance Raman profiles in conjunction with the electronic absorption spectrum for the same species can be used to further define electronic energy levels for the particular molecular species.

B. Chemical, Physical and Electronic Structure

1B.1 Chemical Structure Determination

In the 1930's, Fisher¹³ and co-workers determined the basic structure of chlorophyll and formula as $C_{55}H_{72}MgN_4O_5$. This was confirmed by the complete synthesis carried out by Woodward and co-workers^{14,15} in the 1960's. The configurations at the chiral centres (authors original notation) of the molecule were determined by Ficken, Johns and Linstead¹⁶ (ring IV, methyl and propionic ester group as trans); Burrell, Jackman and Weedon^{17,18} (Phytyl group as 2'-trans-7'R,11'R); and Wolf, Brockman, Biere and Inhoffen¹⁹ (relative configuration, methoxycarbonyl group C_{10} trans to propionic ester side chain (C_7)).

The absolute configuration of the Chl a molecule became known with the determination of the absolute configuration at C₇ and C₈ by Fleming in 1967²⁰ (methyl at C₈ and hydrogen at C₇ both cis to carbomethoxy group at C₁₀). Chl a thus has the configuration 7S, 8S, 10R, 7'R, and 11'R as shown in Figure 1. Chlorophyll b (Chl b) has the same configuration as Chl a²¹. Chl a and Chl a' are isomers of the respective molecules which have undergone epimerization at C₁₀. These results were confirmed by X-ray crystallographic determinations^{22,23}.

1B.2 Physical Structure Determination

Studies of chlorophyll analogues by X-ray crystallographic methods have identified some structural properties which may effect the molecular bonding properties of porphyrins and chlorins. Individual properties of the pigments have been attributed to²⁴: (1) the chemistry and stereochemistry of the pyrrole-bonded side chains, (2) the effect of macrocyclic distortion on π -bonding with metal insertion in the free base, (3) the environment of the porphyrin in the biological system, and (4) the distortion and dynamic flexibility of the porphyrin ring macrocycle from non-planarity.

In order to attribute physical properties to Chl a structure, a number of free base and metal substituted porphyrin molecules have been studied. The porphyrin skeleton is formed by four pyrrole rings connected by bridging methine carbon atoms (C_m) to form the porphine molecule (Figure 2). Analogue molecules have provided information about the macrocyclic skeleton, the effects of substitution at methine (C_m) and pyrrole positions (C_p), metal insertion, asymmetric ligand

Figure 1

Structures and numbering system for the Chlorophylls and related pigments. Macrocyclic rings identified by Roman numerals. Abbreviations given in the List of Symbols and Abbreviations.

- Chl a: $R_1 = \text{CH}_3$, $R_2 = \text{C}_{20}\text{H}_{39}$ (phytyl)
 Chl b: $R_1 = \text{CHO}$, $R_2 = \text{C}_{20}\text{H}_{39}$
 EtChl a: $R_1 = \text{CH}_3$, $R_2 = \text{C}_2\text{H}_5$ (ethyl)
 MeChl a: $R_1 = \text{CH}_3$, $R_2 = \text{CH}_3$ (methyl)
 Pheo a: two hydrogen atoms instead of Mg, vinyl group about the C_2--C_3 position rotated 180° , R_1 and R_2 as for Chl a
 BChl a: $R_1 = \text{CH}_3$, $R_2 = \text{C}_{20}\text{H}_{39}$, ring IV double bond over C_7--C_8 , hydrogen at C_7 and C_8 removed
 PChl: $R_1 = \text{CH}_3$, $R_2 = \text{C}_{20}\text{H}_{39}$, ring II and ring IV double bonds over C_3--C_4 and C_7--C_8 , hydrogens at carbons 3, 7 and 8 removed
 MePPb: metal ion Mg removed, phytol chain replaced by methyl ester group

Figure 2

Macrocyclic skeleton of the porphines. Carbon atom positions C_m (methine) and C_b are used in the text.

Porphine: $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = H$
 TPP: $R_1 = R_2 = R_3 = R_4 = C_6H_5$ (phenyl)
 $R_5 = R_6 = H$
 TPrP: $R_1 = R_2 = R_3 = R_4 = C_3H_7$ (propyl)
 $R_5 = R_6 = H$
 FeTPP: $R_1 = R_2 = R_3 = R_4 = C_6H_5$ (phenyl)
 N_1 and N_3 bonded to Fe directly
 CuTPrP: $R_1 = R_2 = R_3 = R_4 = C_3H_7$
 N_1 and N_3 bonded to Cu directly
 OEP: $R_1 = R_2 = \dots = R_7 = R_8 = C_2H_5$

substitution, axial coordination of ligands onto the central metal above and below the macrocycle plane, interstitial coordination, the addition of ring V, the reduction of ring IV pyrrole to form chlorin, and the addition of a short phytyl 'tail' on ring IV.

Ethyl chlorophyllide a (EChlde a)²² is the porphyrin most homologous to Chl a which has been crystalised for X-ray structural determinations and differs only by the presence of an ethyl group instead of phytyl. In the crystal unit cell there are two water molecules per EChlde. The Mg is five coordinate and displaced 0.39Å above the plane defined by N₁, N₂ and N₃ atoms, in the same direction as the methyl ester group of ring V. EChlde exhibits many of the structural and physical parameters observed for the other molecules studied by x-ray crystallographic methods²⁴⁻³². The fifth coordination site is occupied by water, the second water is hydrogen bonded to the axial water and the carbonyl oxygen of the methyl ester group of ring V. The metal to nitrogen bond lengths are shortest between rings I and III and longest between rings II and IV, an effect common to VODPEF³¹ and NiDPE³⁰. The C--C bond lengths on the ethyl groups (1.49Å, 1.47Å) are shorter than normal single bonds (1.54Å). As well the bond length of the vinyl group on ring I (1.27Å) is shorter than a normal double bond (1.34Å). As with MePPb³², the vinyl group is not planar with ring I. Ring IV deviates from the relative plane of rings I, II and III -- all of the carbon atoms, especially the C₇ and C₈ are 'puckered' up towards the metal. The other pyrrole rings are all puckered away from the metal. The keto carbonyl of ring V is less than 0.02Å from the plane of Ring III. This may allow conjugation between the carbonyl double bond and the chlorin

π -system. The C_{23} is removed $0.51\text{\AA}$ towards the nearest EChlde molecule in the crystal lattice. Unlike MePPb, the direction of the vinyl bond differs by a two fold rotation. The displacement of the vinyl group from the ring I plane may isolate it from conjugation.

Some of the physical properties of Chl a may be extrapolated from EChlde since Chl a differs only by a longer phytol chain. The direction of macrocyclic distortion is determined by the preferred metal coordination geometry and whether the metal is coordinated to a fifth ligand. Ligands may be bonded axially, as well as hydrogen bonded to the periphery of the molecule. The diagonal distance between rings I and III is always shorter than between rings II and IV for porphyrins or chlorins with a fifth ring. In the chlorins, ring IV is saturated over C_7-C_8 thus substituents on this ring should be removed from the macrocyclic conjugation sphere. Ring V is coplanar with ring III, and its substituents may be involved in conjugation with the macrocycle since they lie on carbon atoms adjacent to the macrocycle and because ring III is held closer to the central metal. The ethyl group of Chl a may be involved in the π -system since its bond lengths are intermediate between single and double hydrocarbon bond lengths, whereas the vinyl group is likely not conjugated to the macrocycle since it is far from being planar with ring I.

These physical properties may help in classifying the electronic behaviour of Chl a in different solvents as well as electronic behavior upon aggregation. The observed optical absorption transitions represent porphyrin-ring $\pi \rightarrow \pi^*$ transitions, thus changes in the macrocyclic conjugation will affect the energy of the transitions. Aggregation of

Chl a, whether indirectly through other molecules (like water) or directly through self-assembly, should also influence the energies of electronic transitions.

1B.3 Electronic Structure

The electronic absorption spectrum of free-base porphyrin (porphine) is characterized by a spectrum containing four bands, whereas metallated porphyrins, chlorins and dibasic cations ($2H$, $2H^+$ on pyrrole nitrogens) have two absorption bands. This change is accompanied by an increase in symmetry from D_{2h} (x , y not degenerate) to D_{4h} (x , y degenerate).

The empirical electronic properties of D_{2h} type porphyrins are as follows^{33,34}: (1) two strong bands and two weak bands in the region 500--600 nm, $Q_x(0,0)$, $Q_y(0,0)$, $Q_x(1,0)$ and $Q_y(1,0)$ where x and y represent directions of polarization. The lower energy bands (0,0) are separated from the higher (1,0) by about 1250 cm^{-1} ; (2) bands of weak intensity labelled N, L, and M at higher energy to the Q bands; and (3) a very strong band between 380--420 nm assigned to the second excited state singlet, $B(0,0)$. Bands are not well resolved in this region for x and y polarization. A vibronic labelled $B(1,0)$ may be observed at higher energy. This region is sometimes called the 'Soret' region.

In D_{4h} type porphyrins the electronic absorption spectra appear simplified. As x and y directions become degenerate, only one strong band, the electronic origin $Q(0,0)$, and one weaker vibronic band $Q(1,0)$ are observed. The B_x and B_y bands also become formally degenerate but since they are poorly resolved even in the D_{2h} porphyrins, little change is observed in this region.

As predicted from X-ray structure, all alkyl substituted free base porphyrins have essentially the same spectra³³. The substitution of electron withdrawing groups, like vinyl groups at the pyrrole position or ketone groups on the alkyl methine position, also have minimal effect. Ligation directly onto the conjugation pathway or the reduction of one pyrrole ring of porphine to form chlorin causes large optical effects. Metal insertion can have two effects on the electronic absorption spectrum³³, either minor perturbation of the conjugation of the macrocycle (i.e. Mg^{2+} , closed shell orbitals) or severe perturbation (i.e. $Fe(III)$).

With substitution of different metals into the porphyrin ring, in general, there is little change in the absorption spectra but large change in the emission³³. The coordination geometry of the metal only affects the π -structure if it causes a rearrangement of electrons. Large effects are seen if the metal electrons directly interact with the ring. The presence of counter-ions or neutral ligands on the metal, as well as hydrogen (or otherwise)-bonded ligands causes spectral shifts qualitatively associated with the movement of electron density from the metal-ligand unit to the ring³³.

The position of the electronic levels of the various porphyrins, as well as transition probabilities, can be of interest in determining the effects of substitution onto the porphyrin ring. Factors making theoretical treatments difficult³⁵ are due to: molecular tautomerism, the presence of heteroatoms (nitrogen and carbon), the interference of σ electrons, ring strain, and assumptions of full aromaticity and that the molecular ground and excited states are adequately described by

single electron configurations. The porphyrins are too large for exact or ab initio molecular orbital calculations. Methods used in the past have been mainly empirical and semi-empirical (where the values of some or all of the required integrals are provided by experimentally determined quantities). Both π -electron and σ -electron methods have been used. The π -electron method is highly applicable to the porphyrins because they are planar and highly conjugated and because it is assumed that the longest wavelength electronic bands arise from transitions involving π -electrons only. However, σ -electron methods are necessary when dealing with metal substituted porphyrins because most metals are σ bonded to the porphyrin macrocycle.

C. Spectroscopy and Structure: Ligation and aggregation of Chlorophyll a in Solution

1C.1 Ligation and Association

In solutions of solvents like ether, ethanol and pyridine, it is well known that Chl a is present mainly in monomeric form or in low n-number aggregates³⁶. In contrast, with solvents such as hexane, carbon tetrachloride and benzene, Chl a aggregates at very low concentrations³⁷. This aggregation behaviour is necessary in order to fulfill the coordination requirements of the magnesium atom in the porphyrin structure. The extent of aggregation is dependent on the ability of the solvent or other nucleophiles to coordinate with the metal and to solvate the active functional groups of the porphyrin macrocycle. Low temperatures have been used to induce aggregation³⁸ and

to force specific ligation^{39,40,9}. Different coordinated forms of Chl a can be present in the same solution. Electrophilicity, equilibria, concentration and solvent properties can be used in the assignment of absorption and emission spectra.

In the literature of the chlorophylls, there are numerous conflicting observations and assignments for similar samples. Some of this confusion began with the use of moderately concentrated samples (greater than $10^{-5}M$) or because samples contained variable water and/or extraneous nucleophile contaminations. Now, these problems are recognized by many researchers, but for some experimental work still no special effort has been made to dry solvents. This neglect can lead to the additional complication of assignments made for bands where the effects of ligation, association and solvation are not resolved.

Anhydrous magnesium metal salt solutions can be prepared under extreme conditions but take up water over the space of hours even in a nitrogen dry box⁴¹. Under stringent drying conditions (drybox, co-distillation, and heating to 60° for 30 minutes under vacuum), Chl a is stable as the monohydrate⁴². The difficulty in removing the water coordinated to the magnesium has been noted for other metallo-porphyrins⁴³. Gas phase spectral data are available for some porphyrins^{43,44} but little other data are available for any 4-coordinate metallo-porphyrins because of the difficulty in removing the fifth ligand while not denaturing the molecule. Moreover, there is a risk that a nucleophile will undergo exchange with the ligand during the measurement⁴³. Additional nucleophiles (water or other) are present as trace impurities in hydrocarbon solvent solutions unless the solvents

are purified and dried. At best, the water content of non-polar solutions at concentrations of 10^{-6} - 10^{-4} M cannot be reduced to a level lower than the order of the chlorophyll concentration^{45,46,47,48} (i.e. a minimum of 1:1). A water molecule may already be bound during the extraction process when the Chl a is removed from the plant matrix^{9,49}. Since water molecules cannot be eliminated completely, it is necessary to have reproducible water contents in each sample.

Solvents can also be prepared with maximum concentrations of water by saturating the solvent with water. At high solute concentrations, this may lead to the formation of dihydrate, colloidal (polymeric) aggregates of Chl a (absorbance at 743 nm)⁵⁰ or the precipitation of crystalline material (also absorbance at 743 nm)⁴², or demetallation⁵¹. In these water-saturated solutions, solvent-solute-nucleophile interactions occur with maximum association. Solutions with intermediate water concentrations produce water-ligated species which may be somewhat difficult to assign until the functional groups that are associated have been identified.

The first water molecule bonded to the central magnesium of magnesium porphyrins is necessary to fulfill the coordination requirements of the metal ion. Additional ligands may have several possible bonding sites. For Chl a, these sites may include bonding to the keto-carbonyl or carbomethoxy groups of ring V or to the ketone of the phytyl chain linked to ring IV or a second ligand bonding onto the magnesium (not necessarily equivalent to the first). Separating the effect of ligation at each site has been the source of much discussion. The use of model compounds which differ from Chl a only with certain

functional groups may aid to differentiate these effects.

Octaethylporphyrin (OEP) and magnesium octaethylporphyrin (MgOEP) can serve as chlorophyll model compounds in order to determine several effects: (1) the spectral shift associated with changing from rigorously dried solvent to water-saturated solution (solvation), where the shift is not associated with bonding on the metal (using OEP); and (2) the spectral shift for the same solvents, accompanied by a change in the metal coordination from penta- (dried solvent) to hexa-coordinated (water-saturated solvent) (using MgOEP).

The production of hexa-coordinate magnesium porphyrins is not an unknown phenomena. The observation of hexa-coordinated Chl a (under conditions where demetallation does not apparently result) has been reported by a number of authors. Katz et al.⁵² calculate the constant of formation of a hexa-coordinate Chl a species (the central magnesium being coordinated) as approximately 100 times smaller than the formation of a penta-coordinate species from aggregated Chl a. Evans and Katz^{8,11} state that the formation of the Chl a hexa-coordinated species will happen only under very 'forcing conditions', such as occurs with an excess of the ligand orders of magnitude above the Chl a concentration. BChl a also has been observed in more than one state of ligation at high and low BChl a concentrations^{8,53,54}. BChl a is usually pentacoordinate and aggregates at concentrations greater than 10^{-5} M, but the use of a strong base such as n-butylamine causes complete disaggregation and a red-shift in the absorbance band position from 582 (dilute solution, BChl a.L₁) to 612 (disaggregated, concentrated solution, BChl a.L₂)⁸. Miller and Dorough⁵⁵ report that magnesium tetraphenyl chlorin in

benzene also undergoes an analogous band shift associated with a change from penta- to hexa-coordination with the addition of pyridine as a nucleophile.

1C.2 Early Experimental Results

Shipman et al.⁹ have observed the visible absorption spectrum of 10^{-5} M Chl a in diethyl ether (a monoligated adduct with ether) and deconvoluted the gaussian electronic contributions from Q_x and Q_y transitions. The results of this calculation placed electronic origins at 659 nm, $Q_y(0,0)$; 613 nm, $Q_y(0,1)$; 575 nm, $Q_x(0,0)$ and $Q_y(0,2)$; and 530 nm, $Q_x(0,1)$ with relative intensity contributions of 84.0:13.0:7.1:3.8 respectively⁹. The splitting between $Q_y(0,0)$ and $(0,1)$ energy levels of 1140 cm^{-1} was accounted for by assuming that the Q_y transition was coupled to one or more of the C---C and/or C---N stretching modes.

An equilibrium shift from penta- to hexa-coordinated species can be caused by lowering the solution temperature and using weak nucleophiles rather than strong nucleophiles at room temperature. In Chl a solutions frozen to 2°K prepared using solvents not completely exempt of water, Clarke et al.³⁹ found the fluorescence maximum of an n-octane solution was dependent on excitation wavelength. With 514.5 nm laser excitation, the fluorescence maximum observed was 670 nm, whereas with 457.9 nm excitation, the observed fluorescence maximum was 680 nm (CW Ar^+ laser). A fluorescence band at 720 nm was also observed at both excitation wavelengths. These resolved fluorescence bands at 670 and 680 nm were interpreted as representing 'mono-' and 'biligated' species

respectively, both complexed with water. Clarke et al.³⁹ also observed that the fluorescence at 680 nm was at least an order of magnitude stronger than that at 670 nm. Clarke's study was made at low temperature where biligation is favoured, thus possibly increasing the fluorescence intensity from this species. Clarke also prepared Chl a solutions of 10^{-6} M in n-octane with pyridine in excess over the Chl a concentration at low temperatures and found at both excitation wavelengths only one main fluorescence band was present (680 nm) which was attributed to one hexa-coordinated Chl a species. A very small amount of the 670 nm species could be detected just within experimental error, under the stronger 680 nm band shoulder.

Clarke's³⁹ assignment of the (10 nm) red-shifted fluorescence band to hexa-coordinated Chl a may be incomplete since it neglects the concurrent effect of associated ligands. The band positions of water-ligated hexa-coordinated Chl a and pyridine-ligated hexa-coordinate Chl a may be degenerate since the bonding involves different interactions of the ligands with the π -electronic structure of the porphyrin macrocycle.

Kooyman et al.⁴⁰ also carried out low temperature fluorescence studies and concluded that the main ligand interaction with Chl a occurred at the central magnesium. In those experiments (solvent n-octane, water less than 10^{-3} M, always at 4.2°K), solutions of 10^{-6} M Chl a were observed to have an intense fluorescence band at 669 nm and a shoulder at 687 nm. A 10^{-5} M Chl a solution with an added concentration 5×10^{-3} M pyridine had only one fluorescence band at 686 nm. [Presumably, these two fluorescence profiles are observed with the same

excitation wavelength; 'properly filtered blue light from a super high pressure 900 W Xe arc for excitation']. The peak 669 nm was assigned to a mono-ligated, monomeric Chl a (the low temperature 669 nm fluorescence almost coincides with the room temperature absorption maximum at 663 nm in ether, and the Soret absorption band of Chl a in ether almost coincides with the 4.2°K excitation spectrum when monitoring the 669 nm fluorescence). In agreement with the assignment of Evans et al.⁸ The fluorescence at 686 nm was assigned to a Chl a species biligated with pyridine. By the similarity of the magnitude of the red-shift, the fluorescence at 687 nm was assigned to Chl a 'biligated' with water (an 18 nm shift from the penta-coordinated species band position). For a control sample of pheophytin a, no new red-shifted band was observed after the addition of pyridine to the solution, thus the large red-shift for Chl a was attributed to electronic perturbations caused by the addition of a second ligand bonded to the metal. Kooyman et al.⁴⁰ does not state whether minor red-shifts were observed with the addition of a strong nucleophile (pyridine) to the Pheo a solution. This would have provided insights into the magnitude of perturbation caused by associated ligands alone (except where these second-sphere ligands are attached to the metal-bonded ligand and to a functional group on the macrocycle at the same time).

Shipman et al.⁹ predicted that over 99% of the Chl a would be present as the disolvate species upon hexa-coordination of the Chl a by pyridine, (pure pyridine solution, equilibrium calculation⁸ carried out for the conversion of monoligate to biligate). The bands in the red region of the absorption spectrum for this solution are red-shifted, but

the Q_x bands are further shifted to lower energy than the Q_y (using the penta-coordinate Chl a monomer band assignments of Shipman et al.⁹). After deconvolution, electronic band origins were located at 670 nm, $Q_y(0,0)$ and 639 nm, $Q_x(0,0)$ for the hexa-coordinated Chl a species. Other solvents forming hexa-coordinated Chl a species have an assignment of $Q_x(0,0)$ positions at 633 nm (toluene as bulk solvent titrated with pyridine)⁸, 629 nm (tetrahydrofuran)^{56,57} and 623 nm (dioxane)⁵⁶. For these bands, the (0,0) positions have not shifted as far as with pyridine as solvent.

For solvents within the same group (polar versus non-polar), fluorescence lifetimes for BChl are approximately the same⁵⁴. A shorter lifetime in certain solvents may indicate that there is more intersystem crossing (ISC) when the Chl a has more than one bound water molecule, whether bound to the magnesium or to other peripheral sites⁵⁴. The intersystem crossing to the triplet states is controlled by spin orbit coupling involving singlet states associated with the Mg--N centre complex. Reduction of this interaction lowers the energy of the lowest singlet and allows the S_1 state to be more accessible to spin orbit coupling and increases the triplet decay rate⁵⁴. The attachment of a second magnesium-bound ligand increases the intersystem crossing constant by almost a factor of two from the value for just one ligand³⁹. Chl a solutions with added pyridine have an overall triplet lifetime somewhat shorter than hydrocarbons like n-octane where only water molecule impurities are provided as competitive nucleophiles for both penta- and hexa-coordinated species. This may be explained by a greater attraction of the magnesium for the pyridine molecule (a better electron

donor than water) compared to the porphyrin macrocycle.

1C.3 Electronic Origin of the Spectral Red Shift upon Ligation

The cause of the shift of the Q_y energy levels to lower energy (red-shift) concomitant with the change from monoligated species to biligated/solvated species has been attributed to many causes. The reason for unequal energy changes accompanying change in solvents is less well understood. It is recognized from X-ray diffraction analysis that when penta-coordinated, the magnesium occupies an out-of-plane position located about 0.4Å above the macrocycle towards the bonding ligand²². In hexa-coordinated Chl a it has been assumed that the addition of the second ligand returns the metal to lie on the plane^{9,53}, however no X-ray data for hexa-coordinated chlorophyll or other analogue molecules are yet available (and thus this assumption is untested). Moving the magnesium to a coplanar position would presumably result in more interaction between the porphyrin ring π -structure and the metal orbitals. Evidence that the attachment of a second axial ligand does not significantly change the physical structure comes from nitrogen S-orbital spectra for penta- and hexa-coordinated Chl a⁴² where the hydration does not significantly alter electron density on the four pyrrole nitrogen.

Kooyman⁴⁰ interprets the red-shift caused by attaching an electron donating ligand to the chlorophyll as being equivalent to replacing the metal by another which is less electronegative. The axial ligands bonded to the metal provide needed electron charge for the metal, thus the metal has a smaller charge withdrawing effect on the porphyrin ring.

For porphyrins, this produces a stabilization of the S_1 energy if the effect of configuration interaction is taken into account^{58,59} and, in an analogous manner can cause a red shift of the Chl a bands⁴⁰.

The red shift can also be understood in a qualitative manner using a FMO model⁴⁰. Ligation on the magnesium decreases the π -interaction with the porphyrin ring and thus effectively causes a net displacement towards the periphery of the ring and lowers the energy of S_1 ⁴⁰. This may be an oversimplification because the FMO model is valid only for cases of weak solvent-solute interactions (i.e. hydrogen bonding) and is invalid for π -electron perturbation by solvation effects on side chains⁴⁰. For these reasons, an alternative explanation of the red-shift may be necessary.

1.C4 Aggregate Models of Chlorophyll a

Chl a has the unique property of having both nucleophilic and electrophilic sites which can allow the molecule to act as electron acceptor or electron donor. As discussed previously, nucleophiles (alcohols, ketones, amines, ethers, water etc.) can compete to coordinate with the magnesium ion of the Chl a molecule as well as in associating with other sites on the molecule. Aggregation can proceed directly via covalent bonds between Chl a molecules (internal interaction, self-aggregation) or indirectly through ligands (external interaction). The method of aggregation depends on factors such as the functional groups of the Chl a molecule involved, the presence of trace nucleophiles, the solvent system, the Chl a concentration and temperature. All of these can influence the electronic properties of

the aggregates from those of monomeric Chl a.

The magnesium ion is the most electrophilic site of the Chl a molecule due to the coordination unsaturation. The metal ion acts as an electron acceptor from external ligands. In this geometry, the porphyrin ring provides insufficient electron density to saturate the metal ion, and thus a purely tetra-coordinate magnesium porphyrin species is not observed in solution or vapour phase^{43,44}. The displacement of the metal ion about 5Å out of the molecular plane facilitates the approach of electron donor molecules which can provide necessary charge to stabilize the metal ion. Bulky electron donors may be hindered from effective metal bonding by the π electron cloud of the macrocycle delocalized over the molecule.

A resonant π charge delocalization is seen across most parts of the Chl a molecule. The methine hydrogens at α , β and δ positions are acidic, and the methyl and ethyl groups at positions C₁, C₃, C₄ and C₅ are connected to the macrocycle by bond lengths shorter than normal paraffinic bonds (1.54Å)²². These properties indicate that electronic charge is withdrawn into the macrocyclic π system. The ethyl group at C₂ (not co-planar with the macrocycle), methyl and phytol ester groups of ring IV (ring IV bond lengths longer than other pyrrole rings) are apparently isolated from resonance. It is generally accepted that ring V is able to delocalize charge into/out of the π -macrocycle despite ring V skeletal bond lengths ranging from 1.43 to 1.57Å²². Ring V and the ketone at C₉ are co-planar with rings I, II and III. Evidence suggests that the ring V carbomethoxy ketone is much less acidic (a better electron donor) than the C₁₀ ketone⁴⁹.

Ballschmiter et al.³⁷ have determined chlorophyll aggregate sizes using vapour phase osmometry. In rigorously dried solvents like CCl_4 , benzene and 1,2-dichloroethane the anhydrous Chl a oligomers $(\text{Chl } \underline{a})_n$ never exceed $n=4$ in 0.1M solutions (Chl a preparation given by Strain et al.⁶⁰ such that much of the magnesium bonded water molecules are removed and chlorophyll is forced to self-coordinate through endogenous interactions). In these solvents at lower dilutions, Chl a molecules are at least dimeric even with extrapolation to infinite dilution. Large oligomers with n values of approximately 10 in 0.1M cyclohexane, and 20 in 0.1M n -hexane can be found. In 10^{-6} M dry hexane solution, the n value is about 4. These data contrast with Chl a that is still bonded to water molecules on the magnesium³⁷. For 0.4M Chl a in CCl_4 , the anhydrous preparation has n values of approximately 2.45 compared to 1.78 for the mono-hydrated Chl a in the less rigorously dried solvent. In a 2.3×10^{-4} M benzene solution, the aggregation number was 2.06--2.36 for the dry Chl a and 1.93 for the undried (penta-coordinated) Chl a. In aliphatic hydrocarbons at the high concentrations studied, the monohydrated Chl a formed aggregates of micellar size with the composition $(\text{Chl} \cdot \text{H}_2\text{O})_n$.

A knowledge of the physical geometry of the various dimer complexes is necessary because it influences properties of the 'supermolecule' complex. The absorption and fluorescence transitions observed for Chl a all involve $\pi \rightarrow \pi^*$ transitions of the porphyrin skeleton. The π -macrocycle is influenced by delocalized electron charge on substituent functional groups. The close proximity of electron donating or accepting groups such as the π -macrocycle of

another Chl a molecule or electron rich functional groups like the C₁₀ ketone of another Chl a molecule will affect the π charge of the macrocycle. Studies in search of the Chl a special pair dimer concluded from IR⁴⁹ and ESR⁴⁰ that the two Chl a molecules are held in plane parallel orientation via two ligands (each Chl a having both electron donor and electron acceptor capabilities), CD⁶¹ and NMR⁶² data indicate that the macrocycles of the dimer are tilted at an angle of about 40° (unequal molecular properties, donor-acceptor). Whether in plane-parallel orientation or otherwise, π -charge may be delocalized from one molecule to another as long as the molecules are within a sufficiently close distance. Depending on the size of this interaction, and upon the timescale of the interaction, the individual molecules of the dimer may be resolved and characterized.

Several authors have suggested different structures for a donor-acceptor Chl a dimer based on various spectroscopic evidence. Katz and Norris⁶² proposed a photoactive reaction centre dimer consisting of two Chl a molecules linked through a water molecule by hydrogen and coordination bonds (Figure 3). These dimer molecules are unequal with ring V ketone and carbomethoxy groups of one Chl a hydrogen bonded to a water molecule which is coordinated to another Chl a molecule. This basic interaction forms the repeating dimeric sub-unit proposed for the hydrated oligomer with absorbance at 743 nm and fluorescence at 743 nm.

Fong⁶³ has proposed a similar donor-acceptor dimer to Katz and Norris, differing in that only the ring V ketone of the donor molecule is involved and that there is no intermediate water molecule (Figure 4).

Figure 3

Donor-acceptor dimer model of Katz and Norris.⁶² Magnesium of one chlorophyll molecule linked through a water molecule to another chlorophyll molecule at the ring V ketone and carbomethoxy positions.

Figure 4

Donor-acceptor dimer model of Fong.⁶³ Magnesium of one chlorophyll molecule linked directly to the ring V ketone oxygen of another chlorophyll molecule.

This structure can also form anhydrous Chl a oligomers (absorbance 678 nm)⁶². Kooyman and Schaafsma⁶⁴ have more recently discussed this type of (Chl a)₂ dimer.

Experimental⁴⁹ and theoretical⁶⁵ evidence has shown that the ring V ketone is a better donor of electron density than the ring V carbomethoxy ketone. Low temperature studies⁴⁹ of the Chl a.ethanol adduct in toluene have found that it is the ring V ketone which is hydrogen bonded to the ligand (in this case ethanol). The carbomethoxy ketone becomes involved only when the ethanol is present in large excess.

1.C5 Raman Spectroscopy of Chlorophyll a

Intensity and depolarization ratios for some Raman bands of Chl a, Chl b, Pheo a and Pheo b have been extensively studied using coherent-wavelength (CW) laser excitation and highly concentrated pigment solutions⁶⁶. The band assignments and proposed states of association for typical in vitro systems of chlorophyll and pheophytin have subsequently been used as the main source of vibrational band identification. Dilute solutions containing monomeric BChl a have been investigated using coordination sensitive bands as a probe in order to determine the correlation between absorption bands and resonance Raman spectra⁶⁷. BChl a has also been investigated by resonance Raman spectroscopy⁶⁸ and bands indicative of ligation interactions as well as hydrogen-bonding interactions identified. Several types of aggregated BChl a species have also been studied -- each with resonance Raman spectra differing from that of the monomer through frequency shifts and or band intensity changes. The wavelength-selective capabilities of

lasers have also allowed the study of in vivo systems such as the triplet states of carotenoids present in bacterial reaction centres⁶⁹. Here Raman spectra and frequency shifts have been used to monitor the isomerization reactions of specific molecular conformations.

Certain authors have classified the resonance Raman spectra and infra-red spectra of Chl a and Chl b into two main groups depending on solvent and interpreted the variations as being the product of differing states of metal coordination⁷⁰ (one or two ligands). Infra-red measurements have been used to confirm these Raman data. These ideas have been expanded upon to encompass vibrational studies of Chl a and to form a 'library' of coordination state marker bands⁷¹.

CHAPTER II

2A.1 Preparation/Isolation of Chlorophyll from Spinach

Chl a was isolated from fresh spinach using a column chromatographic technique similar to that of Omata and Murata⁷². Approximately 1 kg of fresh spinach was shredded, washed with cold distilled water and then scalded with boiling distilled water until the leaves softened (1-2 minutes)⁷³. The water was squeezed out of the leaves, and the leaves were cooled, then immersed in a minimum volume of ice cold acetone to which $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ had been added (3.5 mg per 20 ml acetone). This mixture was stored on ice in the dark for several hours until the acetone became dark green, then the leaf material was removed. To precipitate the pigments from the acetone, 1,4-dioxane was added (2.5 ml/20 ml acetone) followed by distilled water (about 4 ml/20 ml acetone) until a green precipitate was observed to form. The colloidal dioxane/water precipitate⁷⁴ was allowed to settle out of solution in the dark for 4-8 hours, then was concentrated to a minimum volume by centrifugation (5000 rpm for 20 minutes) while being refrigerated at 5°C. The precipitate was dissolved in ethanol and passed through a fine glass filter and then evaporated to dryness under vacuum leaving a waxy, black solid. This solid was washed with 20 ml aliquots of hexane to remove soluble chlorophylls and leave behind less soluble pigments (such as some carotenoids). The hexane solution was dried under vacuum, forming a hard black solid.

Column material for chromatography was prepared from aqueous suspensions of Sepharose CL-6B (a cross linked agarose) and DEAE-Sepharose CL-6B (the diethylamine derivative of the cross linked agarose). The DEAE-Sepharose was prepared by washing a 50 ml suspension successively with distilled water, 1 M sodium acetate, distilled water, and acetone by filtration and re-suspension. Sepharose CL-6B was prepared by successively washing 100 ml of suspension with distilled water, acetone, acetone/hexane mixture (2:1 v/v), acetone/hexane (1:2), hexane/2-propanol (10:1) and hexane/2-propanol (20:1). Water cooled glass columns (1-2 cm in diameter) were packed to a height of 3-4 cm, stirred with a glass rod, and allowed to settle under solvent flow to ensure uniform packing.

After use, the columns were regenerated to remove adsorbents. The DEAE-Sepharose column was washed with 80% aqueous acetone containing 1% ammonium acetate, followed by acetone. The Sepharose CL-6B was washed successively with acetone, methanol, and hexane/2-propanol (20:1). With the last solvent each column was repacked by stirring and settling under solvent flow before use. Columns could generally be used five times before they were discarded. Preparations approaching final purity were placed only on previously unused columns.

Small amounts of solid crude pigment extract were dissolved in 1 ml acetone and applied to the first column, DEAE-Sepharose. The column was developed with acetone at a flow rate of approximately 2 ml/min until all of the pheophytin (grey band) and carotenes (yellow-orange band) were eluted. Much of the fatty materials were removed here by retention on the top of the column. The adsorbed chlorophylls were developed

after changing the solvent to acetone/methanol mixture (10:3) but were not resolved on this column. This eluent was immediately evaporated under vacuum at room temperature and stored in the dark while the column was regenerated and further separations made. To ensure complete removal of carotenes, the crude chlorophyll was re-applied to this column twice.

To separate Chl a and Chl b, the crude Chl a was dissolved in 0.5 ml aliquots of hexane/2-propanol (20:1) and applied to the Sepharose CL-6B column. Chl a was eluted by development with the same solvent at a flow rate of about 1 ml/min. The crude Chl a eluent was dried under vacuum and stored in the dark while the column was regenerated and more of the crude Chl a solution applied. The crude Chl a was passed through this column until no trace of Chl b could be seen, then 2 or 3 more times on a previously unused Sepharose CL-6B column until the sample met standard purity criteria (see section 2A.2).

The preparation of several samples of Chl a could be carried out in one day, but generally supplies for several months were prepared at one time. The solid Chl a was stored in glass under a dry nitrogen atmosphere in a dark glove box. Chl a stored in this way was sometimes re-purified on the Sepharose CL-6B column before use. Further details including the preparation of Chl b are given by Omata and Murata⁷².

2A.2 Evaluation of Purity and Identification of Pigments

The purity of Chl a was established by absorption spectroscopy, thin layer chromatography, and reverse phase high performance liquid

chromatography. No single technique can establish both the purity and identity of the pigment. The ratios of the absorbance maxima of blue and red absorption bands may be used to establish purity⁷³ of individual pigments since these values are well established (Table 1). This method is less reliable if the pigments are impure⁷⁵, especially if uncoloured substances are present (proteins, lipids etc.). Absorbance methods will not resolve some similarly coloured pigments such as the isomers Chl a and Chl a'. The Chl a prepared for these studies had an absorbance ratio (in an ether solution) blue/red of 1.28-1.30 after purification.

Thin layer chromatography (TLC) on an adsorbant like Silica Gel G (Merck, used without activation) can be used to monitor the complete isolation of Chl a from crude plant extracts^{76,77} with the choice of suitable solvents. The main components of the crude extract are separated well with hexane/acetone/t-butyl alcohol (90:5:5)⁷⁸. Mixtures of pure Chl a, Chl a', Chl b and Chl b' are resolved with a solvent of hexane/acetone/t-butyl alcohol (15:2:2). The 'pure' Chl a prepared by the method given here had only two spots on the developed TLC plate (Fig. 5), identified as Chl a and its isomer Chl a'.⁷⁹ Immediately after isolation, the pure Chl a showed only a small amount of Chl a'. The thin layer chromatogram of solutions containing Chl a in oxygen-free hexane stored in the dark for several days showed that no large changes in the proportions of the isomers was seen (qualitative). Interactions with the TLC plate surface may produce more Chl a' on the TLC than is present in the dry, isolated Chl a, or in the hexane solutions.

Reverse phase high performance liquid chromatography⁸⁰ (rp-HPLC) was used to qualitatively evaluate the presence of impurities/degradation

products in Chl a directly after preparation, after storage of hexane solutions, when exposed to light or oxygen, and when stored in hexane for prolonged periods (to 1 month). A Perkin Elmer Series 4 Liquid Chromatograph with 10- μ size μ Bondapak C18 column (Waters Associates P/N 27324, inside diameter 3.9 mm X 30 cm, 3000 plates/column) and a Perkin Elmer LC-85 Spectrophotometric Detector (flow cell) with output from a Perkin Elmer LCI-100 Laboratory Computing Integrator were used. The column was equilibrated for 2 minutes under a 2.0 ml/min acetonitrile/water flow (94:6) before sample injection (Rheodyne sample injector, 10 μ L loop, a sample volume of about 10 μ L Chl a in hexane, injected with a precision syringe, concentration adjusted for signal strength). A one-step solvent program was used to separate the Chl a and Chl a'. The total program was run for 22 minutes with absorbance measured at 436 nm. The results are summarized in Table 2. The C18 column was cleaned with 100% tetrahydrofuran. The reproducibility of retention times was within 0.1 minutes on sequential separations when the column was re-equilibrated between stationary and initial mobile phases. The mobile phase properties and other chromatographic properties are included in the legend of Table 2. These results were measured over several weeks, and thus the retention times were not always reproducible. However retention time ratios have a constant value for known substances and can be used for purity evaluations⁸¹. Hexane solvent bands at 2, 6 and 13 minutes are observed, the former two are probably impurities. The hexane band is not seen in the chlorophyll runs because its relative absorption is far less than chlorophyll.

At 436 nm, Chl a is the main absorbing species with a relatively

constant amount of Chl a' in hexane 'stock' solutions. The ratio of retention times of Chl a'/Chl a in non-degraded samples is 1.18 ± 0.02 (additional results not given here). In hexane solutions it appears that an equilibrium is reached between Chl a and Chl a' in a ratio of 3:1, however the amount of the Chl a' isomer may actually be lower in the prepared Chl a and hexane 'stock' solution since it has been observed that the water in the rp-HPLC solvent can cause isomerization/oxidation in the chlorophylls⁸¹. The use of 1% aqueous sodium ascorbate in the developing solvent provides mild buffering from acid catalysed reactions on any free silica surfaces (Cl8) as well as acting as an antioxidant and minimizing isomerization⁸¹. Aging the Chl a stock solution for a month or exposure to air and sunlight, results in the appearance of a third band, detected 1.5 minutes before the Chl a (i.e. more polar), which has been attributed to (an) unspecified allomerization product(s)^{80,82}.

These rp-HPLC results indicate that fresh Chl a hexane stock solutions may be stored for short time periods in the absence of oxygen and light without the formation of impurities absorbing around 436 nm. Similar results, including retention time ratios, were monitored with 400 nm absorption. Observations at 685, 665 and 590 nm did not reveal any absorbing species.

Table 1: Specific absorption coefficients of Chl a in ether^{73,78}

	red absorption band		blue absorption band		
	maximum wavelength (nm)	extinction coefficient $\times 10^{-3}$ $\text{Lmol}^{-1}\text{cm}^{-1}$	maximum wavelength (nm)	extinction coefficient $\times 10^{-3}$ $\text{Lmol}^{-1}\text{cm}^{-1}$	ratio blue/red
Chl <u>a</u>	660.5	96.6	428.5	125.1	1.30
Chl <u>b</u>	642.0	61.8	442.5	175.3	2.84
proto- chlorophyll <u>a</u>	623.0	36.9	432.0	305.9	8.14

Figure 5: Examples of thin layer chromatograms

solvent hexane:acetone:2-propanol (15:2:2)

(a) crude spinach extract (b) after DEAE Sepharose column (twice through) (c) after Sepharose CL6B column (five times), most pure

Table 2: Representative results of reverse phase HPLC on C18 column

The solvent used was acetonitrile/water (94:6). Peaks are assigned as follows: (●) Chl a; (○) Chl a'; (*) possible allomerization product(ref-80) (x) solvent band, hexane; (◊) solvent impurity. Only the largest peaks are indicated, minor peaks (<1% area) make up the rest of the 100%.

sample	peak number	percent area	retention time (min)	assign-ment	Ratio of R_f
A. fresh Chl <u>a</u> , stored in the dark with no oxygen	1	100.	9.92	●	—
B. fresh Chl <u>a</u> in hexane several days in the dark, no oxygen	1-23	-----	-----		
	24	54.414	11.02	●	1.17
	25	18.973	12.90	○	
	26-99	-----	-----		
	1	74.298	9.32	●	1.18
	2	25.702	11.02	○	
C. laser irradiated, several days old, no oxygen	1-3	-----	-----		
	4	51.972	9.73	●	1.20
	5	18.168	11.64	○	
	6-18	-----	-----		
	1-4	-----	-----		
	5	57.013	10.04	●	1.19
	6	21.017	11.93	○	
	7-31	-----	-----		
D. Chl <u>a</u> exposed to air (oxygen) and sunlight	1	22.221	1.84	◊	1.15
	2	67.498	9.46	●	
	3	10.281	10.85	○	
E. old Chl <u>a</u> stock solution, stored in the dark for several weeks	1	0.212	1.88	◊	1.19
	2	9.093	7.66	◊	
	3	66.63	9.22	●	
	4	24.060	10.93	○	
F. hexane	1	15.440	1.97	◊	—
	2	1.657	5.76	◊	
	3	82.902	12.74	x	

2A.3 Stock Solution Preparation

Chl a solutions used for fluorescence, absorption and Raman studies were prepared in a dry glove box under nitrogen atmosphere, with minimum exposure to light. Glassware and sample cells were oven-dried at $>100^{\circ}\text{C}$ for several days before use. Solvents (hexane: Caledon ACS spectrograde, and CCl_4 : Baker Photrex) were used with water content as received ('wet'), water saturated, or dried (by passing twice through a column of neutral alumina (Woelm, activity 1). Pyridine, lutidine (2,6-dimethylpyridine) and collidine (2,4,6-trimethylpyridine) were prepared from spectral grade solvents by drying over KOH pellets and then distilling under reduced pressure⁸³. All solvents were degassed by purging with nitrogen and were stored under nitrogen atmosphere. The solvents had no emission in the vicinity of the chlorophyll fluorescence.

Solid Chl a was dried to remove water by co-distillation with CCl_4 at room temperature. Initially, it was believed that repeated codistillation with rigorously dried CCl_4 under high vacuum could be effective in removing coordinated water from chlorophyll⁸⁴. It is now known that this procedure is effective in removing water to a minimum amount which is approximately equal to the Chl a concentration⁸⁵. The coordinated water molecule⁴¹ may or may not be⁸⁵ removed from the magnesium by more rigorous methods of treatment, but at the expense of degradation and instability (impossible to prevent the re-absorption of water). For the studies carried out in this report, the water concentration was minimized to approach the 1:1 limit in order to avoid degradation and because this provided a chlorophyll sample of known

coordination. Stock solutions of the desired concentration and solvent were prepared and stored in Teflon stopcock sealed vacuum flasks until use. For solvents other than CCl_4 , the Chl a solid was co-distilled two times with the dried solvent before stock solution preparation. Diethyl-ether solutions for absorption measurements were prepared in open air, and measured immediately. Concentrations of the stock solutions were measured by diluting a known quantity of the stock solution into ether (ca. $10 \mu\text{L}/10\text{mL}$), and the concentration of Chl a as monomer calculated using the 660 nm absorption coefficient listed in Table 1. Absorption spectra were measured on a Varian DMS 90 UV-visible spectrophotometer.

Absorption, fluorescence, and Raman measurements on the stock solutions were made using quartz cells (Helma 220/221-QS Suprasil, absorption linear and about 82% transmission over the visible region). Cells were filled under a nitrogen atmosphere in the dry box and sealed with vacuum-tight Teflon stopcocks (Rotaflo).

2A.4 Absorption Spectra Measurements

Absorption spectra were recorded using a DMS-90 Spectrophotometer or a HP 8450A UV/VIS Spectrophotometer using 1, 2 or 10 mm quartz cells fitted with Teflon stopcocks.

2A.5 Fluorescence and Raman Scattering Measurements

Fluorescence from Chl a solutions was produced by illuminating the samples with light from continuous wave (CW) or pulsed laser sources. Raman and fluorescence measurements were made by directing the incident

beam to enter the sample cell at an angle of about 55° to the cell face (unless otherwise stated). This angle was experimentally found to produce the strongest signals from solutions of moderate concentration (around 10^{-4}M) and is the angle at which the re-absorption of emitted light should be minimized⁸⁶. Light was focussed onto the cell with a 5 cm cylindrical lens to produce a stripe across the cell front. Emission was collected using a double lens systems and was focussed into the monochromator. Signal processing was carried out by a gated averaging boxcar integrator and signal output was plotted by a chart recorder (output linear with either energy or wavelength depending on the monochromator used). Fluorescence spectra shown in this work are as recorded and have not been corrected for detector sensitivity. Relevant parts of the apparatus are described as follows.

2A.5.1 light sources

Pulsed laser excitation was used from either a Lambda Physik FL2000 dye laser pumped by a M2000 Nitrogen gas laser (1 MW peak power), or Lumonics EHD-330 Dye Laser pumped by a Lumonics TE-430-2 Excimer Laser (20 MW peak power). By the use of a suitable laser dye/solvent combination, any visible wavelength could be produced from either of the dye lasers. The nitrogen or excimer lasers act as narrow band ultra-violet generators to excite fluorescence from organic dyes in the dye laser. Each dye lases over only a short range (about 20 nm), influenced by the length of the resonating cavity. The maximum power output from the dye laser is not strictly governed by the power of the nitrogen or excimer pump source, but is rather governed by the dye solution stability and the critical saturation value of the dye (maximum

power output regardless of excitation). The power of the dye laser can be enhanced by increasing the pump power. When required, this lasing was spatially dispersed with a Pellin-Broca prism and the strongest lasing portion was selected using an iris. This portion of strongest lasing was essentially monochromatic (bandwidth about 5 cm^{-1} or narrower). Laser pulses from both these systems were of nano-second duration with full-width half-maxima and repetition rates of 3.5 ns and 25 hz respectively (nitrogen laser) or 7 ns and 25 hz (excimer laser).

The CW laser used was a Coherent Radiation CR-4 Supergraphite Ion Laser, mechanically chopped (square wave) at 250 Hz. This Ar^+ laser produces discrete line emission at nine wavelengths: 454.5 nm, 457.9 nm, 462.8 nm, 472.7 nm, 476.5 nm, 488.0 nm, 496.5 nm, 501.7 nm and 514.5 nm

2A.5.2 light detection

Light emitted or scattered from the samples was collected with a double lens system and focussed into the slit of a Jobin Yvon Ramanor HG-2 Double Monochromator (triple pass holographic gratings, rejection value for light 20 cm^{-1} from the reference frequency $1:10^{-8}$ ⁸⁷). The scanning monochromator allowed high resolution of the scattered light. Light photons of the desired energy were detected by an RCA 31034-A water-cooled photomultiplier tube (PMT). This monochromator scans at a rate linear with energy (cm^{-1}).

2A.5.3 signal processing

A Stanford Research SR250 gated, averaging boxcar integrator (3 ns gate) was used to process signals from the PMT. As all the lasers used were either pulsed or chopped, the boxcar had to be triggered to detect the emission signal at a time when there was a signal (within ns of the

laser pulse). This was done through the use of a fast photo-diode circuit illuminated by the super-radiance from within the dye laser or with part of the chopped light from the CW laser. The averaged output was plotted on an x-y chart recorder. The boxcar was used because of its time-resolving capabilities. Sampling of the emission signal could be made over the width of the excitation pulse by varying the timing of the 3 ns gate or after the excitation pulse had ended as long as there was sufficient emission. This essentially produced a three-parameter signal resolution capability with intensity, time, and wavelength information. Excitation and emission time-resolution become useful for differentiating the photophysical behavior of species with similar fluorescence lifetimes and/or band positions. Fluorescence band profiles were measured 2 ns after the laser pulse maximum (unless otherwise stated) since the fluorescence is delayed. Raman bands were measured at the maximum of the laser pulse.

2A.5.4 power measurements

Power measurements were made with a Photodyne model 66XLS Power/Energy Meter (model 350, Si sampling head). Only unfocussed light was placed on the photodiode to avoid saturating the diode with the pulsed light or damaging the sampling head. Laser powers used in calculations were corrected for wavelength sensitivity of the power-metre according to the manufacturers calibration curve.

2A.5.5 techniques

The apparatus used for measuring fluorescence induced by chopped CW and pulsed lasers are shown schematically in Figs. 6 and 7 respectively. In Fig. 7 both pulsed laser systems are shown, only one of which was

used for each set of experiments. Fluorescence band profiles were measured by fixing the excitation wavelength and time gate and then scanning over the fluorescence band at a constant rate (usually $200 \text{ cm}^{-1} \text{ min}^{-1}$). Measurements were generally made with a minimum of laser excitation power in order to avoid photo-degradation (less than $20 \text{ } \mu\text{W}$ measured power). The power of the laser was reduced with neutral density filters.

The lifetime of the fluorescence bands were measured by using laser excitation with an energy at which the fluorescence produced was due to the species desired (or as close to this optimal condition as possible). At the same excitation wavelength to be used on the sample, but without the sample present, the laser pulse characteristics were first measured by setting the monochromator to the energy of the exciting line. Very narrow detection slits were used for the laser pulse measurement to avoid damaging or saturating the PMT as the it would be receiving maximum power at all times, and saturation of the PMT could cause non-linear effects. The laser emission intensity was measured on the x-y chart recorder as a function of gate timing (3 ns gate, measurements made at 1 ns intervals) over the whole pulse. Measurements for the sample fluorescence decay time were made by adjusting the monochromator to an energy at which fluorescence from the desired species was present. Then the intensity measurements were made as a function of time over the complete fluorescence band. Calculations of lifetime were made using a modified phase-plane method⁸⁸.

Simultaneous measurements of fluorescence and transmission were made using a sampling setup arrangement shown in Fig. 8. For these

measurements only a 1 mm quartz sample cell was used. A spherical lens was used to focus light at a point within the Chl a solution. The light collected by the power meter was unfocussed and as such represents only the averaged power over time, less the amount scattered by the quartz cell. Measurements were also made with the power meter in its 'integrating mode' but these did not result in any change in the transmission curves.

Fluorescence excitation profiles were measured with the incident light normal to the (1 cm X 1 cm) sample cell, and the light was collected at 90° to the incident laser beam. Minimum laser power levels were used (less than 20 μ W) to avoid sample degradation. This was monitored by measuring the maximum fluorescence before and after the complete scan and looking for changes in intensity. However, at these power levels no such changes were observed. Entire fluorescence bands were measured at 1 nm intervals in excitation, generally from $15,500\text{ cm}^{-1}$ to $14,000\text{ cm}^{-1}$. Power measurements were made at each excitation wavelength before and after the scans were made in order to detect any changes in laser power which might cause misleading power-dependent intensity measurements. Excitation profile calculations were made from the fluorescence bands recorded by measuring the fluorescence intensity at a specific wavelength, and normalizing this intensity for laser power at each excitation wavelength. The resulting value was then plotted as a function of laser excitation wavelength. These calculations were made for several positions over the fluorescence band. The purpose of making more than one calculation on each fluorescence profile was to ensure that the same behavior was

followed over the whole band. Deviation in behavior for measurements on different parts of the fluorescence band might indicate that more than one species was present because they would not (necessarily) follow the same excitation profiles or have degenerate origins. Vibronic bands should follow the same excitation profiles as their originating species.

Fluorescence excitation profiles were measured in a 'transmission' geometry with fluorescence collected at 90° to the incident laser beam direction rather than using the front reflection technique in order to avoid interference patterns observed in front reflection with 1-5 mm sample cells for dilute solutions (10^{-5} M or less). Even at these dilute concentrations, fluorescence re-absorption problems were significant. Interference patterns in some Raman excitation profiles⁹⁴ were attributed to non-linearities caused by significant population of the first excited singlet state as the absorbance maximum was approached, consecutive absorption from the populated excited states and changes in the refractive index of the sample at the absorbance maximum with bleaching of the ground state.

Raman excitation profiles were not measured with the same apparatus arrangement used for the fluorescence excitation profiles. The more highly concentrated samples (around $1-5 \times 10^{-4}$ M) necessary to generate Raman scattering with sufficiently low noise levels were also subject to re-absorption errors. Power levels of around 0.50 mW were necessary for good signal to noise ratios. Power and fluorescence intensity were measured before and after every scan in order to monitor laser and sample stability. For Raman measurements the front reflection technique was used, as shown in Fig. 8. A double-iris arrangement was

used to ensure reproducible placement of the laser beam on the sample cell. At each excitation wavelength measured, the Raman scattering bands of hexane (1435 cm^{-1}) and Chl a $\text{C}=\text{C}$ and $\text{C}=\text{N}$ (two mixed/degenerate bands at 1565 and 1545 cm^{-1}) were scanned consecutively at a rate of $10\text{ cm}^{-1}\text{min}^{-1}$. At each new wavelength, a different section of the sample cell was irradiated. The reproducibility of the Raman scattering detection alignment was tested by several measurements with the same excitation wavelength after moving the sample cell to a new area as was done when the wavelength was changed. No appreciable error was found by using this technique. Calculations of band intensity were made by integrating the area under the Raman bands several times to minimize error. The excitation profile was calculated by dividing the area of the Chl a band by the area of the hexane band for each excitation wavelength in order to normalize for focussed power. The hexane band area was used rather than the measured intensity (power meter) since the Raman band intensity of hexane behaves in a linear fashion with power and wavelength over the excitation range measured and is thus a reasonable standard. Since the bands due to the Chl a overlap, measurements were made on the area of the overlapping bands as well as with the two bands resolved from each other. The two bands were resolved by subtraction of the stronger (the non-overlapping side reflected and subtracted from the total band shape). Either method produced the same excitation profiles.

Figure 6

Apparatus for fluorescence measurements using chopped CW laser excitation.

Figure 7

Apparatus used for laser induced fluorescence and Raman spectroscopic measurements. Either the Nitrogen laser or Excimer laser sources were used as light sources. A common light emission detection system is shown.

Figure 8

Apparatus for measurement of simultaneous fluorescence and transmission power readings. Reflected and emitted light from the sample cell is collected at a 90° geometry for fluorescence excitation profile measurements. For Raman excitation profile measurements, a front reflection geometry is used.

CHAPTER III

A. MONOMER SOLUTIONS

3A.1 Absorption Spectrum of Monomeric Chl a Solutions

The room temperature absorption spectrum of a 10^{-7} M solution of Chl a in dried hexane is shown in Fig. 9. The hexane solvent for this solution was dried more rigorously than usual by passing it three times through a column of alumina (Woelm, activity 1) followed by storage over molecular sieves (3Å), in order to lower the water content of the solution to trace amounts. This solution has two main absorption bands at 659 and 426 nm. As well, weaker absorption bands on the high energy side of the main bands can also be seen (labelled η_1 and η_2). The increase in baseline towards the ultra-violet region is due to absorbance by an uncorrected solvent background.

3A.2 Fluorescence Spectrum of Monomeric Chl a Solutions

The pulsed laser induced fluorescence of a 10^{-7} M solution of Chl a in dried hexane is shown in Fig. 10. The band has a full width at half-height (FWHH) of 350 cm^{-1} and a maximum intensity at about 664 nm ($15,060 \text{ cm}^{-1}$). The fluorescence band maximum and bandwidth do not change with excitation varying between 415 and 459 nm.

3A.3 Fluorescence Excitation Profiles for Monomeric Chl a

In figures 11 and 12, the fluorescence excitation profiles for 10^{-7} M Chl a in hexane exhibit maxima at 660.6 nm and 426-427 nm.

Several different positions on the fluorescence bands were used to calculate the excitation profiles. For example, the profiles in Fig. 11 were calculated using intensities measured on the shoulder of the 664 nm emission (Fig. 10) at $14,800\text{ cm}^{-1}$ (675 nm) and $14,900\text{ cm}^{-1}$ (671 nm).

Figure 9

Absorption spectrum of 10^{-7} M Chl *a* in dried, deoxygenated hexane at room temperature. Nominal concentration of 2.7×10^{-7} M (using the extinction coefficient of blue band in ether). Solvent dried under nitrogen atmosphere by passing through alumina (Woelm, activity 1) three times, followed by storage over 3Å molecular sieves.

Figure 10

Fluorescence spectrum of a 10^{-7} M Chl *a* solution in dried hexane produced using pulsed laser excitation at 436 nm (fluorescence maximum invariant with excitation wavelength between 415 and 459 nm). The absorbance spectrum of this solution is shown in Fig. 9.

Figure 11

Fluorescence excitation profile in the Q_y region for a 10^{-7} M solution of Chl a in dried hexane detected on the shoulder of the 664 nm emission: 671 nm (O, upper curve) and 675 nm (□, lower curve). Absorption spectrum for the same solution is shown in Fig. 9).

Figure 12

Fluorescence excitation profile in the Soret region for a 10^{-7} M solution of Chl a in dried hexane. Absorption spectrum for the same solution is shown in Fig. 9. Measurements are made over the fluorescence band at 662 nm (○, upper curve), 666 nm (●, middle curve), and 671 nm (□, bottom curve). The apparent relative intensity increase at wavelengths below 415 nm is due to decreasing sensitivity of the power meter.

3A.4 Monomeric Solutions of Chl a in Wet Hexane

The spectral properties of a 10^{-7} M solution of Chl a in water-saturated hexane are as follows (spectra not shown): absorbance 670 nm and 413 nm; pulsed laser induced fluorescence at 673.4 nm (with an excitation wavelength of 436 nm), FWHH 171 cm^{-1} , fluorescence band maximum invariant with excitation wavelength over the range 410-459 nm; fluorescence excitation profile maxima at 670.1 nm and 413 nm (calculated from measurements made on the fluorescence band at 673.4 nm).

A different water-saturated hexane solution was prepared by diluting Chl a to 10^{-8} M, allowing this solution to equilibrate overnight, then concentrating the solution by evaporating some hexane with nitrogen flow until an optimal concentration of 10^{-6} M was reached. This solution exhibited absorption maxima at 409 and 667 nm with a blue:red band intensity ratio of 2.04:1 (spectrum not shown). Excitation spectra were not measured for this solution.

Absorbance and fluorescence spectra were measured in three additional solvents, pyridine, lutidine (2,6-dimethylpyridine) and collidine (2,4,6-trimethylpyridine) (spectra not shown). For these experiments, Chl a concentrations were 10^{-4} M. In these solvents the Chl a is entirely in the monomeric state due to the strong nucleophilic nature of the solvents. Laser excitation at 442 nm (Nitrogen laser) was used to produce Chl a fluorescence. Results were as follows: pyridine, absorbance maxima at 443 nm and 671 nm, fluorescence at 679 nm; lutidine, absorbance maxima at 433 nm and 666 nm, fluorescence at 676 nm; collidine, absorbance maxima at 434 and 667 nm, fluorescence at

676 nm. The FWHH of the three fluorescence bands were the same, 450 cm^{-1} .

3A.5 Fluorescence Lifetimes

Fluorescence lifetimes were not measured for the dry and water-saturated 10^{-7} M Chl a hexane solutions depicted in Figs. 9 and 14. The fluorescence spectra induced by pulsed laser excitation at 436 nm for a similar series of solutions, 10^{-6} M Chl a in dried hexane, intermediate water content (solvent opened only in a dry box under nitrogen atmosphere), and water-saturated hexane are shown in Fig. 13. The band maxima and lifetimes are summarized in Table 3. The fluorescence lifetimes of the Chl a species in dry hexane and water-saturated hexane are 6.2 and 6.7 ns, within the 0.5 ns error for this method of calculation.

Figure 13

Fluorescence profiles of 10^{-6} M Chl a in de-oxygenated hexane with varying amounts of water, (A) solvent dried (passed through alumina three times, (B) solvent undried, used as received and (C) water saturated. Pulsed laser excitation at 436 nm. Fluorescence lifetimes measured for these bands were (A) 6.2 ns, (B) 5.3 ns and (C) 6.7 ns.

Table 3 Fluorescence Band Positions and Lifetimes Observed
for a Solution of Monomeric 10^{-6} M Chl a

pulsed laser excitation wavelength (nm)	fluorescence detected for measurement (nm)	lifetime of fluorescence (ns) $\pm$ 0.5 ns	solvent
436	662	6.2	dried hexane
436	664	5.3	undried hexane
436	672	6.7	water-saturated hexane

3A.6 Absorbance Spectra of Octaethylporphyrin and Magnesium

Octaethylporphyrin

The results of preliminary measurements of the absorption spectra for 10^{-6} M octaethylporphyrin (OEP) and magnesium octaethylporphyrin (MgOEP) in dry and water-saturated hexane are given in Table 4. These measurements were made to determine the magnitude of the shift in band position between solutions with rigorously dried and water-saturated solvents for a chlorophyll analogue lacking in external-ring substituents. Details of the solvent drying procedure are given in section 2A.3.

After several hours in the water-saturated hexane, the MgOEP solution was observed to have changed by exhibiting new absorption bands. In particular, the absorbance band at 624 nm was observed to increase with time.

Table 4 Absorbance Band Maxima for Octaethylporphyrin (OEP) and Magnesium Octaethylporphyrin (MgOEP)

	absorbance maxima [*] dried hexane (nm)		absorbance maxima [*] water-saturated hexane (nm)	
	Soret	Q	Soret	Q
OEP	402	492 (0,1) 526 (0,0) 568 (0,1) 624 (0,0)	402	492 (0,1) 526 (0,0) 568 (0,1) 624 (0,0)
MgOEP	402	540 (0,1) 576 (0,0)	403	542 (0,1) 578 (0,0)

* HP Spectrophotometer

3A.7 Discussion: Monomer Solutions

In the figures depicting the dilute Chl a solutions (Figs. 9-12), spectroscopic evidence suggests that only one species is observed. The Chl a species with absorbance maxima at 426 nm and 659 nm have been characterized by other authors⁹. The main red absorption band of a monomeric penta-coordinated Chl a species at 659 nm has been assigned as the Q_y Stoke's shifted (0,0) transition since the Q_y oscillator strength of this transition is calculated to be about five times larger than the Q_x oscillator strength⁹. The Q_x absorbance band is located at a higher energy than the Q_y band and is difficult to resolve because it overlaps with the vibronic band of the $Q_y(0,1)$ transition. The Soret band at 426 nm has vibronic bands on the high energy side usually labelled as η_1 and η_2 . Recently, η_1 has been identified as a real electronic level situated at 409 nm from resonance excitation profiles in the Soret region⁸⁹.

The observed fluorescence maxima at 664 nm for this solution can be attributed to the same species observed with absorbance maxima⁵⁷ at 659 nm and 426 nm. The pulsed laser induced fluorescence excitation spectra for the 10^{-7} M solution of Chl a has: one maximum at about 661 nm observed with intensities measured at two positions across the fluorescence band with laser excitation in the Q_y region; and one maximum at about 426.5 nm observed with intensities measured at three positions across the fluorescence band with excitation in the Soret region. The coincidence of these two sets of maxima measured at five wavelength positions suggests strongly that the fluorescence band is produced by only one Chl a species. If another Chl a species was

present in the solution, this would be indicated by the presence of extra band maxima on the excitation profiles. Absorbance methods are able to monitor only the bulk species in solution, whereas the use of wavelength selective excitation techniques in conjunction with fluorescence detection can be used to discriminate between species which may have similar absorbance properties but different fluorescence properties. For instance, if two species were present in the solution, the absorbance properties of one might 'mask' those of the other because of differences in extinction coefficient even if the second species had significantly different absorbance maxima. The emission properties of these species would possibly be different, and thus monitoring the fluorescence excitation profile at different wavelengths on the fluorescence band could result in more than one maxima on the excitation profile. Narrow-band excitation can more easily resolve minority species because the absorbance of each species will vary with wavelength and minority species can be selectively resolved in the presence of greater amounts of other species which have slightly different spectroscopic properties. Absorbance spectroscopy (using broad band excitation) is lacking in this respect.

The rigorous drying of the Chl a and the solvent for the 10^{-7} M solutions should have minimized the water content of the Chl a solutions to approach the lower limit of one water molecule per Chl a molecule⁸⁵. Solvent drying procedures cannot lower the water below 10^{-7} M. Removal of the remaining water was not attempted because of chlorophyll instability when the magnesium is tetra-coordinate, and because the dryness of such a tetra-coordinate magnesium complex cannot be

maintained^{41,85}. As well, adventitious nucleophiles in the solvent not removed by the drying or purification procedures would coordinate with the magnesium immediately upon solution preparation. The driest solutions of 10^{-7} M Chl a used for this study must predominantly contain a bulk species consisting of Chl a with a penta-coordinated magnesium atom having water as the fifth ligand. The presence of water molecules hydrogen-bonded to other electronic sites on the macrocycle or side chains is unlikely in this solution due to the low water content and because water present in the solution will most likely bond to more labile coordinate-unsaturated sites (i.e. the magnesium). The spectral contributions from other species present are likely to be minimal. Solvation shifts would be minor because the hydrocarbon solvent, hexane, has no functional groups which can actively bond to the chlorophyll.

The effect of different solvents on Chl a was investigated using the solvent series pyridine, lutidine and collidine in order to simulate the small environmental changes associated with different ligands, such as might be encountered when Chl a forms aggregates. These three solvents are sufficiently polar to cause the Chl a to exist only in the monomeric state when dissolved⁴⁴. Because the molecules are strongly nucleophilic, it is expected that they will primarily attach to only the metal atom rather than any of the other peripheral sites such as the ring V carbonyl group. The different solvents each have characteristic Chl a spectra.

Solid state X-ray crystallographic studies have shown that some metalloporphyrins have additional ligands strongly bonded to the metal atom. In the case of FeTPP, the metal to ligand (water) bond is shorter

in length than metal to pyrrole nitrogen lengths²⁶. MgPc also crystallizes with one ligand (water) attached to the metal atom even in the presence of stronger nucleophilic species (pyridine) which associate with functional groups on the ring periphery rather than the metal²⁹. The magnesium in EChlide remains out-of-plane with the rest of the macrocycle²² cm^{-1} with one water molecule situated above the magnesium. No additional stabilization apparently results from two ligands directly bonded to the metal, one on either side of the macrocycle.

The observed optical transitions for metalloporphyrin molecules like Chl a are usually considered to be characteristic $\pi \rightarrow \pi^*$ porphyrin ring transitions. The simple empirical electronic methods which qualitatively predict metalloporphyrin spectra do not usually include the effect of the metal atom. The presence of different metals in the porphyrin ring core, and extra stabilizing molecules (whether solvent or impurity molecule) ligated on the metal may change the electronic properties.

The absorbance and fluorescence properties of Chl a in lutidine and collidine are similar, but in pyridine the spectroscopic behaviour is very different (Section 3A.4). In a solution of pyridine, Chl a forms a penta-coordinated or hexa-coordinated^{8,11} species with one or two pyridine molecules. The hexa-coordinate species is present in about 1% of the quantity⁵² of the penta-coordinate species. Under the same conditions where hexa-coordination might occur, the Chl a molecule may be unstable and lose the metal atom to form pheophytin. The band maxima observed for the Chl a in pyridine (absorbance at 443 nm and 671 nm, fluorescence at 679 nm) are typical of penta-coordinated Chl a. The

hexa-coordinated pyridinated Chl a species has a red-shifted Q_y absorbance band (about 680 nm) which is seen only under very forcing conditions. Band positions for Chl a in collidine (absorbance at 434 nm and 667 nm, fluorescence at 676 nm) result in energy levels higher than those with pyridine as solvent. Band positions in lutidine (absorbance at 433 nm and 666 nm, fluorescence at 676 nm) are at an even higher energy. These results suggest that the three solvent molecules cause the energy level observed in emission to be stabilized in the order pyridine > collidine > lutidine. Although both lutidine and collidine would seem to have the same steric hindrance to approaching the magnesium on the porphyrin ring, the collidine is able to stabilize the ring more than the lutidine. The presence of an extra methyl group on the collidine not present on the lutidine results in an alteration in electron donating ability sufficient to cause small changes in energy level positions.

The energy level stabilization provided by one pyridine molecule bonding to the magnesium atom is much larger than that provided by either lutidine or collidine. The addition of two or three methyl groups onto the resonance ring of pyridine to form these other solvents seriously changes the magnitude of interaction possible with each ligand. No evidence suggests that lutidine and collidine form hexa-coordinated Chl a species with these solvents. The similarity in bandwidth for the fluorescence bands in the three solvents also suggests that only one species is present. The differences in bonding interaction are expected to be important for predicting spectroscopic band positions of different aggregate species.

The model compounds OEP and MgOEP (Fig. 2) are useful in identifying the magnitude of absorption band position shifts accompanying changes from dry to water-saturated hexane. The positions of the absorption bands for OEP and MgOEP are given in Table 4. For OEP neither of the Q bands (resolved into Q_x and Q_y components since the molecule has D_{4h} symmetry) nor the Soret bands shift with change in solvent. As there are no peripheral electrophilic groups on the OEP molecule, the water added to the solvent has no location to strongly hydrogen-bond and disrupt electronic structure. The porphyrin ring has excess electron charge distributed above and below the ring (resonance effect), but the aliphatic ethyl groups at the C_b ring positions (Fig. 2) are isolated from ring resonance by their distant location. Any hydrogen bonding on the aliphatic groups at these positions thus has minimal effect on porphyrin ring resonance structure.

The absorption bands of MgOEP all undergo a 1-2 nm shift accompanying the change from dry to water-saturated hexane solvent. The 2 nm shift must be due to changes in the porphyrin ring caused by the the metal-ion. Since MgOEP is penta-coordinated in dry solvents for the same reasons as Chl a (instability of the magnesium in the porphyrin ring when tetra-coordinate), the 2 nm shift in band position observed in the water-saturated solvent may be due to the addition of a sixth ligand (water) onto the metal. The new bands appearing in the spectrum of the MgOEP wet-hexane solution as the solution is aged are degenerate in band position with OEP in dry or water-saturated hexane. This alteration seems to indicate that the MgOEP is slowly demetallated in the water-saturated solvent to produce OEP (the Chl a equivalent to

pheophytin). This process may be undetected but present in solutions where the water content is reduced to a minimum. Other physical changes in the MgOEP ring periphery may also be induced.

In the 10^{-7} M Chl a solution with water-saturated hexane only one species was observed in both absorption and fluorescence, using experimental techniques analogous to those used for the dried hexane solution. This one species has the following properties: absorbance maxima at 413 nm and 670 nm, fluorescence maximum at 673.4 nm, and fluorescence excitation profile maxima in the red and blue regions at about 670 nm and 413 nm. The Q_y absorption band (taken as the strongest red absorption) and the fluorescence band were observed in this study to undergo 11 nm (249 cm^{-1}) and 9.4 nm (213 cm^{-1}) shifts to higher energy, when the solvent of the dilute monomeric solution was changed from dry to water-saturated. The magnitude of this red-shift is significantly larger than observed for MgOEP undergoing a similar solvent change.

In the absorption spectra of water-saturated Chl a solutions, the Q_y band is red-shifted and the Soret band is blue-shifted relative to band positions from the spectra of dry hexane solutions at similar 10^{-7} M concentrations. The Q_y red-shift may be attributed to association of impurity molecules (water) with electrophilic sites on the Chl a molecule which are absent on the OEP/MgOEP skeleton. Alternatively, the change in electronic properties may be due to a physical alteration of the Chl a caused by the presence of water molecule impurities. In water-saturated hexane (about 5.6% water, the amount present in a hexane water azeotrope⁹⁰, the nucleophile content is always far larger than the Chl a concentration. Under these conditions maximum ligation

and association by the water molecules are liable to occur, and physical alterations of the chlorophyll to liberate magnesium or removal of the phytyl group become possible⁵¹.

A second water-saturated solution prepared by concentrating a 10^{-8} M Chl a solution to 10^{-6} M was found to have absorption maxima at 409 nm and 667 nm, with a blue:red band intensity ratio of 2.04. Pheophytin a and Pheophorbide a both have absorption band positions at these same locations with band ratios of 2.09^{51,78}. The spectra observed for the 10^{-7} M (Fig. 13) and 10^{-6} M Chl a water-saturated hexane solutions may be identified as pheophytin by band position, band ratio, and by the apparent cause of the chemical alteration of the Chl a to pheophytin⁹⁹. Demetallation may have proceeded in the dilute solution due to the excess water acting as a weak acid. It is unlikely that the species observed is pheophorbide because its formation from Chl a requires either enzymatic hydrolysis or the action of a strong acid⁷⁸, both of which were absent for these experiments.

The assignment of Shipman et al.⁹ for $Q_y(0,0)$ origins at 659 nm to a penta-coordinated Chl a species agrees with the observations made in this work for dilute dry-hexane solutions. It is important to note that Shipmans band positions and the results observed here are identical despite Shipmans method involving the deconvolution of absorption bands for spectra of 10^{-5} M solutions (Chl a begins to aggregate even at these concentrations in dry solutions). The work in this report involved solutions that were either one or two orders of magnitude more dilute, approaching the detection limit of the absorbance spectrometer in terms of accurate band positions and noise levels.

The measured fluorescence lifetime of the penta-coordinated Chl a monomer species in dry hexane was found to have a fluorescence lifetime of 6.2 ± 0.5 ns (Table 3). This is within the range of lifetime values reported in the literature for Chl a monomers in other solvents^{39,54}. The 6.7 ± 0.5 ns lifetime measured for the Chl a in a water-saturated hexane solution is in agreement with values reported for Pheo a⁹¹.

For Chl a in undried hexane, the 5.3 ± 0.5 ns lifetime measured is more difficult to explain, and the 2 nm band shift in the position of the fluorescence from that of Chl a in a dry hexane solution (Fig. 13) is smaller than the reported 10 nm shift observed with change from penta- to hexa-coordination³⁹. A shortening in lifetime accompanying change from penta- to hexa-coordination has been observed for BChl a⁵⁴. For solvents where BChl a is penta-coordinated (wet toluene, hexane, CHCl_3 , and CCl_4), the fluorescence lifetime from the lowest singlet is between 3.1 and 3.2 ns. This lifetime decreases to between 2.6 and 2.9 ns in solvents where the BChl a is hexa-coordinated (methanol, ethanol, 1-propanol). Decreases in fluorescence lifetime have been noted in solvents with heavy atoms (CHCl_3 , CCl_4) possibly due to increases in intersystem crossing. This effect is smaller in magnitude than the lifetime decrease observed accompanying change from penta- to hexa-coordination in chlorophyll species. Connolly⁵⁴ has also observed that in solvents like alcohols the fluorescence lifetimes are smaller. Alcohol solvents produce a shorter lifetime than hydrocarbon solvents for BPheo a (BChl a with the magnesium removed) providing additional evidence of hydrogen bonding interactions that shorten the fluorescence lifetime. At present, there is insufficient evidence to attribute the

decrease in Chl a lifetime from 6.2 ns to 5.3 ns to either bi-ligation or association alone.

3A.7 Energy Level Diagram of Monomeric Chlorophyll a

An approximate energy level diagram for monomeric Chl a assembled from the data found in absorption and fluorescence spectra and fluorescence excitation profiles is shown in Figure 14.

The position of the lowest singlet energy level S_1 is located at approximately 661 nm ($15,137\text{ cm}^{-1}$), the average of the absorption maximum at 659 nm ($15,174\text{ cm}^{-1}$) and fluorescence band maximum at 664 nm ($15,060\text{ cm}^{-1}$). A certain error is intrinsic to this calculation because both of these optical transitions are Stokes shifted (i.e. (0,1) and (1,0) rather than (0,0) transitions), and thus the energies of absorption and fluorescence are not equivalent. The size of the error will depend on differences between excited state vibrations and ground state vibrations. The location of the maximum in the fluorescence excitation profile for the monomeric Chl a at 660.6 nm, which also indicates an approximate maximum in the absorption, does not change the position of the energy level to a large degree.

The position of the Soret band (S_n) at 426 nm ($23,474\text{ cm}^{-1}$) is an approximate location taken from absorption spectra. An absorption maximum was observed in the fluorescence excitation profile of the monomer at $426.5 \pm 0.5\text{ nm}$ ($23,446\text{ cm}^{-1}$) which is in fair agreement with this value for the Soret band.

The Chl a is apparently stabilized by strongly metal-bonding ligands such as pyridine, as seen by the large shift to lower energy of absorption and fluorescence bands. A smaller stabilization is seen with

more weakly bonding, or sterically hindered ligands like collidine and lutidine. The effect of ligation onto peripheral functional groups can be seen with Chl a in solutions where small excesses of nucleophilic water species are present (undried solvent solutions). In this case, the excess nucleophile is not sufficient to cause hexa-coordination and subsequent removal of the magnesium from the chlorophyll to form pheophytin, but is sufficient to cause small energy stabilizations and small changes in fluorescence lifetimes. It is expected that the interactions on peripheral sites will be important in determining the spectroscopic behaviour of Chl a aggregates. Additional spectroscopic techniques will be of benefit in resolving these changes.

Figure 14

Energy level diagram of monomeric Chl *a* as derived from absorbance, fluorescence and fluorescence excitation profile measurements for Chl *a* in hexane.

B. SOLUTIONS OF HIGHER CONCENTRATION

3B. Absorption Spectrum of Concentrated Chl a Solutions

A series of four room-temperature absorption spectra for hexane solutions over the concentration range of 10^{-7} -- 10^{-4} M Chl a are shown in Fig. 15 A-D. The Chl a for these solutions was dried by codistillation in the usual way (see section 2A.3). Hexane was used either undried from freshly opened bottles or dried by passing through alumina. All solvents were degassed.

Figure 15A (same spectrum as Fig. 9, included here for reference) is the absorption spectrum for a 10^{-7} M Chl a solution in dried hexane, with maxima in the Soret region at 426 nm and in the Q_y region at 659 nm. The result of an increase in concentration to 10^{-6} M (undried hexane) can be seen in Fig. 15B, with Soret band found at 428 nm and Q_y band at 659 nm with a poorly resolved band on the shoulder of the Q_y band at lower energy. After a further increase in Chl a concentration to about 5×10^{-6} M (Fig. 15C), the Soret band maintains the same position, whereas the Q_y band shifts to 664 nm and the shoulder at 672 nm increases in intensity. At Chl a concentrations between 10^{-5} and 10^{-4} M in dried hexane (Fig. 15D) the absorption reaches a limiting spectrum with the Soret band positioned at about 430 nm, a weak shoulder to lower energy at about 450 nm⁹², and Q_y bands at about 664 nm and 672 nm (not deconvoluted). At higher concentrations band positions remain constant but broadening is observed.

Figure 15

Room-temperature absorption spectra for solutions of Chl a_6 in hexane at concentrations of (A) 10^{-6} M (dried hexane), (B) 10^{-6} M (undried hexane), (C) 5×10^{-6} M (dried hexane) and (D) 10^{-4} M (dried hexane). Spectra (A) and (C) recorded on the HP 8450 A UV/VIS Spectrophotometer, (B) and (D) on the Varian DMS-90 Spectrophotometer.

3B.2 Pulsed Laser and CW Induced Fluorescence Spectra

Figure 16 shows the pulsed-laser induced fluorescence profiles from a 5×10^{-6} M Chl a hexane solution (absorption spectrum of the same solution in Fig. 15C). With excitation at 422 nm, a fluorescence band at 664 nm is observed. As the excitation wavelength is shifted across the Soret absorption maximum towards the red, the 664 nm band shifts to lower energy. Concurrently, a band at about 685 nm increases in intensity reaching a maximum with excitation at 457 nm where it is relatively isolated from the 664 nm band. As the excitation wavelength is moved to lower energy, the main bands and vibronic bands shift to lower energy.

Figures 17-20 show the chopped CW laser-induced fluorescence spectra of Chl a solutions with concentrations ranging from 10^{-5} M (Fig. 17) and 2×10^{-4} M (Fig. 18) to 10^{-3} M (Fig. 19 and 20). The CW induced spectra of Fig. 17 resemble the pulsed-laser induced spectra of Fig. 16 showing the positions of three unresolved fluorescence bands. However for the 10^{-5} M solution, the fluorescence profiles show much broader bands shifted to lower energy (red-shifted) and situated at 668 nm, 671 nm and 687 nm. With pulsed-laser induced excitation at 457 nm (Fig. 16) and CW excitation at 457.9 nm (Fig. 17), the emission bands are located at approximately the same position, and both show a shoulder situated at lower energy.

In Fig. 18, a weak band is seen on the high energy side of the 672 nm fluorescence band with most excitation wavelengths. The 672 nm band observed with 514.5 nm excitation, is not observed with excitation closer to the Soret maximum absorption. A broad unresolved fluorescence

Figure 16

Typical fluorescence spectra for a solution of $3 \times 10^{-6} \text{ M}$ Chl *a* in dry hexane, pulsed laser excitation wavelengths are to the right of each spectrum. Incident laser power on the sample was maintained at about 0.020 mW for all excitation wavelengths with a very defocussed beam. Numbers to the left of each spectrum are intensity multipliers to scale the spectra to equivalent laser power.

Figure 17

Fluorescence spectra for a solution of 10^{-5} M Chl a in dry hexane produced with chopped CW Ar⁺ laser, excitation wavelength to the right of each spectrum.

Figure 18

Fluorescence spectra for a solution of 2×10^{-4} M Chl *a* in dry hexane produced with chopped CW Ar⁺ laser, excitation wavelength to the right of each spectrum.

Figure 19

Fluorescence spectra for a solution of 10^{-3} M Chl *a* in dry hexane produced with chopped CW Ar^+ laser, excitation wavelength to the right of each spectrum. (Solid Chl *a* dried prior to solution preparation by codistillation with dried benzene).

Figure 20

Fluorescence spectra for a solution of 10^{-3} M Chl a in dry hexane produced with chopped CW Ar⁺ laser, excitation wavelength to the right of each spectrum.

band at 690 nm is visible with all excitation wavelengths and is best isolated with excitation at 454.5 nm. In the vibronic region, the maximum at about 720 nm shifts to about 745 nm at the same time as the 672 nm band decreases in intensity and the intensity of the vibronic band decreases (relative to the strongest fluorescence).

Figures 19 and 20 show similar fluorescence spectra for highly concentrated Chl a solutions prepared by different drying procedures (solid Chl a codistilled with benzene (Fig. 19) or CCl₄ (Fig. 20)). With excitation at 454.5 nm, the main emission bands are symmetric and broad, the maximum situated at 692 nm (Fig. 19) or 690 nm (Fig. 20). With change in excitation away from the Soret maximum, the emission undergoes a continuous shift (rather than unique bands appearing) towards lower energy producing a very broad unresolved band series. In Fig. 19, weaker emission in the 675 nm region is present with some excitation wavelengths.

3B.3 Fluorescence Lifetimes for Aggregated Chl a

Fluorescence lifetimes were measured for the 5×10^{-6} M Chl a hexane solution shown in Fig. 9 and Fig. 15C using excitation wavelengths with fluorescence bands isolated as much as possible. These results are summarized in Table 5.

3D.4 Fluorescence Excitation Profiles

Fluorescence excitation profiles (Figs. 21A and 21B) were measured across the Soret region for a 5×10^{-6} M Chl a solution in dry hexane (absorption spectrum of same solution Fig. 15C and fluorescence Fig. 16). Fluorescence intensities were measured at two positions, $15,060 \text{ cm}^{-1}$ (664 nm) and $14,600 \text{ cm}^{-1}$ (685 nm). Intensities were normalized for variations in laser power at each excitation wavelength.

The profile measured at 664 nm (Fig. 21A) reaches a maximum at an excitation wavelength of about 427 nm. In Fig. 21B, the profile measured at 685 nm reaches a maximum intensity with excitation energy of about 440 ± 5 nm.

Figure 21

Fluorescence excitation profiles for a solution of 5×10^{-6} M Chl $\bar{a}$ in dry hexane measured at fluorescence wavelengths of 664 nm in (A) and 685 nm in (B) (same solution, absorption spectrum Fig. 15C). The pulsed laser induced fluorescence profiles (same shown Fig. 16) were recorded at 1 nm excitation wavelength intervals and were normalized for variations in laser power.

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Table 5 Fluorescence Lifetimes Observed for a 10^{-6} M Chl a Solution

pulsed laser excitation wavelength (nm)	fluorescence detected for measurement (nm)	lifetimes of fluorescence (ns) $\pm$ 0.5 ns
420	664	6.8
436	664 *	3.3
	685 *	4.7
459	685	5.9

* fluorescence bands at these wavelengths were not deconvoluted for the intensity measurements, thus lifetimes do not necessarily represent the most accurate value for the underlying band

Figure 21

Fluorescence excitation profiles for a solution of 5×10^{-6} M Chl a in dry hexane measured at fluorescence wavelengths of 664 nm in (A) and 685 nm in (B) (same solution, absorption spectrum Fig. 15C). The pulsed laser induced fluorescence profiles (some shown Fig. 16) were recorded at 1 nm excitation wavelength intervals and were normalized for variations in laser power.

3B.5 Two-photon Experiments

The two-photon induced blue fluorescence from a 2×10^{-4} M Chl a solution in undried hexane is shown in Fig. 22. The fluorescence maximum is observed at about 455 nm with a FWHH of about 45 nm. The contour of the fluorescence band does not change when the laser excitation is varied between 662 nm and 690 nm. Within the spectral resolution of this experiment, the fluorescence reaches a maximum intensity with red laser excitation which is in resonance with the Q_y ($S_1 \leftarrow S_0$) absorption band system of the Chl a (inset Fig. 22).

3B.6 Raman Scattering Measurements

Raman scattering bands induced by pulsed laser were recorded for the 1565 cm^{-1} and 1545 cm^{-1} $\text{C}=\text{C}/\text{C}=\text{N}$ stretching modes of Chl a using the intensity of the solvent Raman shift at 1440 cm^{-1} as an internal standard. The fluorescence characterizing this solution is shown in Fig. 23A (436 nm excitation) along with a typical Raman band profile in Fig. 23B (excitation 447 nm) illustrating the noise level found using laser power levels of about 0.50 mW.

Figure 24 shows the Raman excitation profile of the 1565 cm^{-1} band for the same solution shown in Fig. 23. A similar excitation profile (shape, maxima locations) was found whether the intensities of 1565 cm^{-1} or 1545 cm^{-1} bands, areas of the two bands together, or areas of the individual bands (deconvoluted) were used for this calculation. The Raman excitation profile has two maxima at 435.2 nm and 448.2 nm.

Figure 22

Consecutive two-photon-induced fluorescence spectra for a $2 \times 10^{-4} \text{ M}$ solution of Chl a in undried hexane taken in front reflection geometry. Pulsed laser excitation wavelengths are indicated to the left of each spectra. Inset shows the absorbance spectra of the solution (solid line) and the excitation wavelength positions and relative intensities of the observed fluorescence (dotted line).

Figure 23

(A) Fluorescence spectra of a 10^{-4} M solution of Chl *a* in dry hexane, pulsed laser excitation at 436 nm.
 (B) Raman scattering induced by pulsed laser excitation at 447.0 nm. Energy shifts ($\Delta\nu$) from the excitation line are given, C=C/C=N vibrations of Chl *a* are indicated at 1565 and 1545 cm^{-1} , hexane band at 1440 cm^{-1} .

Figure 24

Excitation profile of the 1565 cm^{-1} Raman band from a $1 \times 10^{-4}\text{ M}$ solution of Chl a in dry hexane produced using pulsed laser excitation at 0.5 to 1 nm intervals. Raman band area was normalized for laser power using the hexane band at 1440 cm^{-1} . Fluorescence and typical Raman spectra for this solution are shown in Figure 23.

The fluorescence from a 5×10^{-4} M solution of Chl a in dry hexane with pulsed laser excitation at 436 nm is shown in Fig. 25A. Figure 25B shows typical Raman bands recorded for this solution (excitation 433.7 nm). The fluorescence spectra differs from that of Fig. 23 by having additional intensity in the 685 nm region. The Raman excitation profile for solutions like this 5×10^{-4} M Chl a solution are similar to that observed by de Wilton et al.^{93,94}, shown in Fig. 26. The Raman band intensity for Fig. 26 was normalized for variations in laser power against the 2900 cm^{-1} band of hexane (rather than the 1440 cm^{-1} band as used for Fig. 24). In Fig. 26 the Raman excitation profiles measured for Chl a vibrational bands at 1565 cm^{-1} and 1189 cm^{-1} both have maximum intensity at about 434 nm with two areas of sudden decrease in intensity at 435.4 nm and 434.1 nm. Raman band intensity measured was linear with laser power⁷⁹ whether measured at the maximum or in the 'holes' of the profile.

Figure 25

(A) Fluorescence spectra of a $5 \times 10^{-4} \text{ M}$ solution of Chl *a* in dry hexane, pulsed laser excitation at 436 nm.

(B) Raman scattering induced by pulsed laser excitation at 433.7 nm. Energy shifts (Δ) from the excitation line are given, C=C/C=N vibrations of Chl *a* are indicated at 1565 and 1545 cm^{-1} , hexane band at 1440 cm^{-1} .

Figure 26

Excitation profile of the 1565 and 1189 cm⁻¹ Raman bands of a 3×10^{-4} M solution of in dry hexane, pulsed laser excitation. Raman band areas normalized for laser power using the 2900 cm⁻¹ band of hexane. This picture is copied with permission of the authors (ref-94).

3B.7 Discussion: Aggregates of Chl a

The electronic properties of Chl a in solutions with concentrations greater than 10^{-6} M in hexane cannot be attributed to monomeric Chl a species alone. With increasing concentration, the position of new absorption and fluorescence bands, band widths at half height, and fluorescence lifetimes differ from the monomer. Extensive evidence reported in the literature (NMR⁶², IR^{49,70,71}, optical CD⁶¹ and fluorescence^{62,76,93-99}), provides indications that equilibrium mixtures of dimers and higher oligomers are responsible in part for these changes and that the size of the aggregates in non-polar solvents is highly variable.

The spectra of Fig. 15 depict changes in the position of absorption bands with increasing chlorophyll concentration. The species depicted in Fig. 15A with absorbance maxima at 426 nm and 659 nm was assigned to a penta-coordinated monomer (section 3.A7). At Chl a concentrations greater than 10^{-7} M, the absorption bands shift to lower energy and broaden. Large changes in Chl a concentration result in only small changes in the absorption spectra^{9,62,96}. The Soret band is seen to shift from 426-->428-->430 nm and the Q_y band from 659-->664 nm with the appearance of another band on the shoulder of the 664 nm band at about 672 nm. The four vibronic bands of the Q_y transition remain distinctly visible and do not shift at the concentrations used. The first vibronic of the Soret band, η_1 , maintains approximately the same height ratio to the Soret band with the changes in concentration (about 1.4:1). The red-shift and band broadening with increasing concentration are an effect of aggregation where π -interaction is a predominant

association method. According to Renge and Awaarma¹⁰⁰ all four absorption spectra of Figs. 15A-D can be classified as L_1 -type, or characteristic of Chl a with the magnesium penta-coordinated with a weakly coordinating ligand such as water. These type of spectra are not characteristic of solvents which are able to strongly hydrogen bond (such as alcohols) where an (apparent) dampening of the vibronic bands in the red region results.

Intepretation of the fluorescence spectra produced from solutions of Chl a at concentrations greater than 10^{-5} M may be incorrect unless the distortions caused by self-absorption are recognized. This interference is difficult if not impossible to eliminate or minimize for solutions of high concentration because of restrictions imposed by sample cell and detection geometries. Fluorescence profiles of the types contained in Figs. 16-20 must be examined along with the absorption spectrum of the corresponding solution. As the concentration of the Chl a is increased, the absorption bands shift and broaden and cause the emitted light to be reabsorbed. This problem is in part due to the small Stokes shift between the absorbed and emitted light. The fluorescence is reduced in intensity before it leaves the sample cell by scattering and reabsorption of the emitted light by other groundstate molecules within the solution. Reabsorbtion becomes severe in solutions of high concentration and can result in the observation of almost any fluorescence spectrum caused by inaccurate positioning of the sample¹⁰¹. Reabsorption is judged to be a severe problem in the 10^{-5} M solutions of Figs. 19 and 20 even though front reflection irradiation/emission collection geometry was used.

In the fluorescence spectra of Figs. 16-20, the $Q_{x,y}$ regions show larger changes with increasing Chl a concentration than the corresponding absorption spectra. In Fig. 16, fluorescence bands are resolved with selective excitation. For 10^{-7} M Chl a monomeric solutions (Fig. 10) no change in fluorescence maximum (662 nm or 662 nm) was seen with change in excitation wavelength over the range 415 nm to 459 nm. However, new bands are observed with change in excitation wavelength for the 5×10^{-6} M solution of Fig. 16. As well, for this solution with pulsed laser excitation at 422 nm a fluorescence lifetime of 6.8 ± 0.5 ns is observed for the 662 nm band. This lifetime is within error the the same as the 6.2 ns lifetime observed for the 662 nm fluorescence from the 10^{-6} M solution with excitation at 436 nm (Fig. 13). The 10^{-6} M solution in Fig. 13 is clearly different from that of the 5×10^{-6} M solution in Fig. 16 for similar excitation wavelengths. The shift in monomer band position from 662 nm to 664 nm may be in part due to the effect of extra ligation. The species observed in Fig. 16 may be (mistakenly) assigned to the monomeric penta-coordinated Chl a species since the lifetimes are the same within the experimental error of ± 0.5 ns and the band positions are similar. Alternatively, the emission band may be due to association of a Chl a molecule with a molecule other than water, such as another Chl a molecule.

In the 5×10^{-6} M Chl a solution, the presence of low n-number aggregates is indicated by changes in absorption, fluorescence, and fluorescence lifetimes. The properties observed for this solution do not duplicate those of more dilute monomeric solutions. With efficient Chl a drying techniques and increasing the concentration of solutions

prepared, the metallo-porphyrin may be forced to aggregate in order to meet coordination requirements of some unhydrated molecules. Aggregation may also result from collisions between molecules in solutions of high concentration. These 'aggregates' need only exist for the duration of the measurement, or have an equilibrium of reasonable magnitude in order to be detected. Since the fluorescence measurements are of ns duration, observed species must be present for this sort of time.

The presence of more than one Chl a species in the fluorescence profiles observed in Fig. 16 are indicated by the variations in the fluorescence lifetime with different excitation wavelengths. Lifetimes measured for overlapping bands are not accurate for making single, unique assignments. This can best be accomplished in circumstances where only one species is present. These interferences did not allow lifetime measurements (other than for monomeric Chl a) to be used in resolving Chl a species in solutions of mixed species.

As the lowest aggregate unit, the dimer is seen as the building block for all higher aggregates. A donor/acceptor type of Chl a dimer would consist of two electronically non-identical molecules, the donor molecule bonding through a peripheral functional group (such as the ring V ketone) to the magnesium of an acceptor molecule (Fig. 27). The constituent monomers of the donor/acceptor complex should have absorption and fluorescence properties which differ from the free monomers and from each other. The donor molecule would still likely have a magnesium-bound water molecule to stabilize the metal ion in the planar porphyrin ring, but cannot be directly compared to the

Figure 27

Simple model of a donor/acceptor type of Chl a dimer. The donor molecule (left) is bonding through a peripheral functional group to the magnesium of the acceptor molecule (right).

penta-coordinated monohydrate monomer because the keto (and/or) acetyl group of the donor is donating charge to the magnesium atom of the acceptor molecule. The acceptor molecule may or may not retain a metal-bonded water molecule since necessary stabilization energy is provided by the other charge-donating Chl a molecule.

The interaction of a donor/acceptor dimer should have only a minimal effect on electronic properties³³ where the peripheral bonding sites are not strongly involved in ring conjugation. The difference in size of associated molecule (water vs. Chl a) should not produce large change in electronic properties of the acceptor molecule because the amount of charge donation possible from the ring V functional groups is limited by the minor participation of these functional groups with the π -macrocycle of the same molecule. Ring V is almost coplanar with the porphyrin macrocycle²² which would tend to permit charge delocalization from the macrocycle. However, the C-C bond lengths of this ring are sufficiently large²² to restrict charge delocalization from the porphyrin ring to functional groups off the C₉ and C₁₀ positions and thus the charge donating ability of the donor may not differ extensively from a water molecule. The donor molecule would be more different from the monomer because of charge donation to the magnesium of the other Chl a molecule. The donor would probably have spectroscopic properties which differed considerably from the acceptor molecule despite the weak bonding by these functional groups because the conjugation sphere is more directly influenced.

Based on the prediction of different spectroscopic properties for acceptor and donor species from the penta-coordinated Chl a monomer, the

Q_y emission at 664 nm is assigned to the 'acceptor' molecule with absorbance maximum at about 664 nm. The Q_y emission at 685 nm is assigned to a 'donor' species with absorbance maximum at about 672 nm (the absorbance bands of Fig. 15D have not been deconvoluted because of errors associated with these calculations¹²). Further analysis of fluorescence excitation spectra and two-photon induced blue emission leads to the conclusion that the emissions at 664 nm and 685 nm are correlated and represent a single aggregated dimer species.

Other measurements have been made to characterize the $5 \times 10^{-6} \text{ M}$ Chl a solutions of Figs. 15 and 16 which indicate additional optical parameters for the donor/acceptor dimer. These solutions may contain the monomer as well as aggregated forms of Chl a. Fluorescence excitation profiles for the Soret region ($S_1 \rightarrow S_n$ or S_3) are shown in Fig. 21. The acceptor species with fluorescence at 664 nm has an excitation profile maximum at about 427 nm. This band maximum is very similar to that of the penta-coordinated monomer at about 426 nm. The donor species with fluorescence at 685 nm has an excitation profile maximum at 440 ± 5 nm. An asymmetry in the shoulder of the Soret band absorption spectrum of the $5 \times 10^{-6} \text{ M}$ solution (Fig. 15C) may indicate the presence of a weak absorption at 440 ± 5 nm. The second derivative spectrum of a solution similar to this indicates the presence of an origin in this region⁹².

Measurements made for these studies and others^{95,99,102} have shown under the optical conditions necessary to observe blue fluorescence from Chl a solutions, that population saturation in the lowest excited state singlet energy level (S_1) is present. A blue emission is recorded with

focussed red laser excitation from a 2×10^{-4} M Chl a solution (Fig. 22) with a band maximum at about 455 ± 10 nm. Under the conditions used, the blue emission was observed to follow the contour of absorption in the Q_y region of a similar solution (Fig. 22 insert). In previous observations, monochromatic transmission experiments¹⁰² with pulsed laser radiation tuned to the red spectral region produced a populated excited state under the conditions necessary to record the blue fluorescence. The two-photon excitation profile follows the absorption spectrum, thus it can be concluded that the blue fluorescence results from the consecutive absorption of red laser photons from the populated S_1 donor and (perhaps) S_1 acceptor levels of the donor/acceptor molecule and fluorescence from the lower of the S_3 energy levels (depicted in Fig. 28). The maximum of the blue fluorescence at 455 ± 10 nm can be used in the estimation of the location of the S_3 level of the donor species in the donor/acceptor complex. The emission is assigned to the donor species because this species shows a lower energy level in the Q_y region than the acceptor. Vibrational relaxational processes will likely cause conversion of the energy absorbed by the acceptor to the donor faster than the process allowing emission from the S_3 level of the acceptor molecule and thus will not permit an emission from the S_3 of the acceptor to be observed. The maximum at 455 nm is estimated with a 10 nm error because the band shape is distorted due to reabsorption of the emitted light by the Soret absorption band. Because the spectroscopic absorption properties of Chl a in lutidine are more similar to the acceptor species than those observed in pyridine, the location of the S_3 origin for the acceptor molecule in hexane may be

similar to that observed for monomeric Chl a in lutidine.

An estimation of the location of the S_3 energy level for the acceptor molecule is made here by assuming that the spectroscopic features of the acceptor molecule are similar to a pyridinated monomeric species. The blue fluorescence induced by focussed red-laser excitation from a 10^{-4} M solution of Chl a titrated with pyridine⁹⁵ is located at 444 nm and the Soret absorption band of the pyridinated solution is at 427 nm. The electronic origin $S_3(0,0)$ of the pyridinated Chl a complex can be estimated as the average of fluorescence and absorption energy levels at about 435 nm. The two-photon induced blue fluorescence of monomeric Chl a in lutidine¹⁰¹ is found at 444 nm and has a Soret absorption at 433 nm. By a similar estimation, the S_3 origin of monomeric Chl a in lutidine is at about 439 nm and is close to the maximum in the resonance Raman profile for this solution¹⁰¹.

In the most dilute Chl a solution (Fig. 9) the position of the Soret band for the monohydrated Chl a was at 426 nm. A two-photon induced blue fluorescence could not be detected from this solution. In solvents where the Chl a is penta-coordinated^{95,103} (i.e. lutidine) the Stokes shift is between 11 and 14 nm. For the monohydrate complex an electronic origin is predicted between 431 nm and 433 nm. Attempts to carry out Raman excitation profiles on the monomer are difficult because the emission is very weak. The Raman excitation profile of the 10^{-4} M solution (Fig. 24) shows two areas of enhancement at 435.2 nm and 448.2 nm. For the 10^{-4} M solution, no intensity enhancement was found with other measurements between 426 nm and 435 nm. The Soret absorption maximum of this solution at 430 nm (Fig. 15D) or the monomer at 426 nm

were not seen in the enhancements of the Raman excitation profile. Assuming that the Stokes shift of the acceptor is similar in magnitude to that of the monomer, the electronic origin of the acceptor is expected between 435 nm and 437 nm. The maximum in the profile at 435.2 nm is thus assigned to the position of the $S_3(0,0)$ origin of the acceptor, and the maximum at 448.2 nm is assigned to the $S_3(0,0)$ origin of the donor molecule. This latter assignment is in agreement with assignment of the blue fluorescence at $455 \text{ nm} \pm 10 \text{ nm}$ to the donor molecule. Additional evidence from other Raman excitation profiles using the 1705 cm^{-1} (free keto group of the acceptor molecule)⁶⁶ and 1655 cm^{-1} band (Mg-bound C=O group on ring V of the donor molecule)⁹² indicates enhancement at 435.2 and 448.2 nm with excitation into the vibronic band at 404.9 nm. The above results of absorbance, fluorescence, and resonance Raman spectroscopy are combined to yield the approximate energy level diagram of the donor/acceptor complex as described in section 3B.8. It is clear from the above discussion that this energy level diagram cannot be obtained if the data from the various absorbance and emission processes is taken alone. It may be concluded that the main contribution of the research described in this thesis is the construction of this energy level diagram.

A different Raman excitation profile (Fig. 26) was observed by de Wilton et al.^{93,95} and has recently come under re-examination⁹⁹. The fluorescence spectra of the donor/acceptor complex (Fig. 23) differs from that of a solution (Fig. 25) used to repeat a similar experiment to Fig. 26 by having extra intensity in the 685 nm region. The Raman excitation profile of the donor/acceptor solution shown in Fig. 26 was

calculated using the 1440 cm^{-1} band of hexane as an internal standard (indication of laser power reaching the sample) whereas the Raman excitation profile of Fig. 26 was calculated using the 2900 cm^{-1} band of hexane as internal standard. Holes in the profile of Fig. 24 may be due to excited state absorption effects which are different for the 2900 cm^{-1} band than for the 1440 cm^{-1} band.

As well as the donor-acceptor Chl a complex, other aggregated species composed of donor-acceptor sub-units may be present in the hexane solution. The reaction centre dimer of Katz and Norris⁶² has two Chl a molecules in the basic sub-unit, linked through a water. These dimer molecules are unequal with ring V ketone and carbomethoxy groups of one Chl a molecule coordinated to another Chl a molecule, and form the basic sub-unit proposed for a hydrated oligomer with fluorescence at 743 nm. Another dimer linked by water molecules between the carbomethoxy group of C_{10} has been assigned a 726 nm fluorescence^{38,104}. A basic dimer sub-unit composed of Chl a molecules directly linked through the ring V ketone of a donor molecule with no intermediate water molecule has been proposed by Fong⁶³. Aggregation of dimers of this sort results in an absorbance at 678 nm.

Another type of dimer formed by the cofacial interaction of Chl a molecules lined up in a stacked configuration would have spectra differing from the monomer because the conjugation sphere of the porphyrin is even more directly involved in the bonding. In such an aggregate the Chl a molecules may retain the metal-bonded water molecules. Changes in the form of stabilization of the porphyrin ring

have been calculated theoretically³³ to shift the optical transitions to lower energy. Interactions between the individual molecules of a cofacial dimer may be equal since bonding is reciprocal, and can be expected to produce a larger shift to lower energy for the lowest singlet energy levels than that observed for the donor-acceptor aggregate (where only the acceptor molecule is likely to shift to lower energy). This type of aggregation may also be used to form higher oligomers.

The chopped CW laser induced fluorescence spectra for hexane solutions of Chl a at concentrations greater than $5 \times 10^{-6} \text{ M}$ show a decrease in fluorescence intensity and a gradual shift in band position towards lower energy with increasing concentration. In Fig. 17 the 10^{-5} M solution has fluorescence band maxima around 667 nm and 687 nm observed with different excitation energies. At a concentration of $2 \times 10^{-4} \text{ M}$ in Fig. 18, bands with maxima at 672 nm and 690 nm are observed. With excitation closer to the Soret maximum (454.5 nm), a band is located at about 750 nm in the vibronic region. With excitation further away from the Soret maximum at 514.5 nm, a new fluorescence band is observed at 672 nm with an asymmetry on the high energy side of the band. At this wavelength the main intensity of the vibronic band is shifted to about 725 nm.

At even higher Chl a concentrations (Figs. 19 and 20), the fluorescence profiles contain mainly bands located at 690 nm or 692 nm. A larger intensity is observed in the vibronic region for these solutions relative to the other main fluorescence bands. Solutions of Chl a at concentrations as high as 10^{-3} M are likely to contain all or

any of the possible aggregated forms of Chl a, and thus do not necessarily provide information useful for making unique spectroscopic assignments.

3B.8 Energy Level Diagram of Dimeric Chlorophyll a

From the conglomeration of information on the donor/acceptor Chl a complex, an electronic energy level diagram can be assembled for the complex in hexane (Fig. 28). The location of the $S_1(0,0)$ level of the donor molecule can be calculated as the average of absorbance estimated at 672 nm ($14,881\text{ cm}^{-1}$) and fluorescence maxima at 685 nm ($14,599\text{ cm}^{-1}$). This average at 678 nm ($14,740\text{ cm}^{-1}$) may be subject to some error because of differences in size between of unequal (0,1) energy gaps for the ground and first excited states. The location of the $S_1(0,0)$ level of the acceptor molecule can similarly be estimated at about 664 nm ($15,060\text{ cm}^{-1}$) by the average of absorbance (664 nm, $15,060\text{ cm}^{-1}$) and estimated 1 nm Stokes shifted fluorescence (665 nm, $15,040\text{ cm}^{-1}$). The location of the $S_0 \rightarrow S_1$ absorbance is subject to error because the Q_y bands of the donor and acceptor species were overlapping.

A location of the Q_x (S_2) energy level of the donor and acceptor is not indicated in this diagram because these transitions are usually not observed. For instance the Q_x absorbance of the monomer band is located at an energy approximately degenerate with the first vibronic of the Q_y band. This vibronic band is weak in intensity compared to the Q_y band. The absence of observed Q_x transitions may be due to energy relaxation to other moments in the molecule.

The $S_3(0,0)$ level of the donor molecule is taken from the location

of the enhancement observed in the resonance Raman profile at 448 nm ($22,320\text{ cm}^{-1}$). This origin is in agreement with values predicted from the average of the two photon induced blue emission at $455 \pm 10\text{ nm}$ ($21,978\text{ cm}^{-1}$) and absorbance at $440 \pm 5\text{ nm}$ ($22,727\text{ cm}^{-1}$) from the fluorescence excitation profile.

The $S_3(0,0)$ energy level of the acceptor molecule is located from the enhancement observed at 435.8 nm in the resonance Raman profile ($22,946\text{ cm}^{-1}$). This value is similar to that predicted for a pyridinated monomer at 435 nm^{95} ($22,989\text{ cm}^{-1}$) and a monomer ligated with lutidine at 439 nm^{101} ($22,779\text{ cm}^{-1}$).

Figure 28

Energy level diagram for dimeric chlorophyll a. Approximate energies of singlet states are given in wavenumbers (cm^{-1}). Energies of acceptor (A) and donor (D) molecules are indicated.

C. CONCLUSION

The energy level diagrams shown in Figure 14 and 28 for monomer and dimeric species of Chlorophyll a in hexane solution have been calculated based upon spectroscopic and chemical behaviour. The singlet energy level assignments for a pentacoordinated monomeric Chl a species are based upon spectra produced from dilute Chl a solutions in hexane including absorbance, continuous and pulsed laser-induced fluorescence and fluorescence excitation profiles in the Q_y and Soret regions. Additional information from water-saturated hexane solutions, Chl a in pyridine, collidine and lutidine solutions, and octaethylporphyrin and magnesium octaethylporphyrin in hexane was used. A hexa-coordinated Chl a species was not observed in water-saturated hexane solutions, instead the Chl a was degraded to form pheophytin under this environment. In the presence of a small excess of water above the Chl a concentration, the fluorescence spectra and lifetimes of the Chl a species were changed. This may indicate the presence of an electrophilic ligand associated with functional groups on the Chl a ring periphery. These associations may further stabilize the molecule.

The energy level assignments for a donor/acceptor Chl a complex are based upon the spectra produced for monomeric Chl a solutions as well as solutions containing higher aggregates of Chl a using measurements of continuous and pulsed laser fluorescence, and fluorescence excitation profiles in the Soret region, blue fluorescence produced by two-photon consecutive absorption processes and Paman

produced by two-photon consecutive absorption processes and Raman excitation profiles in the Soret region. Evidence supports the assignment of these properties to an approximately T-shaped dimer where the two molecules are not identical. The acceptor molecule of this dimer physically and spectroscopically resembles the monomer more than the donor. The charge donation of the magnesium-bound water ligand of the acceptor molecule is replaced by a nucleophilic group from the donor molecule (probably one of the functional groups on ring V). The donor molecule probably still remains penta-coordinated with water and has spectroscopic properties different from acceptor and monomer.

Appendix I

The simplest porphyrin molecule, porphine (Fig. 2) differs from chlorophyll by the presence of a double bond over the C_7-C_8 position on ring IV and other peripheral differences. Two porphyrins substituted at the methine positions have been studied, triclinic tetraphenyl porphyrin²⁴ (TPP) and tetra-n-propyl porphyrin²⁵ (TPrP).

The changes induced by metal insertion into the porphyrin have been studied with Fe(III) in TPP²⁶ and Cu(II) in TPrP²⁷. In its crystalline form, FeTPP has two molecules in the unit cell each coordinated strongly through a fifth ligand (oxygen) by a bond shorter than the metal--pyrrole nitrogen (M--N) bond lengths.

The methine substituted porphyrins, TPrP and TPP, are of interest because of the way in which substitutions at the methine C_m positions influence the π -conjugation system. The natural porphyrins are substituted at the C_p positions, which may or may not be included in ring conjugation depending on variable physical parameters such as bond lengths. Another molecule studied by x-ray crystallography, octaethyl porphyrin²⁸ (OEP), is symmetrically substituted at the eight C_p positions and is found in two crystal morphologies (triclinic and tetragonal).

A monohydrated dipyridinated magnesium phthalocyanin (MgPc) has been studied by Fisher et al.²⁹. This structure is analogous to chlorophyll in that it has nitrogen at the methine positions and that the magnesium is coordinated to a water molecule. Under anhydrous conditions, MgPc 'crystallizes' with an isomorphous structure whereas with water and pyridine present it crystallizes with an asymmetric unit cell consisting of one MgPc, one water (axial bound) and two extraneous

pyridine molecules. The water molecule is held at a distance of 2.022 Å (comparable to other experimental Mg--OH₂ bonds²⁹ such as MgSO₄·7H₂O, 2.06 Å; Mg(NH₄)₂(SO₄)₂·6H₂O, 2.07 Å).

Pettersen³⁰ has also studied Ni(II) deoxophylloerythrinmethylester (NiDPE), which is somewhat more homologous to chlorophyll structure (a propionate side chain of ring IV) and VODPEP³¹ (vanadyl deoxophylloerythroetioporphyrin, 1,2-dichloroethane solvate, with an ethyl group at the same position).

The macrocyclic configuration of porphine is significantly changed when ring IV is reduced to form a chlorin macrocycle, saturating (and lengthening) the C₇--C₈ bond. Fisher *et. al.*³² have studied the physical properties of crystallized methyl pheophorbide a (MePPb, Fig. 1). MePPb is a Chl a derivative with the magnesium removed and hydrogen on ring I and III nitrogen, and the chlorophyll phytyl chain (C₂₀H₃₉) replaced by the methyl ester group. The absolute configuration of MePPb is the same as Chl a.

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