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Full Name of Author — Nom complet de l'auteur

ANGELA CLAIRE de WILTON

Date of Birth — Date de naissance

17 MAY 1956

Country of Birth — Lieu de naissance

ENGLAND

Permanent Address — Résidence fixe

355 CAMBRIDGE STREET NORTH
OTTAWA
ONTARIO CANADA K1R 7B3

Title of Thesis — Titre de la thèse

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DR. J. A. KONINGSTEIN

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A STUDY
OF THE PULSED LASER INDUCED FLUORESCENCE
& RESONANCE RAMAN SCATTERING
OF THE DIMERS OF CHLOROPHYLL A AND BACTERIOCHLOROPHYLL A IN SOLUTION

by

Angela Claire de Wilton, B.Sc.

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

Carleton University

Ottawa, Ontario

Canada

5 November 1984

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The Faculty of Graduate Studies and Research
acceptance of the thesis, submitted by
Angela Claire de Wilton, B.Sc.
in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

.....
Chairman, Department of Chemistry

.....
Thesis Supervisor

.....
External Examiner

Carleton University

4 January 1985

ABSTRACT

Analysis of the absorption spectra of solutions of Chlorophyll a (Chl a) or Bacteriochlorophyll a (BChl a) in dry non-polar solvents showed that the absorption bands of the dimers are red shifted with respect to the monomeric absorption bands. To overcome the problem of the weak dimeric fluorescence bands being masked by the strong monomeric fluorescence, selective excitation of dimeric fluorescence was accomplished using a tunable pulsed dye laser to excite the solutions at wavelengths in resonance with the absorption bands of the dimeric species. The red fluorescence spectra of dimeric Chl a in hexane is characterised by two bands: the stronger band at 688nm has a shorter lifetime than the monomeric fluorescence at 668nm and the weaker, narrower band at 625nm has a lifetime comparable to that of the monomer. The fluorescence spectrum of dimeric BChl a has a band at 783nm and a weak band at 583nm. In each case the longer wavelength band is assigned to the dimer Q_y transition and the other band to the dimer Q_x emission. Q_x emission was not observed for the monomeric chlorophylls in solution. The long lifetime of the Q_x fluorescence is discussed in terms of non-radiative relaxation. Information on the dynamics of energy relaxation was obtained from a study of the photoquenching of the red fluorescence and the laser intensity dependence of the transmission.

Blue fluorescence from the Chl a dimer was observed either with direct excitation in the region of the Soret absorption band or following two photon absorption at wavelengths in resonance with the Q_y absorption band.

A novel hole burning phenomenon in the resonance Raman excitation profiles of some normal modes of the Chl a dimer in hexane was also attributed to non-linear absorption effects, and indicated that there are two electronic origins at $\sim 435\text{nm}$, separated by $\sim 60\text{cm}^{-1}$. The latter may be related to an exciton splitting of dimer levels in the Soret region.

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{nm}	Einstein coefficient for spontaneous emission	α	polarizability
Ar ⁺ laser	Argon ion laser	$(\alpha_{\rho\sigma})_{nm}$	ρ th component of the polarizability tensor for the transition n to m
A	Absorbance		
B_{nm}, B_{mn}	Einstein coefficients for induced absorption and emission	Γ_{nm}	damping factor for the transition n to m
B_x, B_y	Absorption transitions in the blue region of the chlorophyll spectra	ϵ	absorption coefficient
BChl <u>a</u>	Bacteriochlorophyll <u>a</u>	ϵ_M	molar absorption coefficient
BPheo <u>a</u>	Bacteriopheophytin <u>a</u>	ϵ_0	permittivity of free space
BVir	Bacterioviridin or 2-acetyl-2-devinyl Chl <u>a</u>	$\kappa(\omega)$	imaginary part of the complex refractive index
Chl <u>a</u>	Chlorophyll <u>a</u>	λ	wavelength
Chl <u>b</u>	Chlorophyll <u>b</u>	λ_l	laser wavelength
c	concentration, speed of light	μs	microsecond ($10^{-6}s$)
cw	continuous wave	ν_{nm}	frequency of transition from n to m
e	exponent, electronic charge as a subscript-denotes excited state	ν	excitation frequency
E_n	Energy of nth level	ν_l	laser frequency
ENDOR	Electron nuclear double resonance	$\rho(\nu_{nm})$	density of radiation field at frequency ν_{nm}
ESR	Electron spin resonance	Φ_{fl}	fluorescence yield
FWHM	Full width at half maximum of a spectral band	$\sigma_{nm}(\nu)$	cross section for transition n to m
f_{nm}	oscillator strength for a transition from state n to state m	τ_{rad}	radiative lifetime
h	Planck's constant	τ_{fl}	fluorescence lifetime
He-Cd laser	Helium-cadmium laser	τ_{abs}	absolute lifetime
HPLC	High performance liquid chromatography	$\omega = 2\pi\nu$	angular frequency
I	Intensity		
I_l	Laser intensity		
I_o	Incident intensity		

LIST OF SYMBOLS AND ABBREVIATIONS

(continued)

I_{Raman}	Raman intensity	PMT	Photomultiplier tube
k	Rate constant	ps	picosecond (10^{-12} s)
k_{fl}	Fluorescence rate constant	Pheo a	Pheophytin a
$k_{\text{non-rad}}$	non-radiative rate constant	Protochl a	Protochlorophyll a
l, l_i	electronic orbital angular momentum quantum number	Q, q	Normal mode
LASER	Light amplification by stimulated emission of radiation	$Q_g^q(\lambda)$	Raman cross section of normal mode q in the ground state at wavelength
M	Molar concentration	$Q_e^q(\lambda)$	Raman cross section of normal mode q in the excited state at wavelength
m_e	mass of electron	$Q_x \cdot Q_y$	Absorption transitions of the chlorophylls in the red spectral region
$N(\omega)$	Complex refractive index	REP	Raman excitation profile
$n'(\omega)$	real part of complex refractive index	S	Spin multiplicity
n	as subscript to denote state n	s, s_i	Electron spin quantum number
N_n, N_m	Population of state n, m	S_n	nth excited singlet state
$N_n(\lambda)$	Wavelength dependent population of state n	T_n	nth excited triplet state
N_e, N_g	Population of excited, ground state	T	Transmission
ns	nanosecond (10^{-9} s)	V, mV	Volt, millivolt
N_2 laser	Nitrogen laser	W, mW, MW	Watt, milliwatt, Megawatt
OMA	Optical multichannel analyser	x	as subscripts to denote polarisation of a transition
		y	

"Why grasse is greene, or why our blood is red,
Are mysteries which none have reached unto."

From "Of the Progresse of the Soule"

by John Donne (1612)

CHAPTER 1

INTRODUCTION

The chlorophylls are the primary photoreceptors in photosynthetic green plants, algae and bacteria.^{1,2,3} Since the discovery of the two major chlorophylls, Chlorophyll a and Chlorophyll b, over a hundred years ago,⁴ the photosynthetic pigments have been the subject of extensive research. An abundance of data has accumulated on the properties of the chlorophylls^{1,5} and their role in photosynthesis.^{2,3,6} Spectroscopic evidence suggests that the unique photoactivity of the chlorophylls in vivo may be due both to chlorophyll-chlorophyll interactions between pairs (dimers) of chlorophyll molecules^{7,8} and to specific environmental interactions with the lipoprotein matrix of the photosynthetic organism.^{9,10} Relatively little is known about the fundamental photophysics of the photosynthetic pigments. The nature of the excited states of the chlorophylls which take part in the primary processes of photosynthesis remains the subject of much discussion.^{11,12,13,14}

Over the last three decades the coordination properties of the chlorophylls in solution have been established.¹⁵ Investigations of the spectroscopic properties of dimeric, oligomeric and aggregated chlorophylls in vitro are now directed towards an understanding of the nature of interaction of two or more chlorophyll molecules and the effect of aggregation on the electronic excited states of the chlorophylls in relation to their characteristics in vivo.^{14,16,17}

Apart from the relevance of spectroscopic studies of the chlorophylls to the mechanism of the initial processes of photosynthesis itself, the photophysics of the chlorophylls in vitro involve phenomena common to many man-made systems for conversion of solar energy.¹⁸ The photosynthetic pigments therefore provide useful models for experimentally testing general theories of electronic excitation and energy transfer.

A recent innovation in chlorophyll research is the application of pulsed laser spectroscopy.^{19,20,22,21} With the added dimensions of time resolution and frequency selectivity, new spectroscopic techniques can now provide a direct probe of excited states of the chlorophylls, both in vivo and in vitro.

The aim of this thesis is to demonstrate the potential of nanosecond pulsed laser spectroscopy to yield new information on the electronic energy levels, interaction energies and dynamics of energy conversion in dimeric forms of two important chlorophylls, Chlorophyll a and the bacterial equivalent Bacteriochlorophyll a.

The roles of monomeric, dimeric and aggregated chlorophylls in photosynthesis are discussed in the following chapter and the possible models for the photosynthetic reaction centres are described. It will be seen that the simple chlorophyll dimers are unlikely models for the photosynthetic reaction centres. However, there are several fundamental spectroscopic problems concerning the dimeric and oligomeric chlorophylls in solution which demand attention.

The chlorophyll-chlorophyll interactions in these structures have been investigated by infrared absorption,¹⁵ nuclear magnetic resonance,^{15,23,24} electron spin resonance, electron nuclear double

resonance,⁸ visible absorption,¹⁵ circular dichroism and magnetic circular dichroism spectroscopy.²⁵ Selected results from the extensive literature, which are relevant to the understanding of the work in this thesis, are discussed in Chapters 2 and 3. In spite of the intensive research, the structure of the Chlorophyll a dimer in solution has not been definitively determined. The interpretation of the absorption spectrum of dimeric forms of the chlorophylls in terms of exciton interactions between the two chlorophyll molecules^{26,27,28} and the magnitudes of the chlorophyll-chlorophyll interaction energies therefore remain a source of contention (see Chapter 3).^{25,29} Another interesting controversy in the literature rests on whether or not dimeric and oligomeric chlorophylls are fluorescent.^{15,30,31,32,33}

The demonstration of fluorescence from dimeric chlorophylls in solution forms the basis of this thesis. Although the dimeric fluorescence spectra are observed only with difficulty under ordinary conditions, particularly in the presence of highly fluorescent monomeric chlorophylls, the use of wavelength selective excitation with a tunable pulsed laser and a sensitive detector shows that the dimers of Chlorophyll a and Bacteriochlorophyll a are certainly fluorescent. Non-linear spectroscopic techniques, including power dependent studies of the photoquenching of normal red fluorescence and analysis of blue fluorescence produced from more highly excited states by two photon absorption, are used to provide information on the energy levels and the lifetimes of the dimeric chlorophylls.

Pulsed resonance Raman scattering studies also show promise

for the investigation of chlorophyll-chlorophyll interaction energies. The novel hole burning phenomenon observed in the study of the excitation profiles of Raman active normal modes of Chlorophyll a appears to be a new technique for determining the origins of electronic levels, but it remains to be seen whether this is a general phenomenon in excited state Raman spectroscopy or another unique property of the chlorophylls.

A large part of the research described in this thesis has been published in several papers by the author as the work progressed.^{21,34-40}

CHAPTER 2

THE STRUCTURES, PROPERTIES AND FUNCTIONS OF THE CHLOROPHYLLS

2.1 Structures of the chlorophylls

The chlorophylls are a group of about 10 closely related pigments which are necessary for photosynthesis in green plants, algae and photosynthetic bacteria.^{1,3,41} The most important of these is Chlorophyll a (Chl a) (Figure 2.1A) which is present in all photosynthetic plants which evolve oxygen. It may be accompanied by the auxiliary pigment Chlorophyll b (Chl b) (Figure 2.1B) in vascular green plants and algae. The principle photoreceptor in green and purple photosynthetic bacteria³ is Bacteriochlorophyll a (BChl a) (Figure 2.1C) of which there are two forms: BChl a_p is the common form found in over 85 types of bacteria and BChl a_{gg} is found principally in only 2 bacterial species. The chlorobium chlorophylls are a group of several chlorophylls of uncertain structure (sometimes collectively called bacteriochlorophylls d and e) which usually accompany BChl a. Other supplementary plant chlorophylls are Chlorophylls c₁ and c₂ which are found in some brown algae, and Chlorophyll d which is present in red algae (Figure 2.2).

The structures of the chlorophylls were elucidated by H. Fischer and co-workers.⁴² Their deductive and synthetic work showed that the structure on which the chlorophylls and many other biologically important molecules is based is a cyclic tetrapyrrole, Porphyrin,^a

^a(from the Greek porphura = purple)

Structures of some of the minor chlorophylls and a biosynthetic precursor protōchlorophyll.

also known as porphine or porphin. The structures of the chlorophylls are usually named and numbered according to the system of Fischer,⁴³ based on the parent-molecule porphyrin (Figure 2.3A). This is the numbering system used in this thesis. The revised nomenclature adopted at the Commission on Nomenclature in Biology in 1960 (Figure 2.3B) is not widely used for chlorophyll derivatives. The IUPAC names for the chlorophylls and their derivatives are based on the magnesium-free parent-molecule pheophytin [e.g. Chl a is called (pheophytinato a) magnesium (II)] but the trivial names are more commonly used.⁴³ The name chlorophyll^b was first given to the mixture of green plant pigments by Pelletier and Caventou in 1818.⁴⁴

All the chlorophylls are complexes of magnesium. Although they are structurally similar to the porphyrins, they contain reduced pyrrole rings and are therefore in a lower oxidation state. The important natural chlorophylls are 3,4-dihydro- or 3,4,7,8-tetrahydro- porphyrins (Figures 2.3C and 2.3D). The side chains are usually methyl, ethyl, vinyl and propionic acid, which are common to all porphyrins. Position 7 in the chlorophylls is esterified with a long chain alcohol [phytol, geranylgeraniol (Figures 2.1 and 2.2) or farnesol^c] which accounts for the hydrophobic nature of these pigments.

The unique feature of the chlorophylls is the cyclopentanone ring conjoint with ring III. The presence of this aliphatic ring V is sufficient to characterise a porphyrin derivative as a chlorophyll, independently of the side chains or the oxidation state of the tetrapyrrole ring.

^bfrom the Greek: chloros=green, phyllon=leaf

^cfarnesol is the esterifying alcohol in the Chlorobium chlorophylls.

9.

(A)

Fischer numbering
system

PORPHYRIN
(PORPHIN)

(B)

revised nomenclature
1960

(C)

DIHYDROPORPHYRIN
(CHLORIN)

(D)

TETRAHYDROPORPHYRIN
(DIHYDROCHLORIN OR BACTERIOCHLORIN)

Figure 2.3

Structures and numbering systems of the parent porphyrins on which the chlorophylls are based.

Biosynthetically, Chl a is derived from protoporphyrin (Figure 2.4).

PROTOPORPHYRIN

Figure 2.4

Both protoporphyrin and its magnesium complex are photoactive. Thus it appears that the structural modifications are necessary for the specific interactions of the chlorophylls with the lipids and proteins in the photosynthetic membrane which give the chlorophylls their other special characteristics. The magnesium ion together with the functional groups on ring V give the chlorophylls unusual electron acceptor-donor properties (see Section 2.3). The long chain ester group may serve to anchor the chlorophyll within the hydrophobic region of the lipoprotein matrix.^{6,10} The oxidation state of the ring and the peripheral substituents provide the electronic structure which gives the chlorophylls their characteristic strong blue and red absorption bands (Section 3.1).

2.2 Derivatives of the Chlorophylls

Most of the chlorophylls occur naturally in the presence of a number of other pigments including carotenoids.⁴¹ After extraction with an organic solvent, separation is usually achieved by chromatography.^d All the photosynthetic pigments are quite labile, particularly in solution, and great care must be taken to obtain chlorophylls of high purity which are free from the many possible alteration products. These may be produced by adverse treatment of the plant material before or during extraction, or when the chlorophyll extracts are exposed to heat, acids or alkalis,⁴¹ certain adsorbents,⁴⁵ bright light or oxygen (particularly in the presence of methanol). The chlorophylls may also undergo enzymatic oxidation or hydrolysis.

The alteration products fall into two groups: the green magnesium-containing compounds and the brown or grey magnesium-free derivatives. Since many of the green products are indistinguishable from the true chlorophylls by visible absorption spectroscopy, this alone should not be used as a criterion for purity or identity. A reaction scheme for some derivatives of Chl a is given in Figure 2.5, which applies similarly for the other chlorophylls.)

The following derivatives are commonly encountered:

Pheophytin: mild treatment of the chlorophylls with acid replaces the metal ion with hydrogens on the nitrogens of rings I and III yielding the corresponding pheophytin. Stronger acid also removes the phytol chain by hydrolysis to give the pheophorbide.

^dChromatography was originally invented by Tswett in 1906 to separate the plant pigments (see ref. 46).

Figure 2.5

Reaction scheme for some commonly encountered derivatives or degradation products of Chlorophyll a. The other chlorophylls react similarly. (Further information on the derivatives is given in Appendix I).

Chlorophyllide: hydrolysis of the phytol ester can be achieved while leaving the magnesium ion in place by using the enzyme chlorophyllase in aqueous acetone. In aqueous alcohols, the alkyl chlorophyllides are formed. With removal of the phytol chain it is possible to crystallize the hydrates of the chlorophylls. This can be useful to simplify the infrared or nuclear magnetic resonance spectra and has little effect on the visible absorption spectra.

The C₁₀ epimers: these stereoisomers of the chlorophylls are designated by a prime [e.g. Chl a']. They are formed reversibly in solutions which are left standing, heated or in the presence of trace amounts of base.^{41,47,48,49} There is rapid reversion to the more stable isomer except at low temperatures. In tetrahydrofuran (THF) or pyridine a substantial amount of the chlorophyll may be present in the diastereoisomeric form. Chl a and Chl a' have very similar properties and are almost identical spectroscopically.⁵⁰ They are separable by chromatography.

Oxidation and allomerization products: under basic conditions in the presence of oxygen or other oxidants, various forms of oxidised chlorophylls may be formed. These are known as allomerization products.^{51, 52,53,54} Oxidation at C₁₀ of ring V is common to all allomerization reactions. The major products are 10-hydroxy-chlorophylls, which are indistinguishable from the parent chlorophylls by absorption spectroscopy. 10-methoxy-chlorophyll and 10-methoxy-lactone chlorophyll are typically produced in anhydrous methanol.

In bacteriochlorophylls, the vinyl group at position 4 is particularly susceptible to photoinduced oxidation in aerated solutions.

The major product is 2-devinyl-2-acetyl-chlorophyll.^{55,56,57}

The structures and some spectroscopic data for these and other common derivatives or alteration products of the chlorophylls are given in Appendix I.

2.3 Coordination interactions and aggregation of the chlorophylls in solution

Coordination interactions with nucleophilic ligands

The central magnesium in the chlorophylls has a coordination number of four: it is bound by the four nitrogens of the tetrapyrrole ring (Figure 2.6A). Four-coordinate magnesium is coordinately unsaturated.¹⁵ The two axial ligand positions above and below the plane of the macrocycle are readily occupied by electron donating ligands. When the chlorophylls are dissolved in a polar (nucleophilic or Lewis base) solvent, they are present as monomeric species with either five-coordination of the magnesium (Figure 2.6B) or six-coordination of the magnesium (Figure 2.6C) depending on the Lewis basicity and steric properties of the ligand. For example, in acetone or ether, the five-coordinate species of Chl a is favoured [i.e. Chl a.L₁] and in the more polar solvents pyridine or tetrahydrofuran the six-coordinate species is predominant [i.e. Chl a.L₂].²⁴ There is evidence that the magnesium ion in the five-coordinate species is displaced slightly out of the plane of the macrocycle towards the ligand, as is commonly observed in other metalloporphyrins.^{24,58,59,60}

If the ligand is bifunctional, such as pyrazine or dioxane, or is capable of hydrogen bonding, such as water, it is possible to form chlorophyll adducts which can reach colloidal dimensions as shown in Figure 2.6D:

Figure 2.6

Coordination of the magnesium ion of the chlorophylls in solution.

A, B and C show how monomeric species may be coordinated with nucleophilic ligands, L. D is representative of an adduct formed with a bifunctional nucleophilic ligand, in this example, dioxane.

The chlorophyll molecules themselves also have nucleophilic oxygen functions: Chl a has a keto C=O group and two ester C=O groups; BChl a has an additional acetyl C=O group. These carbonyl functions may act as electron donors in their own right. Thus the chlorophylls have the uncommon combination of both electron acceptor (Mg) and electron donor (C=O) properties. In the absence of extraneous nucleophilic ligands the chlorophylls can satisfy the coordination requirements of the magnesium ion by forming dimeric or oligomeric structures via a C=O...Mg interaction (Figures 2.7 and 2.8).^{15,29,61}

There are therefore two types of chlorophyll aggregates:¹⁵

- (i) Those which involve extraneous nucleophiles and are formed by "exogamous" interactions, and
- (ii) those in which chlorophyll functions both as an electron donor and an electron acceptor by "endogamous" or direct chlorophyll-chlorophyll interactions.

The nature of both exogamous and endogamous aggregation of the chlorophylls has been established by a number of techniques including vapour phase osmometry,⁶² nuclear magnetic resonance,^{15,23,24,63} infrared absorption,^{15,64} Raman scattering^{65,66} and visible absorption and fluorescence spectroscopy.²⁹ It is the dimers formed by endogamous interactions which are of interest to the work in this thesis. The evidence for dimerization and oligomerization of Chl a and BChl a in solution is summarized in the following section.

Figure 2.7

A possible structure for a Chlorophyll *a* dimer, illustrating a direct keto C=O...Mg linkage. The relative orientation of the rings would be flexible and may be solvent dependent.

Figure 2.8

A and B show a C_2 symmetric dimer structure formed by two reciprocal direct keto $C=O \dots Mg$ linkages; C shows a similar dimeric structure in which the two Chlorophyll molecules are hydrogen bonded via two water molecules. The latter structure was proposed by Katz et al.²⁹ as a model for the photosynthetic reaction centre "special pair" Chlorophyll.

Formation of dimers and oligomers of Chlorophyll a and Bacteriochlorophyll a in solution

Formation of significant amounts of dimeric or oligomeric chlorophylls in dry non-polar solvents requires careful removal of all extraneous nucleophiles from both the chlorophyll and the solvent.⁶⁷ Chlorophylls are normally obtained in the hydrated form following extraction and may contain various non-stoichiometric amounts of water. Dry chlorophylls will not crystallize, but form an amorphous solid, which resembles a very viscous liquid or waxy solid. The hydrates will form adducts which can be precipitated. Anhydrous chlorophylls are highly hydrophilic and short exposure to atmospheric moisture results in absorption of water. Adventitious nucleophiles such as colourless plant lipids, polar impurities concentrated from the large volumes of solvents used for chromatography, or traces of adsorbent may escape removal and have a high affinity for dry chlorophylls.²⁹ However, because of the presence of water in the atmosphere, this is the most difficult nucleophile to avoid.

Two molecules of water may be bound directly to the magnesium ion. Heating the chlorophyll under high vacuum (10^{-5} Torr) for several hours will remove only one water of hydration from $\text{Chl } a \cdot (\text{H}_2\text{O})_2$.²⁹ Removal of the second water molecule requires addition of a dry solvent, such as benzene or carbon tetrachloride, which forms an azeotrope with water, followed by evaporation of the solvent under reduced pressure. Repetition of this codistillation procedure several times can increase the chlorophyll to water ratio to better than 100:1. It appears that in the rigid structure of the solid, the chlorophyll cannot rearrange its structure to replace the

Chl-H₂O interactions with endogenous interactions. In solution the structure is more flexible and with removal of the water into the solvent, oligomerization can take place more easily.²⁹

In general, the presence of extraneous nucleophiles or even polar groups on the surface of the glass or quartz cells may produce some monomeric chlorophylls and reduce the chlorophyll to water ratio in even the most carefully dried solutions.

Vapour phase osmometry has shown that Chl a exists predominantly as a dimer over a wide range of concentrations in dry benzene or carbon tetrachloride (CCl₄).⁶² In less polarizable solvents such as aliphatic hydrocarbons, the aggregation is highly concentration dependant, and there is evidence for formation of oligomers of more than 20 chlorophyll units in concentrated ($\geq 0.1M$) solutions in octane or hexane.

The solvent dependence of the infrared absorption spectrum in the C=O region (1880cm^{-1} - 1550cm^{-1}) is interpreted as providing evidence for direct keto C=O...Mg interactions in the Chl a dimer and oligomer.²⁹ There are three main peaks: the two bands at 1735cm^{-1} (ester C=O) and 1695cm^{-1} (free ring V keto C=O) appear in the monomeric solutions, and the third band at 1652cm^{-1} is present in thoroughly dried solutions. The latter is attributed to magnesium bound keto C=O because the bands at 1695cm^{-1} diminish in intensity as the 1652cm^{-1} increases in intensity.^{15,64} Evidence for this type of interaction is also suggested by ¹³C nuclear magnetic resonance studies.²⁹ A possible dimer structure formed by a direct keto C=O...Mg interaction is shown in Figure 2.7.

The structure of this dimer may be somewhat flexible and the orientation of the two macrocycles may vary both in the angle of tilt to one another and the angle of rotation about the $C=O...Mg$ axis, depending on the solvent.

1H nuclear magnetic resonance measurements indicate that the structure of the dimer in solution is solvent dependent. The spectra of Chl a in carbon tetrachloride or in octane are broad and distorted while in benzene or dichlorethane the bands are much better resolved. Under some conditions it may be possible to form a C_2 symmetric dimer structure, as shown in Figure 2.8A and B, which displays two reciprocal $C=O...Mg$ links.²⁹

A novel structure for the Chl a dimer in solution which involves interaction of both the C_{10} ester $C=O$ and the C_9 keto $C=O$ with the magnesium ion has recently been proposed on the basis of an improved analysis of the 1H and ^{13}C nuclear magnetic resonance data with an refined ring current model.^{68,69} This structure has the advantage that oligomers may be readily formed by extension of the dimeric structure via the same mode of interaction (Figure 2.9).

Kooyman and Schaafsma⁷⁰ also investigated the 1H spin lattice relaxation and ring current shifts for the Chl a dimer in chloroform. They deduced that the most probable geometry, in this solvent, for a dimer interacting via keto $C=O...Mg$ linkages would be such that the two macrocycles were perpendicular to one another. However they did not consider the possibility of interaction with one or more of the other carbonyl functions.

Houssier and Sauer also favoured the C_9 keto $C=O...Mg$ interaction but considered additional interaction via the C_7 ester carbonyl on the

A.

B.

Figure 2.9

A. Structure for the Chlorophyll a dimer in solution proposed by Abraham et al.^{68,69} which involves interaction of both the C₁₀ and C₉ carbonyl groups of one molecule with the magnesium ion of the other molecule. B. Oligomers may be formed by stacking via the same mode of interaction.

side chain.²⁵ On the other hand, Fong et al. interpreted the infrared absorption data to indicate that interaction occurred via the C₁₀ ester function (Figure 2.10A and B).

There has also been a great deal of interest in dimeric forms of Chl a in which the pairs are linked via hydrogen bonding with water, involving either the C₉ keto carbonyl (Figure 2.8B) or the C₁₀ ester carbonyl (Figure 2.10B).^{73,74} These dimeric structures in which the two Chl a molecules are linked via two water molecules [or two other nucleophilic ligands capable of hydrogen bonding such as alcohols] are the only in vitro forms of Chl a which have been observed to show photoreversible electron spin resonance (ESR) signals due to photo-oxidation.⁷⁵ However, the photochemistry of the dry dimers has not been reported, possibly because most electron acceptors are nucleophilic and would disaggregate the dimer in solution.¹⁶

Plasma desorption mass spectroscopic data^{76,77,78,79} has been obtained from films of Chl a cast from several dry solvents, and shows no evidence of attached ligands or water intervening in the direct C=O...Mg linkage. There is however no direct evidence of whether or not the dimeric and oligomeric structures in solution may be 'externally' ligated with water or other ligands which may be released during the mass spectroscopic measurements. A structure of the form shown in Figure 2.11 may be possible.

A.

B.

Figure 2.10

A. Structure for the Chlorophyll a dimer in solution involving a direct C_{10} ester $C=O \dots Mg$ interaction; B. a similar structure in which the two molecules are linked by hydrogen bonding with two water molecules. The latter structure was proposed by Fong et al. as a model for the reaction centre "special pair" form of Chlorophyll a.⁷²

Figure 2.11

Structure to show the way in which two chlorophyll molecules interacting via a direct $C=O \dots Mg$ linkage may be 'externally' hydrated. This type of hydration could limit the size of oligomers formed in non-polar solvents which are not completely dry.

The formation of dimers and oligomers of BChl a in non-polar solvents has not been studied as intensively as the aggregation of Chl a in solution. Although BChl a has an additional acetyl C=O group and shows a slightly greater tendency to aggregate, the behaviour of the two chlorophylls is in many ways analogous. Visible absorption studies have shown that BChl a in carbon tetrachloride exists as a dimer in equilibrium with monomer, as does Chl a in carbon tetrachloride and in benzene.⁸⁰ A recent proton nuclear magnetic resonance and visible absorption study has also shown that BChl a is predominantly dimeric in wet benzene.⁸¹ These results contrast with those from vapour phase osmometry⁶² which suggest that in several dry hydrocarbon solvents BChl a has an aggregation number of 3 at low concentrations. On the other hand, the aggregation number of Chl a in the same solvents is 2 or 4, characteristic of multiples of dimeric units. Nuclear magnetic resonance,⁸¹⁻⁸³ infrared absorption studies and resonance Raman spectroscopy⁶⁶ have also been used to aid the characterisation of interactions in BChl a aggregates.

2.4 The function of the chlorophylls in photosynthesis

There is only one known example of an ecosystem based on chemical synthesis without sunlight.^e All other animal life on earth depends on a food chain in which the primary source of nutrients is produced by photosynthetic organisms.

The ultimate aim of chlorophyll research is to provide a better understanding of photosynthesis in order to develop improved natural or artificial photosynthetic systems and to produce new solar powered energy sources. Photosynthesis is the process by which green plants, algae and bacteria convert carbon dioxide into carbohydrates according to the following generalised equation:

where H_2A is a hydrogen electron donor. For green plants H_2A is water and oxygen is evolved during photosynthesis. Chl a is present in all such plants. Photosynthetic bacteria use sulphur electron donors in the presence of BChl a or BChl b.

The chlorophylls are associated with the primary processes of photosynthesis, which includes the initial absorption of light and its conversion into chemical energy. One feature which makes the photochemistry of the chlorophylls unusual is their ability to undergo reversible photochemistry in the presence of red light whereas

^eThis interesting system exists around hydrothermal vents in the Mid-Oceanic Rift of the Pacific Ocean floor. The warm water around the vents is rich in suspended minerals. Colonies of bacteria which metabolize hydrogen sulfide, CO_2 and O_2 flourish and provide the primary food source for animal life adapted to live at a pressure of 250 Atm., 2 miles underwater in total darkness

most in vitro photochemical reactions are triggered by blue or ultraviolet light.

The majority of the chlorophyll molecules in a photosynthetic organism, called the antenna or light harvesting chlorophylls, simply absorb light and transfer the energy to a relatively few special photoactive chlorophyll molecules which are called the reaction centres. At the reaction centres, the energy is converted into oxidation and reduction capacity involving photochemical charge separation in a reaction centre complex of a special form of chlorophyll, P, associated with electron donors and electron acceptors (see Figure 2.12).

It is now generally accepted that the green plant photosynthetic process involves two distinct, yet interacting, photochemical reaction centres. Photosystem I (PSI) or P700 absorbs light at ~700nm and is the primary source of electrons for reduction of CO₂. Photosystem II (PSII) or P680 absorbs light at ~680nm and generates oxidising power for the abstraction of electrons from water. Since the two systems operate over different redox potentials it is believed that the local environment and interactions of the reaction centre Chl a are responsible for the ability of the same chlorophyll to perform two distinct functions.

The absorption bands of in vivo chlorophylls are red shifted compared to the absorption bands of monomeric chlorophylls in solution, and resemble more the absorption bands of oligomeric chlorophylls in solution. Electron spin resonance (ESR) and electron double nuclear resonance (ENDOR) have also indicated that P700 may be a small Chl a aggregate such as a dimer^{7,8} because the bandwidths are narrowed and

this may be explained by spin delocalization over two or more molecules. On the other hand it has been proposed that P680 is a monomeric Chl a complex.⁸⁵

Whether P700 is in fact a dimeric form of Chl a is far from being settled unambiguously. Wasielewski et al.⁸⁶ have suggested that a monomeric Chl a enol has a similar narrow ESR bandwidth and although the other properties of such a modified form of Chl a are not consistent with those of P700, it is possible to explain the ESR and ENDOR bandwidth changes with a monomeric form of Chl a. Other workers have also come to the same conclusion: a very recent ENDOR study of P700⁺⁸⁷ showed evidence that the spin delocalization could be accounted for by a monomeric species. The authors also noted an error in the previous theoretical analysis of the bandwidth [in which it was found that $\Delta H(\text{polymer}) = \Delta H(\text{monomer}) \times n^{1/2}$ where ΔH is the peak to peak first derivative width of the ESR band and n is the number of molecular units in the polymer].

It is believed that the primary electron acceptor in green plant photosynthesis is a pheophytin which then interacts with a quinone.⁸⁵ Figure 2.12 summarises the primary processes of photosynthesis in green plants.

In the photosynthetic bacteria the situation is a little different. There is only one photosystem, P870, but there is evidence from ESR and ENDOR measurements that here too the photoactive form of BChl a may be a dimer, and that the primary electron acceptor is BPheo a.¹³ Interestingly, the X-ray determination of the structure of a crystalline BChl a protein complex shows that there is hydrophobic interaction between the phytyl chain and some non-polar ring substituents and the protein

but there was no evidence for direct chemical interaction between neighbouring BChl a molecules, which are $\sim 12\text{\AA}$ apart. The major factor determining the orientation of the macrocycles arises from BChl a-protein interactions and not from BChl a-BChl a interactions. Ligation of the magnesium ion also appears to function in this way.¹⁰ It is not known whether this is typical of other chlorophyll-protein complexes.

To complete this section on the function of the Chlorophylls in photosynthesis it should be mentioned that there is a class of photosynthetic bacteria which do not contain chlorophyll. The Halobacteria convert light energy into chemical energy with the aid of the pigment bacteriorhodopsin. Although the efficiency of energy conversion is lower than that of chlorophyll containing organisms, these bacteria are adapted to survive in environments with a high salt concentration, many of them requiring a minimum 15% NaCl concentration (see Ref. 3, Chapter 5).

Figure 2.12 SCHEMATIC REPRESENTATION OF THE PRIMARY PROCESSES OF GREEN PLANT PHOTOSYNTHESIS

PHOTOSYSTEM I

PHOTOSYSTEM II

Key

(P680)= reaction centre form of Chl a, possibly monomeric; (P700)= reaction centre form of Chl a, possibly dimeric; D and Z are electron donors, not yet identified; X = primary electron acceptor of PSI, probably a monomeric Chl a; Q= electron acceptors of PSII; the primary acceptor is probably Pheophytin a and the secondary acceptor is a quinone. * denotes an excited state. The auxiliary pigments are carotenoids and/or phycobilins.

CHAPTER 3

ABSORPTION AND FLUORESCENCE SPECTRA OF THE CHLOROPHYLLS

3.1 Electronic structural effects and the optical absorption transitions

The basic porphyrin structure has at its centre a 16 member ring with approximate 4-fold symmetry containing 18 π electrons (Figure 3.1A).^{88,89,90} This gives rise to the typical porphyrin type absorption spectrum (Figure 3.2A) which is assigned to $\pi-\pi^*$ type transitions. The weaker bands in the 500nm-600nm region are called the Q bands (from Quasi allowed) and the very strong absorption bands in the 380nm-4430nm region are called the B bands or the Soret bands.

Magnesium porphyrin has D_{4h} symmetry and the x and y polarized transitions are therefore degenerate (Figures 3.1B and 3.2A). The presence in the free base of the two hydrogens reduces the 4-fold symmetry to D_{2h} (Figure 3.1A). As a result, the absorption bands are split into non-degenerate pairs of x and y polarized transitions (Q_x^+ , Q_y and B_x , B_y) (Figure 3.2A).

The same reduction in symmetry occurs when the porphyrin structure is reduced to a chlorin or bacteriochlorin, the structures on which the chlorophylls are based (Figure 2.3). In these molecules, the absorption spectrum is significantly affected by the perturbation of the π electron structure of the macrocycle caused by changes in the conjugation due to reduction of one or more of the pyrrole rings (Figures 3.2C and D). The high absorption strength of the red Q bands compared with the Soret absorption bands is a feature peculiar

Figure 3.1

Structures of porphyrin and magnesium-porphyrin, showing the appropriate symmetry of the π -electron system and the definition of the coordinates for defining x and y polarised optical transitions.

to the dihydro- and tetrahydro- porphyrins, including the chlorophylls.

External substitution of the macrocycle which does not significantly perturb the π -electronic structure does not have much effect on the optical absorption transitions. Protochlorophyll a (Figure 2:2) has the same peripheral substituents as Chl a but differs in the oxidation state of ring IV. This makes it a porphyrin type structure, rather than a dihydro-porphyrin, and as expected the Q absorption bands are much weaker than the Soret absorption bands (Figure 3.3A). However, the electronic structure is no longer square symmetric in the chlorophyll due to the presence of Ring V and a C=O substituent and the x and y polarized transitions are non-degenerate.

In Chl a and its magnesium free analogue Pheophytin a (Pheo a) the strength of the Q_y band is increased relative to that of the Soret absorption bands and the Q bands shift to the red (Figure 3.3B). Further reduction of the tetrapyrrole structure to BChl a or Bacteriopheophytin a (BPheo a) results in greater intensification of both the Q_x and Q_y absorption bands and a large red shift of the Q_y transitions into the near infrared region (Figure 3.3C). [Note also the absorption spectrum of the oxidised form of BChl a (2-acetyl-2-devinyl-Chl a) and its reversion back to the chlorin type spectrum in Figure 3.3C].

Figure 3.2

Absorption spectra of (A) porphyrin, (B) chlorin, (C) bacteriochlorin and their magnesium containing analogues to illustrate the effects of perturbation of the π -electron system by reduction of the tetrapyrrole structure and by changing the symmetry.

Figure 3.3

Absorption spectra of (A) Protochlorophyll a (a porphyrin); (B) Chlorophyll a (a dihydro-porphyrin); (C) Bacteriochlorophyll a (a tetrahydro-porphyrin); and the corresponding magnesium-free pheophytins. These spectra illustrate how successive perturbation of the π electron structure shifts the Q bands to the red and increases the absorption strength relative to the Soret bands.

Molecular orbital theory of the porphyrins has been developed by Gouterman and co-workers.⁹¹ Pariser-Parr self-consistent-field molecular orbital calculations were made by Weiss⁸⁹ in a theoretical analysis of absorption, fluorescence polarisation and circular dichroism results for the chlorophylls and related structures. A later study by Christoffersen *et al.* included *ab initio* configuration interaction wavefunctions and energies for ethyl chlorophyllide^{88,92} and ethyl bacteriochlorophyllide.⁹⁰ The latter results for the calculated transition energies are shown for comparison with the experimental absorption spectra of Chl *a* and BChl *a* in Figures 3.4 and 3.5. The polarisations of the calculated transitions of mixed x and y character are denoted by xy or yx depending on whether the transition was found to be predominantly x or y polarised respectively.

Experimentally, the positions of the Q_x bands of the chlorophylls seem to be particularly sensitive to the coordination of the magnesium ion. In hexacoordinated Chl *a* the $Q_x(0-0)$ transition lies at $\sim 625\text{nm}$, between the $Q_y(0-0)$ transition and $Q_y(0-1)$ transitions. In the pentacoordinate monomer, the $Q_x(0-0)$ transition is shifted to the blue and lies underneath the $Q_y(0-1)$ transition.

The effect is even more noticeable in BChl *a* since the Q_x bands are separated from the Q_y bands by $\sim 4000\text{cm}^{-1}$. In the Q_x region, the hexacoordinate species absorbs at $\sim 580\text{nm}$, and the pentacoordinate species absorbs at $\sim 600\text{nm}$.⁹³ All bands of BChl *a* seem to be more sensitive than those of Chl *a* to the effects of solvent on both the absorption strengths and the bandwidths.⁹⁴

Figure 3.4

Comparison of the experimental absorption spectrum of Chlorophyll a (A) (monomeric pyridinate) in hexane with the calculated transition energies ΔE , oscillator strengths and polarizations of ethyl chlorophyllide (B). The lower diagram is adapted from ref. 88.

Figure 3.5

Comparison of the experimental absorption spectrum of BChl a (A) (monomeric pyridinate) in hexane with the calculated transition energies ΔE , oscillator strengths (f) and polarizations of Et-BChl a (B). The lower diagram is adapted from ref. 90.

3.2 Effect of aggregation of the chlorophylls in solution on the visible absorption and fluorescence spectra.

The broadening and red shift of the main red and blue absorption bands of the chlorophylls in solution in dry non-polar solvents has now been accepted to be due to dimerization and oligomerization of the chlorophylls.^{15,80} Even more marked red shifts are observed in the absorption bands of adducts of chlorophylls formed with hydrogen-bonding polar ligands or water^{64,95,96,97} which mimic the absorption spectra of the chlorophylls in vivo^{98,99} (Figure 3.6).

When two chlorophyll molecules in the dimer approach and interact, each electronic level is split by the interaction.^{28,80,100,101} In oligomeric structures of n chlorophyll molecules there are in principle n components for each level of the monomer. Calculations and experiment show that generally the absorption bands of the oligomers are red shifted with respect to those of the monomer, and that the red shift in going from the monomer to the dimer is much larger than the red shift from the dimer to the tetramer or to larger oligomers.¹⁰² Whether or not optical transitions between each of the dimer and oligomer levels are allowed depends on the structure and nature of interaction of the chlorophyll molecules in the aggregate.

Sauer et al.⁸⁰ applied difference absorption spectroscopy to study the monomer-dimer equilibrium for three chlorophylls in solution, and the dimeric absorption spectra were analysed in terms of a simple exciton formalism.^{103,104} Two equal Q_y components were assigned to the dimer, with a splitting of $\sim 300\text{cm}^{-1}$.

Figure 3.6

Comparison of the absorption spectra of in vitro and in vivo Chl a:

(A) a solution of monomeric Chl a; (B) a concentrated (10^{-4} M) oligomeric solution of Chl a; (C) an active extract from a blue-green algae chloroplasts.

Shipman et al.¹⁰⁰ deconvoluted the absorption spectra of dimeric Chl a in solution into a number of Gaussian components and identified two components with the 'donor' and 'acceptor' forms of Chl a in the dimer. Later, the exciton theory of Shipman et al.^{28,101} which took into account environmental shifts was used to analyse the same data. Two dimer Q_y exciton components of unequal contributions; split by 310cm^{-1} were predicted from this model. Other exciton formalisms have been developed by Fong and co-workers^{26,105} and by Freed.²⁷ All these calculations are based on a 2 electron model in which the electronic states of the dimer are calculated by taking into consideration one ground state and two excited states arising from the ground and excited state of the monomer. Thus in this model the ground state splitting is taken to be insignificant, as in an excimer model. To first order, this type of model predicts that there should be one allowed and one forbidden dimer Q_y transition^f corresponding to the monomer Q_y transition.

More recently, Koningstein and Haley¹⁰⁶ have developed the four orbital model of Gouterman⁹¹ using a modified supermolecule model for the Chl a dimer. This method differs from previous treatments because the molecular orbitals of the dimer are calculated rather than the state functions. This method was necessary to develop the selection rules for the electronic Raman activity of the

^f Unless the x and y coordinates of both Chl a molecules in the dimer are parallel to one another, the definition of x and y polarised transitions in a dimer composed of two molecules in arbitrary orientation will not correspond to the definition of x and y polarised transitions in the monomer. The term dimer Q_y transition is therefore intended to indicate the origin of the transition with reference to the monomer transitions in that spectral region rather than the polarisation in this context.

dimer, but also gives a more complete description of the electronic absorption and emission transitions of the dimer. Thus the interaction energy of the two Chl a molecules in the ground state is taken into account by considering the interactions of electrons in the highest occupied and lowest unoccupied molecular orbitals of the two molecules. As shown diagrammatically in Figure 3.7, two allowed and two forbidden transitions in the dimer are expected to correspond to the single allowed Q_y transition in the monomer. Which two dimer transitions are allowed will depend on the structure and symmetry of the dimer.

Figure 3.7

Electronic energy level scheme for a Chl a dimer formed by interaction of two monomers. According to the model of Haley and Koningstein (ref.106) two of the four possible transitions in the dimer are allowed to first order. The splittings in the energy levels are related to the differences in the energy of the highest occupied and lowest unoccupied molecular orbitals of the dimer.

The strong red fluorescence of monomeric Chl a in solution is well known. There have been a number of recent investigations of laser induced fluorescence from monomeric Chl a^{21,104,108,109} and aggregated species of Chl a involving water¹¹⁰ or other nucleophilic ligands.^{111,112} Stimulated emission has been observed from Chl a, BChl a, and Pheo a.¹⁰⁹ Fluorescence^{17,107,113} and stimulated emission¹¹⁴ have also been reported for several covalently linked chlorophyll pairs. Wavelength selective laser excitation of fluorescence has been used to probe specific species in multicomponent systems.²⁰

In spite of a number of reports of red shifted emission from aggregated chlorophylls in non-polar solvents^{31,32,115,116,117,118,119} Chl a dimer and oligomer are generally considered to be essentially non-fluorescent, and the residual fluorescence has been attributed to impurities or hydrated species.^{29,33} This belief is based on the observations of Livingston et al.,^{120,121} over thirty years ago, that the fluorescence yield from a solution of Chl a in a dry hydrocarbon solvent was less than 10% of that of a solution in which the monomeric fluorescence was 'activated' by addition of water or other nucleophilic ligand. At that time the aggregation behaviour of Chl a in solution had not been established, and originally Livingston dismissed the idea of a non-fluorescent dimer in favour of a non-activated monomeric species of Chl a in order to account for the drop in fluorescence intensity. More recently however, it has been restated that the Chl a dimer is non-fluorescent based on these early results.^{30,120,121,122} Although laser light sources and superior detection systems have been available for several years, there appears to have been no thorough or

systematic investigation of the residual weak fluorescence which may arise from dimeric or oligomeric chlorophylls in solution until the present study, described in this thesis, was undertaken.

3.3 Synthetically linked chlorophyll pairs

Several covalently linked pairs of chlorophylls or related molecules have been prepared and their properties reviewed.^{29,123} Some structures and visible absorption spectra are shown in Figure 3.8. The spectral properties of some of the linked pairs have been examined by Bucks and Boxer.¹⁷

The two macrocycles in the cofacial Chl a pair¹⁷ (Figure 3.8A) and the Pyrochlorophyll a cyclophane pair¹²⁴ (Figure 3.8B) are held together in a fairly rigid orientation allowing only for some flexing about the hinge-like linkages. Interaction between the two rings can occur by π -electron overlap but not by acceptor-donor type C=O...Mg interactions. The relative orientations of the macrocycles and the coupling of the x and y polarised components of the optical transition moments results in an absorption spectrum for the cofacial pair in which the Q_y band is blue shifted compared with the corresponding band in the monomer. The cyclophane structure has absorption bands which are very similar to the monomeric units. It may be significant that in the latter structure the transition dipoles of the two rings are antiparallel, whereas in the cofacial pair the transition dipoles are rotated $\sim 90^\circ$ in one macrocycle relative to the other (Figure 3.8 A,B).

The singly linked pair¹²⁵ is interesting because it is more flexible and it is believed that several conformations are possible. In polar solvents, the pair exists in an open form with no significant interaction between the two macrocycles (Figure 3.8D) and each magnesium ion is 5 or 6 coordinated in the presence of nucleophilic ligands. The absorption spectrum then resembles that of the monomer. In dry non-polar solvents, the pair behaves like ordinary chlorophyll and

interacts to form a dimer similar to the structure shown in Figure 2.7 with a direct C=O...Mg linkage. On addition of a hydrogen bonding polar ligand such as water or an alcohol the dimer folds into the structure shown in Figure 3.7D.^{17,125} This conformation has a red shifted, broad Q_y absorption band compared with that of the monomer.

The fluorescence yield and lifetime of the folded dimer are of the same order of magnitude as those of the monomer in most solvents, but an unusual temperature dependence of the fluorescence yield was noted in methylene chloride solutions.¹²⁵ The Stokes shift between the absorption and fluorescence maxima is about $500\text{--}600\text{cm}^{-1}$ in the folded form. The folded form will produce stimulated emission at 733nm in wet benzene when pumped directly with a nitrogen laser at 337nm. Interestingly no stimulated emission was observed in the open conformation although the Chl a monomer itself has a strong stimulated transition at 680nm.¹¹⁴

A similar linked Bacteriochlorophyllide pair was also prepared¹²⁷ The absorption spectrum in the region of the Q_x band shows that the magnesium ion is 5-coordinated in the folded form. This conformation also shows only one set of ^1H nuclear magnetic resonances for each type of proton, which implies that the two rings are equivalent on the nuclear magnetic resonance time scale. In contrast, in dry non-polar solvents, the resonances of the dry dimer are broad and distorted, as observed for the usual Chl a dimers formed in solution.

It has been found that generally the fluorescence lifetimes of the doubly linked pairs of the chlorophylls or porphyrins are comparable to those of the corresponding monomers.^{14,16,17} On the other hand, the singly linked, conformationally mobile pairs tend to

have shorter lifetimes and lower fluorescence yields in the folded conformation.

Figure 3.8

The structures and absorption spectra of some synthetically linked chlorophyll pairs: A. Cofacial Chl *a* pair; B. Pyrochlorophyll *a* cyclophane pair; C. Singly linked Chl *a* pair in the folded form and D. in the open form. In the absorption spectra (adapted from ref. 17) the solid line is the spectrum of the pair, and the dashed line is the spectrum of the corresponding monomer or non-interacting conformation.

CHAPTER 4

SPECTROSCOPIC THEORY: ABSORPTION, FLUORESCENCE AND RESONANCE RAMAN SCATTERING

4.1 Absorption and Emission^{128,129}

From time dependent quantum mechanical calculations it may be shown that the rate of radiative transitions between states m and n of a molecular system are dependent on quantities called the Einstein coefficients A_{nm} , B_{nm} and B_{mn} which represent the transition probabilities. Consider a general case with a system having energies E_m and E_n (Figure 4.1) with non-degenerate levels, which may interact with an external electromagnetic field of density $\rho(\nu_{nm})$ at a frequency $\nu_{nm} = (\nu_n - \nu_m)$. The interaction with the radiation field may cause the system to absorb energy and pass from state m to n or to emit energy and pass from n to m . There are three cases to consider:

(i) Spontaneous emission

A system prepared in a state n may spontaneously emit radiation at a rate proportional to the population of state n , N_n , and the Einstein coefficient A_{nm} for spontaneous emission.

$$-\frac{dN_n}{dt} = N_n A_{nm}$$

stimulated
absorption and emission

$$\rho(\nu_{nm}) \neq 0$$

spontaneous emission

$$\rho(\nu_{nm}) = 0$$

Figure 4.1

Schematic diagram for the interaction of a two level system, with energies E_m and E_n , with a radiation field $\rho(\sigma_{nm})$

(ii) (Induced)^g Absorption

The rate of absorption induced by a radiation field of density $\rho(\nu_{mn})$ is given by

$$-\frac{dN_n}{dt} = N_m B_{mn} \rho(\nu_{mn})$$

(iii) Induced (or stimulated) emission

A radiation field $\rho(\nu_{nm})$ may induce a transition from n to m resulting in emission of radiation of energy $h(\nu_n - \nu_m)$

$$-\frac{dN_n}{dt} = N_n B_{nm} \rho(\nu_{nm})$$

A_{nm} , B_{nm} and B_{mn} are fundamental properties of the system and are independent of whether or not the system is in thermodynamic equilibrium.

It may be shown that the Einstein coefficients are proportional to the square of the transition moments, $\langle m | \mu | n \rangle$ between states m and n

$$\text{for example } B_{nm} \propto |\langle m | \mu | n \rangle|^2$$

where μ is the appropriate operator representing the interaction of the system with the radiation field. The significant interaction for normal optical transitions occurs via a change in electric dipole induced by the radiation field. (In some cases a magnetic dipole, electric quadrupole or other multipole interaction may also be important).

For a system in thermal equilibrium for which a Boltzmann distribution applies

$$\frac{N_n}{N_m} = \frac{e^{-E_n/kT}}{e^{-E_m/kT}} = e^{-h\nu_{nm}/kT}$$

At equilibrium, considering only radiative excitation and decay, the rate of emission is equal to the rate of absorption so that

^gNote that the presence of the radiation field at ν is necessary for all absorption and the term 'induced' is not usually specified.

$$N_n A_{nm} + N_n B_{nm} \rho(\nu_{nm}) = N_m B_{mn} \rho(\nu_{nm})$$

or

$$\rho(\nu_{nm}) = \frac{A_{nm}}{B_{mn} \{e^{-h\nu_{nm}/kT}\} - B_{nm}}$$

Comparing this with Planck's radiation equations for the case of black body radiation

$$\rho(\nu_{nm}) = \frac{8\pi h \nu_{nm}^3}{c^3} \{e^{-h\nu_{nm}/kT} - 1\}^{-1}$$

it can be seen that the Einstein coefficients are related as follows:

$$A_{nm} = \frac{8\pi h \nu_{nm}^3}{c^3} B_{nm} \quad \text{and} \quad B_{nm} = B_{mn}$$

Thus

$$B_{nm} = B_{mn} = \frac{2\pi^2 e^2}{3\epsilon_0 h^2} |\langle m | \mu | n \rangle|^2$$

The probability of induced emission $B_{nm}(\nu)$ is greater than the spontaneous emission probability only if

$$\rho(\nu_{nm}) > \frac{8\pi h \nu_{nm}^3}{c^3} = n(\nu_{nm}) h \nu_{nm}$$

where $n(\nu)$ is the number of molecules in mode ν . Thus in thermal radiation fields spontaneous emission dominates. However, with frequency selective excitation with a high intensity laser, optical pumping of the upper level may create highly non-Boltzmann distributions of the excited state populations and stimulated emission may dominate.

4.2 Non-radiative transitions

In general a multi-level molecular system may decay by non-radiative as well as radiative transitions. Non-radiative transitions may involve energy redistribution among the electronic, vibrational, rotational and translational degrees of freedom within one molecule or intermolecular energy transfer. Figure 4.2 shows a generalized Jablonski diagram for molecular de-excitation processes for a system with excited singlet states, S_1 to S_n , and triplet states, T_0 to T_n , typical of the chlorophylls. The term singlet and triplet refer to the spin multiplicity S of the state: $S = (2s + 1)$ where s is the total electron spin quantum number $s = \sum_i s_i$ and $s_i = \pm \frac{1}{2}$. Thus a state with all electron spins paired is called a singlet state because $S=1$, and a triplet state arises if $S=3$.

A radiative transition between two states of the same spin multiplicity is called fluorescence. Phosphorescence is a radiative transition between states of different multiplicity. Luminescence refers to an emission of unspecified nature.

By dipole selection rules, a change in spin quantum number is forbidden. Thus radiative transitions between states of different spin multiplicity are strictly forbidden. As shown in Figure 4.2 the singlet and triplet states are often depicted diagrammatically as separate manifolds, since formally they do not interact significantly unless the selection rules are broken down by spin-orbit coupling. This occurs if there is coupling of the electronic orbital angular momentum and spin angular momentum, as is often found in large molecules: the orbital angular momentum quantum number l and the spin quantum number s are then no longer independent, the spin multiplicity cannot be strictly defined, so all states may be said to have mixed singlet and triplet character.

Figure 4.2

Generalised Jablonski diagram to show radiative and non-radiative molecular de-excitation processes.

Transitions between states of different multiplicity are then allowed (e.g. phosphorescence) but the radiative lifetimes are typically nanoseconds (ns) to microseconds (μ s), or longer, several orders of magnitude longer than those of the strictly allowed transitions (i.e. fluorescence) which have radiative lifetimes in the order of picoseconds (ps) or nanoseconds (ns).

Non-radiative transitions within the same manifold are called internal conversions, and between manifolds of different spin multiplicity they are called intersystem crossings.

Thermal equilibrations of vibrational levels within an electronic level usually occurs within a few picoseconds. For this discussion, vibrational relaxation is therefore taken to be instantaneous compared with the decay of the zero phonon levels of the excited states because those of interest in the chlorophylls have lifetimes in the range 100ps - 10ns.

Kasha's rule states that emission is always observed from the lowest lying excited singlet state beneath the level excited. Often under ordinary low intensity illumination conditions this empirical rule is observed to be true. However, with laser excitation, significant populations of excited states may be created and the possibility arises of transitions from these states occurring with sufficient intensity to be detected. Observation of emission from the higher excited states of Chlorophyll a is discussed in Chapter 9.

- Following excitation of the system with, for example, a short (ns) laser pulse, the rate equation for the decay of an excited state n by both radiative and non-radiative processes takes the form (overleaf)

$$-\frac{dN_n}{dt} = (k_{\text{radiative}} + k_{\text{non-radiative}})N_n(t) = K N_n(t)$$

where $k_{\text{radiative}}$ is the sum of the rate constants for all radiative transitions from level n (fluorescence and phosphorescence) and $k_{\text{non-radiative}}$ includes all processes of internal conversion, intersystem-crossing and intermolecular energy transfer (if applicable). Clearly this equation represents a first order rate equation characterized by an overall rate constant K or lifetime $1/K = \tau$. The latter is the fluorescence or phosphorescence lifetime and this is the quantity which is directly observable by measurement of an emission decay curve. The fluorescence and phosphorescence intensity is directly proportional to the population $N_n(t)$ of the excited state which decays exponentially with a lifetime dependant on all (radiative and non-radiative) processes which contribute to de-excitation of the state. Fluorescence lifetimes will be denoted by τ_{fl} .

Somewhat confusingly, the lifetime of state m calculated from the absorption cross section is also called the radiative lifetime in some works. In this thesis the lifetime of a state determined from the absorption transition probability will be called the absorption, or absolute, lifetime τ_{abs} .

The ratio of the number of photons emitted as fluorescence to the number of photons absorbed is called the fluorescence quantum yield Φ_{fl} .

4.3 Linear and non-linear absorption¹²⁹

Under the illumination conditions encountered in a typical absorption spectrometer, the number of photons per unit volume incident on the sample is much lower than the number of molecules per unit volume N_n . The attenuation of the incident light intensity I in passing through a length dz of a sample with absorption coefficient ϵ is given by the Beer-Lambert law:

$$dI = I \epsilon dz$$

$$\text{or } I(z) = I_0 e^{-\epsilon z}$$

where I_0 is the incident intensity. The transmission is then defined as the ratio of the transmitted intensity, I , to the incident intensity, I_0 :

$$T = \frac{I}{I_0} = e^{-\epsilon z}$$

These equations imply that the attenuation is independent of the incident intensity.

Another common way of expressing the Beer-Lambert law is in terms of the molar concentration, c , the molar absorption (or extinction) coefficient, ϵ_M , and the absorbance which is defined as $A = -\log T$, so that

$$A = \epsilon_M c z$$

The absorption coefficient ϵ is a macroscopic property of the system which is wavelength dependent. It can be related to the complex refractive index, $N(\omega) = n'(\omega) + i\kappa(\omega)$. The dispersion $n'(\omega)$ is related to the dependence of the phase velocity on the frequency of the radiation, that is the refractive index, and the absorption coefficient is proportional to the imaginary part κ .

In order to link the classical description of absorption and dispersion [Kramers-Kronig equations] to the quantum mechanical description, a semiclassical quantity called the oscillator strength, f_{nm} ,

is introduced. In general, the absorption of a molecule in a state m is distributed among many transitions $m \rightarrow n$ to all higher levels n and each transition contributes a fraction f_{mn} to the total absorption strength. Thus, $\sum_m f_{mn} = p$ where p is the number of electrons contributing to the transitions $m \rightarrow n$.

The relationship between the absorption and dispersion for a damped electronic oscillator may then be described by the Kramers-Heisenberg response function for an applied radiation field of frequency :

$$N(\omega) = \frac{ne^2}{m_e} \sum_m \frac{f_{nm}}{\omega_m^2 - \omega^2 - i\omega\Gamma_{mn}}$$

$$\text{or } \kappa_n(\omega) = \frac{N_n e^2}{2\epsilon_0 m_e} \sum \frac{\omega f_{nm}}{(\omega_{nm}^2 - \omega^2) - \Gamma_{nm}^2 \omega^2}$$

$$\text{and } n'_n(\omega) = 1 + \frac{N_n e^2}{2\epsilon_0 m_e} \sum \frac{(\omega_{nm}^2 - \omega^2) f_{nm}}{(\omega_{nm}^2 - \omega^2) - \Gamma_{nm}^2 \omega^2}$$

It is found from these equations that the absorption coefficient is given by $\epsilon(\omega) = 4\pi\kappa/\lambda$

The oscillator strength f_{mn} is related to the Einstein coefficient B_{mn}

$$f_{mn} = \frac{2m_e \epsilon_0 h \omega_{mn}}{e^2} \cdot B_{mn}$$

and the absorption cross section, σ_{mn} , is simply the absorption per molecule:

$$\epsilon_{mn}(\nu) = \sigma_{mn}(\nu) N_m$$

and

$$\sigma_{\text{total}} = \int_{\nu} \sigma_{mn}(\nu) d\nu = (\lambda^2/8\pi) A_{nm}$$

and therefore these quantities are directly related to the square of the transition moment from n to m .

In principle, the absorption spectrum of a molecular system may be calculated by evaluating the transition moments

$$M_{mn} = \langle m | \mu | n \rangle$$

for transitions between all states n and m of the system. Such a quantum mechanical calculation is extremely complex for a large molecule. However, symmetry arguments can be used to determine whether or not a transition is allowed if calculations of intensities or absorption cross sections are not required.²⁶⁵ For a transition to be allowed, the transition moment must be non-zero. Another way of expressing this is that the product of the symmetry representations of the two states m and n must contain the irreducible representation to which one or more components of the dipole moment operator belongs. The latter transform as the Cartesian coordinates x, y, z . Thus for a system with a centre of symmetry, the dipole moment operator is ungerade (u) and for the transition to be non-zero, the transition must be from a gerade (g) state to an ungerade state or vice versa. Transitions between states of the same parity are therefore forbidden by normal dipole operator selection rules.

Non-linear absorption¹²⁹

With laser excitation it is possible to create significant population of an excited state n which may absorb at the same frequency ν_{mn} as the ground state. In this case the transmission of the sample at ν is given

$$\text{by } \frac{I}{I_0} = e^{-\sigma_{mn}(\nu)N_m} + e^{-\sigma_{nr}(\nu)N_n}$$

The relative populations of the two states, N_n and N_m , are dependent on the incident power and the dynamics of radiative and non-radiative interactions between all levels in the system. In this case

Beer's law is no longer applicable and the transmission of the sample is dependent on the incident intensity. In the general case of a multilevel system in which many levels absorb at the incident frequency

$$\frac{I}{I_0} = \sum_m e^{-\sigma_{mn}(\nu) N_m}$$

In theory, the dynamics of a molecular system may be treated by solving the Master equations for the rates of population and decay of each level to determine the dependence of the excited state populations N_m on the incident intensity. In reality the calculation is intractable for a multilevel system such as chlorophyll unless a realistic approximation to the dynamics of the system can be modelled with only a few levels. For example, Wylie¹³⁴ has examined the non-linear absorption of β -carotene. Figure 4.3 shows a typical non-linear transmission curve for such a system, which can be modelled using a 3-level system. The gradients and plateaux of the curve can be related to the relative magnitudes of the cross sections for the various excitation and decay processes.

Even if most of the parameters are not known, and a theoretical treatment is not possible, experimental determination of the power dependence of the transmission of a sample at the excitation frequency is indispensable in excited state spectroscopy. The optimum conditions for obtaining effective saturation of a particular excited state can be found from a transmission curve as shown in Figure 4.3 because this shows up as a plateau in the curve (ie. in the region in which the excited state is effectively saturated, but there is insufficient incident power to produce significant absorption to the next highest excited state, there is a plateau because Beer's law holds for the excited state absorption).

Figure 4.3

Diagram to show the typical dependence of the transmission of a sample on incident laser power under conditions of optical pumping, for the case in which the cross-section from the ground state to the first excited state is greater than the cross section from the first excited state to the second excited state.

Two photon absorption

Multiphoton absorption processes may occur with significant probabilities at high incident photon fluxes. For optical transitions there are two 2-photon processes of interest to the chlorophyll studies. These are illustrated schematically in Figure 4.4.

Consecutive two photon absorption depends on first creating population of state r , which then absorbs another photon at the same energy, creating an excited state m . The intermediate state is resonant with the radiation field (Figure 4.4A).

In the second case there is no intermediate state (Figure 4.4B). This is true two photon absorption. Since the selection rules for the two photon process allow transitions between states of the same parity, states which are not accessible by one photon absorption may be probed.

The dependence on the intermediate states r arise from the quantum mechanical description of the reaction of the system to a perturbation in terms of eigenfunctions and energies of all possible states of the system. The state r may in fact lie above or below the initial or final states of the system.

The damping constants are inversely proportional to the lifetimes of the states r and Γ_{nm} is related to the width of the transition $m \rightarrow n$. the damping of the state is due ultimately to spontaneous radiative or non-radiative transitions from that state to other states.

The form of the polarizability tensor elements given on the previous page give no indication of which vibrational modes are subject to resonance enhancement. Several groups have approached this problem in different ways. For example, vibronic expansion theories were introduced by Albrecht,¹⁴³ and developed by Tang and Albrecht¹⁴⁴ and by Behringer.¹³⁵ Introducing the vibrational quantum numbers v , v' and v'' for states m , n and r respectively, the form of the polarizability tensor components will now appear as

$$(\alpha_{\rho\sigma})_{m v n v'} = \sum_{r, v''} \left[\frac{\langle m v | M_{\sigma} | r v'' \rangle \langle r v'' | M_{\rho} | n v' \rangle}{E_{r v''} - E_{m v} - E_0 + i \Gamma_{r v''}} + \frac{\langle m v | M_{\rho} | r v'' \rangle \langle r v'' | M_{\sigma} | n v' \rangle}{E_{r v''} - E_{n v'} + E_0 + i \Gamma_{r v''}} \right]$$

Albrecht used the adiabatic approximation to separate the wavefunctions and then expanded the electronic part of the wavefunctions in a Taylor series in nuclear displacements using the Herzberg Teller formalism.

It was then argued that the vibrations subject to resonance enhancement are those which are responsible for mixing of the two relevant electronic states.

Having expanded each term in the equation, Albrecht and Hutley¹⁴³ showed that by considering resonance processes for the cases $n=m$ and $n \neq m$,

4.4 Theory of Light Scattering (Raman) Spectroscopy^{128,135}

When monochromatic radiation at frequency ν_0 is incident on a molecular system, some of the energy is absorbed, some is transmitted without change and some is scattered. Analysis of the frequency of the scattered radiation shows that in addition to the component at ν_0 there may be components at certain frequencies $\nu = \nu_0 \pm \nu_{mn}$ where ν_{mn} is associated with a transition between rotational, vibrational or electronic energy levels in the system. The polarisation of the scattered radiation may also be different from that of the incident radiation.

The elastically scattered radiation at ν_0 is called Rayleigh scattering. The radiation scattered with a change in energy corresponding to a transition energy of the system is called Raman scattering, and was first reported by C.V. Raman and K.S. Krishnan¹³⁶ in 1928. Landsberg and Mandelstam¹³⁷ discovered the effect independently and published an account of their results later in the same year. The Raman effect was predicted by Smekal in 1923.¹³⁸

The spectrum of the scattered radiation of Raman bands or lines constitute the Raman spectrum of the system, with frequencies $(\nu_0 - \nu_{nm})$ called the Stokes bands and frequencies $(\nu_0 + \nu_{nm})$ called the anti-Stokes bands (Figure 4.5).

Rayleigh scattering always accompanies Raman scattering. Typically, Rayleigh scattering is 10^{-3} times the intensity of the incident radiation. Raman intensities are generally 10^{-6} times the intensity of the exciting radiation. Whereas normal absorption and emission are first order processes involving distinct single photon events, Raman scattering is a second order process involving simultaneous annihilation of a photon

Figure 4.5
Schematic level diagrams of Stokes and anti-Stokes Raman scattering

at the incident energy and creation of a second photon at the scattered energy. The wavelength of fluorescence or phosphorescence from a molecule is characteristic of that system and is independent of the excitation frequency. However, Raman bands are not characterised by an absolute frequency, but by the shift, or difference, from the exciting frequency, i.e. $\nu_0 \pm \nu_{mn}$. For this reason luminescence and Raman scattering may be distinguished by changing the excitation frequency: the luminescence bands will remain at the same absolute frequency while the Raman bands will shift by the same amount of energy as the exciting frequency. This means that by appropriate choice of exciting frequency the Raman spectrum of even low frequency ($\sim 100\text{cm}^{-1}$) vibrational and rotational transitions can be observed in the visible region of the spectrum for which efficient light detectors exist.

The theoretical explanation of the Raman effect is considerably more complex than that of the first order absorption and emission processes. Also, normal pre-resonance Raman scattering is inherently a weak second order effect and in the past experimental techniques were developed more slowly than other types of spectroscopy for studying electronic, vibrational or rotational transitions in molecular systems (e.g. ultra-violet-visible absorption, infrared and microwave absorption). Indeed Raman spectroscopy remains a research tool rather than a routine spectroscopic technique.

Progress in resonance Raman scattering increased dramatically with the application of lasers. The high photon fluxes available from laser light sources allow significant Raman intensities to be measured, even allowing for the low quantum yield of Raman scattering. In addition the

resolution of a Raman spectrum is dependant on the bandwidth of the exciting radiation, so a laser is an ideal source.

Of particular importance to modern studies is the resonance Raman effect: this is the enhancement of certain vibrational Raman band intensities by factors of up to $\sim 10^3$ when the exciting frequency approaches that of an allowed electronic transition. Tunable lasers provide a method of investigation of the wavelength dependence of Raman intensities and a plot of the Raman intensity versus the excitation frequency is called a "Raman excitation profile" or REP. The relationship between the electronic absorption spectrum and the Raman excitation profile is now a subject of active research^{139,140} because the REP can provide a great deal of information about electronic and vibrational structure which may not be resolved in a normal absorption spectrum.

Whereas many aspects of the theory of pre-resonance Raman scattering may be treated satisfactorily by classical methods, the explanation of the resonance Raman effect requires a quantum-mechanical approach. However, some qualitative insight into the more important aspects of Raman scattering may be obtained first by considering the classical polarizability theory originally developed by Placzek,¹⁴¹ which was formulated specifically for the non-resonance case.

The classical ground state polarizability theory of the Raman effect

Smekal's original prediction of the Raman effect¹³⁸ was based on quantum mechanical arguments, but soon after the discovery of the phenomenon Cabannes and Rocard showed that the essential features of Raman scattering may be interpreted classically.¹⁴² Subsequently, Placzek developed his polarizability theory for vibrational Raman scattering in which the

vibrational Raman effect originates from a parametric dependence of the molecular polarizability (α) on the nuclear coordinates. The polarizability links the electric moment induced in the scattering molecule, $\underline{P}$, to the electric field of the incident radiation, $\underline{E}$: $\underline{P} = \alpha \underline{E}$

In general α is therefore a tensor

$$\alpha = (\alpha_{\rho\sigma})$$

where ρ and σ are molecule or space fixed cartesian coordinates. Thus

$$\alpha = \begin{pmatrix} \alpha_{xx} & \alpha_{xy} & \alpha_{xz} \\ \alpha_{yx} & \alpha_{yy} & \alpha_{yz} \\ \alpha_{zx} & \alpha_{zy} & \alpha_{zz} \end{pmatrix}$$

The basic idea of Placzeks theory is that α is expanded as a power series in the normal coordinates q_i about $q_i = 0$. Considering for simplicity just one normal mode q :

$$\alpha(q) = \alpha(q=0) + q \left(\frac{\partial \alpha}{\partial q} \right)_0 + \frac{1}{2} q^2 \left(\frac{\partial^2 \alpha}{\partial q^2} \right)_0 + \dots$$

If this series converges, normally only the first few terms need to be considered and the induced dipole moment μ caused by the incident radiation at frequency ω is given by

$$\mu = \alpha_0 E_0 \cos \omega_0 t + \sum_i \frac{1}{2} \left(\frac{\partial \alpha}{\partial q_i} \right)_0 \cdot q_i E \left\{ \cos[(\omega_0 - \omega_i)t - \delta_i] + \cos[(\omega_0 + \omega_i)t + \delta_i] \right\}$$

+ higher order terms

The first term leads to Rayleigh scattering at the same frequency as the incident radiation and is determined by the actual polarizability tensor α . The second term represents Raman scattering at frequencies $(\omega_0 \pm \omega_i)$ which is determined by the derived polarizability tensor. The higher order terms lead to such effects as hyper-Raman scattering.

From the classical radiation theory, the total intensity per molecule from Raman scattering is given by

$$I_{nm} = \frac{2^7 \pi^5}{9c^4} I_0 (\nu_0 \pm \nu_m)^4 \sum_{\rho\sigma} |(\alpha_{\rho\sigma})_{nm}|^2$$

Note the dependence on the fourth power of the exciting frequency.

Since the Raman effect in the classical theory derives from the same polarizability as is responsible for Rayleigh scattering, the tensor is real and symmetric. If the ground state is non-degenerate, the exciting frequency is far from resonance (i.e. the exciting energy is both much greater than a vibrational frequency and much smaller than an electronic transition energy) and in the absence of magnetic perturbations, including spin orbit coupling and other external fields, then the Raman polarizability tensor is frequency independent and a function only of the nuclear coordinates. Of course these conditions are seldom met in practice.

Semiclassical and quantum mechanical approaches to the theory of the resonance Raman effect.¹³⁵

The classical theory cannot be used to explain Raman intensities under near-resonance or resonance conditions when the exciting frequency approaches that of an allowed electronic transition. The quantum mechanical approach is based on the Kramers-Heisenberg-Dirac dispersion equations. By using time-dependent perturbation theory with classical treatment of the radiation field, the polarizability tensor can be related to the wavefunctions and energy levels of a molecular system. In place of the classical induced moment $\underline{P} = \underline{E}$ one considers the transition moments

P_{mn} for the transition $m \rightarrow n$ in question. It is found that the general form of the $\rho\sigma$ th component of the scattering tensor is then given by the following sum over all vibronic states of the molecule

$$(\alpha_{\rho\sigma})_{nm} = \sum_r \left[\frac{\langle m | M_\sigma | r \rangle \langle r | M_\rho | n \rangle}{E_r - E_m - E_0 + i\Gamma_r} + \frac{\langle m | M_\rho | r \rangle \langle r | M_\sigma | n \rangle}{E_r - E_n + E_0 + i\Gamma_r} \right]$$

where m and n are the initial and final states of the molecule and r is an 'intermediate' state (see below). E_0 ($=h\nu_0$) is the energy of the exciting radiation and Γ_r is a damping constant, which prevents the denominator at resonance from reaching zero. The integrated intensity is equivalent to that found classically (equation on page 69).

When $\nu_r - \nu_m - \nu_0$ becomes small, one element in the summation corresponding to the resonant electronic transition becomes dominant. The magnitude of $\alpha_{\rho\sigma}$ is then a function of the oscillator strength of this transition.

Although the general form of the tensor is similar to that found classically, $\underline{P}$ and $\underline{E}$ are complex and the components of the polarizability tensor may also be complex. In practice it is not usually possible to evaluate α but the form of the scattering tensor in terms of the transition probabilities and energy levels of the system can give insight into the mechanism of the process on a molecular level.

The numerators of the transition probability components always contain products of transition moments between the initial state m and state r and between the final state n and state r . If the scattering tensor is to be non-zero there must be at least one state r which has non-zero transition moments to the initial and final states. However this does not mean that scattering involves two distinct steps with r as an intermediate state, and $h\nu_0$ does not have to equal $h\nu_r$.

The dependence on the intermediate states r arise from the quantum mechanical description of the reaction of the system to a perturbation in terms of eigenfunctions and energies of all possible states of the system. The state r may in fact lie above or below the initial or final states of the system.

The damping constants are inversely proportional to the lifetimes of the states r and Γ_{nm} is related to the width of the transition $m \rightarrow n$. the damping of the state is due ultimately to spontaneous radiative or non-radiative transitions from that state to other states.

The form of the polarizability tensor elements given on the previous page give no indication of which vibrational modes are subject to resonance enhancement. Several groups have approached this problem in different ways. For example, vibronic expansion theories were introduced by Albrecht,¹⁴³ and developed by Tang and Albrecht¹⁴⁴ and by Behringer.¹³⁵ Introducing the vibrational quantum numbers v , v' and v'' for states m , n and r respectively, the form of the polarizability tensor components will now appear as

$$(\alpha_{\rho\sigma})_{m v n v'} = \sum_{r, v''} \left[\frac{\langle m v | M_{\sigma} | r v'' \rangle \langle r v'' | M_{\rho} | n v' \rangle}{E_{r v''} - E_{m v} - E_0 + i \Gamma_{r v''}} + \frac{\langle m v | M_{\rho} | r v'' \rangle \langle r v'' | M_{\sigma} | n v' \rangle}{E_{r v''} - E_{n v'} - E_0 + i \Gamma_{r v''}} \right]$$

Albrecht used the adiabatic approximation to separate the wavefunctions and then expanded the electronic part of the wavefunctions in a Taylor series in nuclear displacements using the Herzberg Teller formalism.

It was then argued that the vibrations subject to resonance enhancement are those which are responsible for mixing of the two relevant electronic states.

Having expanded each term in the equation, Albrecht and Hutley¹⁴³ showed that by considering resonance processes for the cases $n=m$ and $n \neq m$,

the polarizability elements could be expressed as the sum of three sets of terms:

$$(\alpha_{p\sigma})_{mn} = A + B + C$$

The A terms include contributions from vibrational interactions with a single excited state by way of Franck-Condon overlap integrals and the B terms involve vibronic mixing of two excited states. The last term if included represents non-adiabatic coupling terms. In pre-resonance Raman scattering the A and B terms are distinguished by their different frequency dependences.

The approach of Peticolas and co-workers was based on third order perturbation theory, including vibronic coupling terms in the Hamiltonian.¹⁴⁶ Warshel and co-workers¹⁴⁷ calculated transition dipole matrix elements using adiabatic wavefunctions which were expanded in displacements of the normal coordinates which leads to a B term which is different from that of Albrecht. Other semiclassical treatments were developed by Shorygin et al., Savin and Verlan.^{148,149}

Jacon et al. developed a purely quantum mechanical theory based on the resolvent operator formalism of quantum electrodynamics.¹⁵⁰

All these theories can be distinguished by the level of approximation in defining the excited states associated with a resonant transition. The simplest approach takes the excited state wavefunctions to be products of crude adiabatic wavefunctions and vibrational wavefunctions. The Condon or A term approximation considers only the diagonal vibronic coupling terms which implies there is no variation in the electronic transition moment with the nuclear coordinates. If changes in force constants are neglected, then the only vibronic effect is the shift in

equilibrium of the excited state potential surface with respect to that of the ground state which is included via the diagonal vibronic coupling terms. When only one electronic state is considered to be present in the resonance region, this set of approximations has come to be known as the standard assumptions.

Deviations from the standard assumptions included variation of electronic transition moments with the nuclear coordinates which lead to the non-Condon terms, or the B and C terms in Albrecht's terminology.

In reality it may be necessary also to consider higher order terms which are related to force constant changes, mixing of vibrational modes, anharmonicity and the existence of more than one electronic state in the resonance region.

In general there are many normal modes and in a large molecule, such as chlorophyll, the many dimensional vibrational subspace and its coupling to an electronic transition that is active in resonance Raman scattering is called the multimode problem. Clearly this leads to a problem of great complexity because of possible coupling of the large number of modes and the prohibitive sum over all vibrational modes.^{145,151}

Siebrand, Zgierski and co-workers¹³⁹ have discussed the multimode problem with respect to Raman excitation profiles. They also considered the effects of two overlapping resonant electronic levels, which may occur for example in a molecular dimer. The latter was shown to lead to strong interference effects in the Raman scattering, which will be discussed briefly in Chapter 10.

In reality a compromise between the number of modes treated and the accuracy of the wavefunctions is made. Usually the true multimode

approach is sacrificed in favour of a single, two or three mode approximation. This limitation has serious consequences because the Raman excitation profiles and the corresponding absorption bands which may be broad because of multimode contributions must be analysed in terms of artificially large damping factors. When the full subspace of vibrational modes is included, certain phenomena are predicted which are not apparent by the superposition of single mode theories.

The most important aspect concerns the relationship between the absorption bandshape and the resonance Raman intensity as a function of wavelength in the region of the absorption band. A major development in the theory of the resonance Raman effect came with the transform methods of Hiznyakov and Tehver.^{135,145,152,153} This theory presents "secondary radiation" in the language of time correlation theory, based on the time integral form of the Kramers-Heisenberg expression for the linear polarizability. The definition of secondary radiation includes all one photon forms of radiation which may emerge from a sample. This approach is interesting because it distinguishes luminescence and scattering processes in terms of dephasing. The distinction between resonance Raman scattering and resonance fluorescence, which has been the source of great discussion in the literature, is described in the following way. Rayleigh and Raman scattered photons are emitted prior to dephasing of the intermediate state and light appearing after dephasing of the intermediate state is described as luminescence.

More significantly, the transform techniques which are now under development by several groups [including Tonks and Page¹⁴⁰ Heller et al.¹⁵³ and Albrecht and co-workers¹⁴⁵] bring out the connection between the optical absorption and resonance Raman excitation profiles.

For example, Tonks and Page show that for linear vibronic coupling the lineshape for the first order Raman excitation profile of a mode f of a multimode system is given by

$$\omega_L (\omega_L - \omega_f)^3 |\Delta(\omega_f) \Phi(\omega_L)|^2$$

where ω_f is the vibrational frequency and

$$\Delta(\omega_f) \Phi(\omega_L) \equiv \Phi(\omega_L) - \Phi(\omega_L - \omega_f)$$

The complex susceptibility is related to the optical absorption through a Kramers-Kronig analysis. Thus, the Raman excitation profile is simply proportional to the square modulus of the difference between the molecular polarizability at the incident frequency ω_L and the molecular polarizability at the scattered frequency $\omega_L - \omega_f$.

The vibronic models and the transform techniques have been shown to yield similar results^{144,155} but the transform techniques have the advantage that multimode effects may be included more readily and the calculations performed rapidly while formally avoiding the vibrational sum-over-states which involves directly evaluating the vibrational overlap integrals.

The relationship between the Raman excitation profile and the electronic absorption spectrum will be further discussed in Chapter 10 with reference to the unusual results obtained for the resonance Raman excitation profile of the Chlorophyll a dimer in solution.

CHAPTER 5

SPECTROSCOPIC INSTRUMENTATION, TECHNIQUES AND SAMPLE PREPARATION

Figure 5.1 shows a schematic diagram of the laser spectroscopy laboratory, which is equipped with lasers, monochromators and detection equipment which may be used interchangeably for time resolved fluorescence or Raman spectroscopy.

5.1 The lasers

Nitrogen laser and dye laser system

The Lambda Physik M2000 nitrogen (N_2) laser produces 3.2ns pulses at 337nm and was operated at a repetition rate of 25Hz. The peak power was 1MW. The N_2 laser pumps a two stage Lambda Physik tunable dye laser, model FL2000, capable of providing wavelengths over the visible range from 380nm to 700nm using a range of laser dyes. The maximum average power obtainable from the dye laser is 5mW, corresponding to a peak power of 2×10^4 W or $\sim 10^{13}$ photons per pulse.

The excimer laser and dye laser system

The Lumonics TE 432-2 excimer laser may be operated with a number of rare gas halide or molecular gas systems to provide ~ 10 ns laser pulses in the ultraviolet region. For this work the XeCl system was chosen since this gives a longer lasting and more stable output. The lasing transition is from a level of the metastable XeCl* excimer produced by electrical discharge excitation of a mixture of Xe and HCl in a buffer gas (He). This system produces ~ 10 ns laser pulses at a variable repetition rate (20-200Hz). The laser was usually operated at 25Hz to prolong the life of the dyes.

2

Figure 5.1

Schematic diagram of lasers and detection systems for nanosecond pulsed laser light scattering and fluorescence spectroscopy.
 M_1 and M_2 are mirrors, D_1 and D_2 are dispersing prisms.

TABLE I

LASER DYES

Laser Dye	Manufacturer	Solvent	Concentration (g l ⁻¹)	Lasing wavelength (nm)
BPBD	Lambda	Toluene	1.4	395-410 ^a
-NPO	Lambda	Toluene	0.075	400 ^a 395-410 ^b
DPS	Lambda	Dioxane	Saturated solution	405 ^a
POPOP	Exciton	Toluene	1.24	416 ^a
Coumarin {120 440}	Lambda	Ethanol	1.09	427-450 ^a
Coumarin 460	Exciton	Ethanol	2.3	460 ^a
Coumarin 152A	Lambda	Ethanol	2.6	500-520 ^a
Coumarin 153	Lambda	Ethanol	3.1	540 ^a
Rhodamine {6G 590}	Lambda	Ethanol	2.3	585-610 ^a
Rhodamine {B 610}	Lambda	Ethanol	2.5	580-620 ^a
Oxazine 170	Lambda	Ethanol	0.16	720 ^a
Nile Blue	Lambda	Ethanol	0.32	696 ^b
Nile Blue + Rhodamine B	Lambda	Ethanol	0.32 2.5	various mixtures can be made which lase in the region 672 - 700nm. ^b

^apumped with the nitrogen laser (337nm).^bpumped with the excimer (XeCl*) laser (308nm).

The excimer laser pumps a Lumonics EPD 330 pulsed dye laser which has a tuning range from 320nm to 950nm. The pulse width is ~ 2 ns narrower than that of the pump laser, with 95% vertical polarization of the 1 x 2mm output beam. The average power output was ~ 5 mW for the blue dyes.

Laser dyes

Table I lists the laser dyes used and their useful tuning ranges.

Helium-Neon laser

A 0.5mW Coherent Radiation helium-neon cw laser was used for alignment and for excitation of the cw fluorescence spectrum of Chl a at 632.8nm. For the latter a Keithley Instruments model 417 high speed, picoammeter was used to measure the signal from the photomultiplier tube.

5.2 Optical systems

Filtering of laser radiation

Since the emission of the laser dyes contains a strong broad fluorescence background (superradiance) underneath the lasing transition, for experiments requiring monochromatic radiation the superradiance must be eliminated. This was done by placing a Pellin-Broca dispersing prism (see appendix 2) directly in front of the output port of the dye laser. If necessary, a tunable grating filter (PTR 100) could also be used, but this resulted in a reduction in power of $\sim 50\%$.

Collection and focussing optics

For fluorescence studies, emission was collected at 90° to the laser beam, and focussed into the entrance slit of the monochromator with two lenses, a large diameter 10cm focal length lens and a smaller 10cm focal length lens directly in front of the entrance of the spectrometer as shown

in Figure 5.2A. Various lenses were used for focussing. For the most intense focussing a 28mm microscope objective lens was used. In some cases the beam was expanded with a long focal length lens.

For the Raman studies with optically dense samples, the 90° scattering geometry was unsatisfactory except at wavelengths at which the sample absorbed only weakly. The alternative arrangement is shown in Figure 5.2B. The laser radiation was focussed with a 10cm focal length cylindrical lens onto one face of the sample cell at an angle of incidence of 60° . The scattered radiation was collected at 90° to this face. The trace of the laser beam on the cell formed a horizontal stripe which lay in the same plane as the optical axis of the collection optics and the entrance slit of the monochromator.

Power measurements and transmission studies

The laser power was measured with a Photodyne 66XLA power meter fitted with a number 350 silicon detector head. This meter was also used as the detector for the power dependent transmission studies. In the latter case the laser radiation was attenuated as necessary using a set of neutral density filters (Ealing, 0.1dB to 3dB). The neutral density filters were calibrated with the power meter at the desired wavelength because at higher powers the attenuation is power dependent and the cw calibration is not reliable. In the earlier experiments a pair of Glan-Thompson polarizing prisms was used for attenuation of the laser radiation.

The power dependent transmission of a sample was simply measured by determining the attenuation of the laser power by the sample contained in a 1mm path length quartz cell. If required, the laser beam was focussed into the sample cell to obtain higher photon densities, and a 10cm focal length lens was used to collect the transmitted light into the power meter.

Figure 5.2

Schematic diagram of collection and focussing optics: A) 90° scattering geometry used for fluorescence spectroscopy; B) 60° angle of incidence used for Raman and fluorescence studies with samples of high optical density.

The monochromators

For the red fluorescence studies, the emission was dispersed with a Jarrell-Ash 3/4 metre single monochromator with gratings blazed for maximum throughput at $\sim 500\text{nm}$. For the Raman experiments the Jarrell-Ash monochromator does not have sufficient rejection of elastically scattered light and the Jobin-Yvon Ramanor HG-2 double monochromator was used. The latter has curved holographic gratings and gives a rejection of 10^{-8} at 20cm^{-1} from the Rayleigh line, and a resolution of 0.5 cm^{-1} at 514.5nm . The scanning range is 25000cm^{-1} to 12500cm^{-1} , but the resolution declines significantly above 22000cm^{-1} .

5.3 Detection systems

Photomultiplier tubes

Selected parameters and response curves for the photomultiplier tubes (PMTs) used in this work are given in Table II. The characteristics of these PMTs are discussed in detail in Chapter 6, where experiments to test their performance under pulsed operation are described.

Boxcar averager and amplifiers

After passing through the monochromator, the dispersed emission was detected with a fast rise-time PMT and if necessary the signal was pre-amplified by a factor of $\times 10$ or $\times 100$ by fast pulse preamplifiers (Le Croy, 101A) to provide a minimum signal of 10mV . The signal was then fed via delay boxes (EG&G DB263) to the PAR Boxcar averager (Model 162 Mainframe) equipped with Model 164 (minimum gate 10ns) and Model 165 (minimum gate 2ns) gated integrators. The signal from the boxcar averager was recorded on a strip chart recorder or fed via an analogue to digital converter to a Motorola 6800 microcomputer which controls the Ramanor HG2

TABLE II

SELECTED PARAMETERS OF PMTs UNDER PULSED AND CONTINUOUS ILLUMINATION

PMT	High voltage supply (V)	Anode pulse rise time (ns)	Anode pulse decay time (experimental) (ns)	Maximum average anode current CW illumination (A)
RCA 7102 ^a	1500	2.4	2.5	10×10^{-6}
RCA C31034A ^b	2000	2.6	2.5	100×10^{-9}
RCA 8850 ^c	2500	2.5	4.5	0.2×10^{-3}
ITT FW 130 ^d	1800	10	25	0.3×10^{-3}

^aRCA Technical Report. 7102-S-65.^bRCA Data Sheet C31034A.^cRCA Electronic Components Data Sheet 8850^dElectron Tube Division (ITT) Data Sheet FW 130

monochromator.

Triggering of the boxcar was accomplished via a signal generated from the N_2 laser pump beam by a ultraviolet sensitive photodiode placed in each of the dye laser compartments. Delay of the signals from the PMT was necessary because the boxcar has a dead time of 70ns after triggering before the gate first opens. This is shown schematically in Figure 5.3. To prevent unstable triggering, the photodiode pulse was checked periodically with a Tektronix 7633 oscilloscope.

5.4 Time resolved spectroscopy

There are two ways of recording spectra with the boxcar averager and monochromator detection system. The gate of the boxcar may be set to open at a specific time in the range 0 to 1000ns or it may be scanned within certain time ranges. Thus a spectrum of emission or scattering intensity may be recorded by setting the gate of the boxcar at a particular delay and scanning the monochromator to obtain a spectrum of intensity versus wavelength, or by setting the monochromator to a certain wavelength and scanning the gate of the boxcar to obtain a plot of intensity as a function of time as for measuring a fluorescence decay curve. This is illustrated in Figure 5.4.

5.5 Absorption spectrometers

Visible and ultraviolet absorption spectra of the chlorophylls were run on a Varian DMS 90 ultraviolet/visible absorption spectrometer. Measurements of the near infrared absorption band of water at 19600Å for checking the effectiveness of solvent drying were run on a Cary 14 absorption spectrometer.

Figure 5.3

Schematic diagram to show the effect of the delay circuitry in the detection system.

Figure 5.4

The time resolved laser spectroscopy system in principle allows a three dimensional map of emission or scattering intensity as a function of both wavelength and time to be built up. In practice spectra can be recorded in two ways: A. The boxcar gate is set to a particular time and a scan of intensity versus wavelength is made, or B. The monochromator is set to a particular wavelength and a scan of intensity versus time is made by scanning the boxcar gate over a fixed time range.

5.6 Sample preparation

The Chl a samples used for the initial experiments were obtained from Sigma Chemicals and used without further purification. However, the results were inconsistent and all other Chl a samples were extracted and purified in the laboratory.

Chl a was extracted from spinach and purified by chromatography first on DEAE Sepharose CL-6B and then on Sepahrose CL-6B according to the method of Omata and Murata.¹⁵⁶ The advantages of this method are discussed in the following section. BChl a was obtained from Porphyrin Products Inc. and its purity was assessed as described on page 90.

The purified chlorophylls were dried by codistillation²⁹ with dried benzene or carbon tetrachloride (Baker Photrex spectral grade). These solvents, and the hexane (Caledon Labs. spectral/HPLC grade), used to make up the sample solutions were dried by adsorptive filtration through neutral activated alumina (Woelm, activity 1).^{157,158} Sample preparation was carried out in a dry nitrogen purged glove-box to exclude moisture and oxygen from the samples. All glassware was dried for at least 24 hours at 200°C. The quartz sample cells which were used for fluorescence and Raman spectroscopy were filled inside the glove-box and sealed with vacuum tight teflon stopcocks. The use of vacuum grease on the vacuum line inside the glove box was kept to a minimum and grease was not allowed to come into contact with the flasks or optical cells containing the solvents or Chl a samples.

The effectiveness of solvent drying with activated alumina was monitored by checking the near infrared absorption band of water at

19600Å.¹⁵⁹ This band was chosen rather than that of the OH stretch at 3000cm^{-1} because it was possible to measure the former with the sample solution remaining sealed in the 10mm quartz optical cell.

For short term use a stock solution of 10^{-3}M Chl a or BChl a was made up in the chosen solvent (benzene, carbon tetrachloride or hexane) and the sample solutions were prepared by dilution of the stock solution as required. The concentrations of the stock solutions and the sample solutions were determined by absorption: the solvent was evaporated under reduced pressure and the chlorophyll was dissolved in a known volume of diethyl ether (Mallinkrodt, anhydrous). The molar absorptivity of Chl a was taken from Seely and Jensen⁹⁴ ($\epsilon_{660} = 8.51 \times 10^4 \text{ l mole}^{-1} \text{ cm}^{-1}$). The chlorophyll concentrations are therefore given as the total concentration of chlorophyll as monomer.

For storage for more than two weeks, the chlorophyll samples were stored as the solid either under nitrogen or on the vacuum line, at room temperature and in the dark.

Solutions of monomeric ligated chlorophylls were prepared by adding measured amounts of either water (distilled, deionized, degassed) or pyridine (BDH Aristar grade) to the solutions in benzene, hexane or carbon tetrachloride using a Hamilton microliter syringe.

Assessment of the method of Omata and Murata¹⁵⁶ for the preparation of chlorophylls from spinach.

In this method the chlorophylls in a crude acetone extract of spinach are precipitated as a colloidal aggregate with dioxane. The chlorophylls are then separated from the remaining carotenoids by column chromatography on DEAE Sepharose CL-6B.^h Chl a, Chl b and their stereoisomers are then separated from the remaining impurities and alteration products on a column of Sepharose CL-6B.^h Repeated chromatography of the Chl a fraction on the latter column yields Chl a of high purity as demonstrated by reverse phase high pressure liquid chromatography (HPLC) on 10 m RP18 silica (acetonitrile, water 96:4).¹⁶⁰ The content of the stereoisomer Chl a' was barely detectable in freshly prepared samples and rose to about 20% in samples which had been left standing for a week. There was no evidence of contamination with Chl b or degradation products.

The method of Omata and Murata was chosen rather than the traditional method of chromatography on sucrose columns⁴¹ because the former procedure was found to be a very quick, efficient and effective method of preparing small quantities (batches of 10mg to 100mg) of Chl a of high purity. It is not recommended that this method be used for large scale preparation because the gel nature of the column

^hSepharose CL-6B is a cross linked agarose gel of particle size 40-210 μ m. DEAE Sepharose CL-6B is the diethylaminoethyl derivative.

materials limits the size of the columns which may be used for optimum performance [2.5cm diameter x 10cm were suitable]. Cooling of the columns with a cold water jacket (10°C) was advantageous both for preserving the chlorophylls and preventing formation of troublesome bubbles in the gels. The structure of the column material is sensitive to the solvent and care must be taken to transfer it from aqueous to dry organic solvents according to the instructions of Omata and Murata. Also bacterial decomposition of the gel must be avoided in samples stored for long periods. Reference 161 contains a number of helpful hints on the use of gel type column materials.

Thin layer chromatography

Before the reverse phase HPLC system was available, purity of the samples was regularly checked by thin layer chromatography (TLC) as detailed below. The TLC method appeared to be an effective and convenient method because the results were later confirmed by the more sensitive HPLC technique.

The Chl a samples were described as pure after repeated chromatography on Sepharose CL-6B when TLC on silica gel G (Merck, used without activation) revealed only two spots: Chl a accompanied by a minor component of the isomeric Chl a' (solvent system: hexane, acetone, t-butanol 90:5:5).¹⁶²

Chromatography of the commercially prepared BChl a sample on Silica gel G (solvent system: hexane, acetone, t-butanol, 9:1:1) showed up 5 minor components in addition to the major spot of BChl a (Figure 5.5A). Purification of the sample on the Sepharose columns was attempted, and although separation of the alteration products was observed, the BChl a fraction appeared to degrade very easily in the solvent mixture

Figure 5.5

Separation of impurities from BChl a samples by A) thin layer chromatography (TLC) on silica gel G and B) reverse phase TLC on kieselguhr impregnated with oleic acid. Zones were identified from chromatographic sequence and absorption and fluorescence spectroscopy: 1 unknown (yellow green); 2 unknown (blue grey); 3 BChl a (blue-green); 4 BVir (yellow green); 5 BChl a' (blue green); 6 Bacteriopheophytin (pink). Since zones 1, 2 and 6 are absent or barely detectable on the reverse phase TLC, and the relative amounts of these impurities increase with exposure time to the silica gel G plate, these impurities are believed to be caused by reaction with the siliceous adsorbent. [see text for solvent systems]

(hexane, 2-propanol 98:2) in the presence of air and light. Setting up the columns inside the glove-box was not feasible at that time. Consequently, the samples were used as received but in order to rule out the presence of impurities which could be responsible for the new fluorescence bands reported in Chapter 8, the zones from a number of TLCs were scraped off the plates, eluted with pyridine, and the absorption and fluorescence spectra were recorded. The absorption spectra are shown in Figure 5.6. These samples were exposed to air and light and therefore it is expected that the proportion of degradation products to BChl a would be much greater than that found in samples prepared in dim light inside the glovebox as were the samples used for the experiments described in Chapter 8.

Siliceous adsorbants have been reported to cause alteration of the chlorophylls⁴⁵ and may contribute to the degradation products observed. A separation on a less reactive reverse-phase thin layer system was therefore also employed.^{163,164} Kieselguhr plates (Merck) were impregnated by dipping in a 1% solution of oleic acid in petroleum ether (b.p. 30° - 60°C) and allowed to dry for 30 minutes at 60°C. The plates were developed by the sandwich plate method using methanol, acetone, water, 20:10:5. This method revealed only 3 impurities in the BChl a sample (Figure 5.5B). Since the two additional spots observed by TLC on the silica gel G plate were absent, and it was observed that the relative amount of these additional spots increased on prolonged exposure of the sample to the siliceous adsorbent, it is believed that zones 1 and 2 are due to degradation products formed by reaction with the adsorbent. It is noted that the results of the reverse phase TLC analysis

Figure 5.6

Absorption spectra of the impurity zones eluted from the silica gel G TLC plate. The identity for each zone is that of the major component. It is noted that complete separation of each component is not achieved: a component absorbing at ~680nm and ~430nm, thought to be BVir or a similar pigment is apparent in each spectrum.

are in agreement with the analysis of the degradation products of BChl a by Kim¹⁶⁵ using a similar reverse phase thin layer technique.

The impurities were identified if possible from known absorption spectra, and the identities and approximate relative abundances of the known impurities are given in Figure 5.6. The emission spectra of the samples eluted from each zone showed fluorescence which corresponded only to the main red absorption band of each impurity; that is, at ~780nm for zones 1,2 and 5, at ~760nm for zone 6 and at ~690nm for zone 4. Zone 4 was identified as Bacterioviridin (BVir) [also known as 2-acetyl-2-devinyl-Chl a or Δ 3,4-Chl a] which is an oxidation product of BChl a.^{52,55,57}

The fluorescence quantum yield of this component is higher than that of BChl a or the other impurities when excited with laser radiation at 400nm. From the relative abundances and quantum yields of the impurities it was estimated that the fluorescence intensities from impurities 1,2,5 and 6 in the unpurified samples of BChl a prepared in a dry nitrogen atmosphere is 10^{-2} that of BChl a, while that of impurity 4, BVir, should be 10^{-1} times that of BChl a. Thus BVir is expected to be the only impurity with detectable fluorescence in the unseparated BChl a sample. It has been noted by several authors that separation of this impurity from BChl a is difficult, and that even highly purified samples of BChl a are not free from fluorescence at 690nm. We observed that this oxidation product accumulated after prolonged irradiation of the BChl a samples, and subsequently, irradiated samples degraded completely if left overnight in the dark, yielding BVir as the major product.

CHAPTER 6DETECTION LIMITATIONS OF PHOTOMULTIPLIER TUBES
IN PULSED LASER EMISSION SPECTROSCOPY²¹

The choice of a light detector in pulsed laser emission spectroscopy depends on the time resolution required. Photomultiplier tubes (PMTs) with rise-times down to ~ 100 ps are available. Picosecond resolution requires a streak camera. Recent developments with doubly intensified vidicon systems for optical multichannel analysers (OMAs) promise to improve the sensitivity of these light detectors. The capability of time resolution and the advantage of simultaneous detection of an extended spectral range results in data acquisition times which are considerably shorter than those obtained by scanning a spectrum with a monochromator and using a PMT detection system. This makes the OMA an attractive system for pulsed laser emission and light scattering spectroscopy. However, at present the sensitivity of OMAs is generally inferior to PMTs in the visible region of the spectrum. A PMT with a fast rise-time and a boxcar averager equipped with gated integrators for time resolution down to 2ns therefore remains a sensitive and versatile system for both fluorescence and Raman spectroscopy in the nanosecond time regime.¹²⁹

The characteristics of PMTs required for use in Raman spectroscopy and in fluorescence spectroscopy are usually rather different. Raman spectroscopy, depending on a weak second order effect, requires a sensitive tube with high gain and low dark current. On the other hand fluorescence spectroscopy demands a more robust tube which can cope with

exposure to relatively high photon fluxes. PMTs have been used for many years in emission and light scattering spectroscopy with continuous wave (cw) excitation and they have been well characterised under these conditions. Now that laser systems producing nanosecond or picosecond light pulses have opened up the field of time resolved spectroscopy, the choice of a PMT depends not only on the spectral response, absolute sensitivity, gain and attainable signal-to-noise ratio, but also on the ability of the detector to process intense light pulses of short duration. In order to follow nanosecond pulses the detection system must have a rise time of ~ 1 ns or less. For a laser system of low repetition rate (10-100 Hz) the PMT must be capable of processing signals generated with a low average power but a high peak power. The maximum anode current specified for cw operation therefore has little significance and care must be taken to avoid saturation effects when using pulsed radiation.

Although the effects of saturation of PMTs due to strong light are well known in ordinary cw spectroscopy, being manifested as non-linear response,¹⁶⁷⁻¹⁷¹ there have been few studies of the effects of saturation on the time response of fast PMTs used in the pulsed mode. Since distortion of the signal in time when measuring fluorescence decays or interpreting time resolved spectra can lead to erroneous determinations of radiative lifetimes it is desirable to assess the performance of the PMT under different conditions of irradiation. A series of experiments to do this are described in section 6.2.

6.2.1 Operation of photomultiplier tubes in the pulsed mode

Pulsed nitrogen lasers and excimer lasers are notoriously good generators of radiofrequency noise, of which PMTs are excellent detectors!

The PMT housing and cables, and preferably, the laser itself should be shielded with Faraday cages. In addition, to avoid artefacts in time resolved spectroscopy caused by ringing or reflections of signals which are detectable on the nanosecond time scale it is essential i) to have a good common ground for all components in the detection system, ii) to check that all connections are properly impedance matched, and iii) that all amplifiers and other electronic circuitry are optimally aligned (adjusted) to process signals cleanly without distortion.

Distortion of the signal pulse by the PMT is due to several factors. The initial velocities of the secondary electrons vary because they are released from different depths in the dynode material and have different initial energies. The initial velocities of the photoelectrons emitted from the photocathode depend on the energy of the incident photons,¹⁷² and the time of flight depends on the position on the cathode from which the photoelectron is emitted. Since the electrons travel paths of slightly different lengths, this also contributes to the broadening of the pulse in time, depending on the geometry of the dynode structure.

Sources of noise include the noise of the incoming radiation, shot noise from fluctuations in amplification and noise of the load resistor. The dark current arises from thermionic emission and may be reduced by cooling the tube.

PMTs are often modified for use in the pulsed mode by changing the resistors in the interdynode chain, to increase the maximum anode current.

6.2 Time dependent photomultiplier response at the onset of saturation

The specifications of the PMTs which were available in the laboratory and were tested for the effects of saturation under pulsed illumination are listed in Table II on page 83.

When an uncooled RCA 7102 PMT (S1 response) was left for several days in the dark at 1500V its dark level in time was recorded as shown in Figure 6.1C. This figure shows a pattern of peaks and valleys in the dark level which occur at specific points in time on the 0-100ns scale of the boxcar averager. These peaks and valleys are characteristic of the detection system as a whole (PMT, delay lines, boxcar averager) and they are reproducible. A change in the high voltage from 1500V to 1200V and then to 0V does not result in major changes in the dark level pattern, but it is noted that the peaks at 14ns, 23ns and 34ns are amplified more in the higher voltage scan. These latter peaks also diminish on cooling the tube to -20°C and it is therefore concluded that these peaks are due to the PMT while the other part of the pattern is characteristic of the system boxcar averager noise and radiofrequency pick-up. Although only part of the pattern is shown in Figure 6.1, similar results were observed over the time range from 0 to 1000ns.

In the next set of experiments the cooled tube was exposed to the red fluorescence of Chl a in solution (10^{-5} M in pyridine). The emission intensity was attenuated to give a low signal (15mV maximum) and the dark and signal levels were recorded (Figure 6.2). It can be seen that the signal is modulated with the same characteristic pattern as the dark level. Subtraction of the dark level yields the decay curve shown in Figure 6.3. Also shown is the position of the laser pulse on the same time scale.

Figure 6.1

Part of the dark level of the RCA 7102 photomultiplier tube as recorded on the 0-100ns scale of the boxcar averager.

Figure 6.2
Dark level and decay of fluorescence signal at 678nm from 10^{-5} M Chl *a* in pyridine
as recorded from the boxcar averager using the RCA 7402 photomultiplier tube.

Figure 6.3

Fluorescence decay (solid line) at 678nm for Chl a in pyridine corrected for the dark level (average of 2 scans, 7102 PMT). The position and shape of the laser pulse in time as recorded by the boxcar averager are also shown (dashed line).

Since the laser excitation pulse has decayed to a low intensity at about $t=35\text{ns}$, a good estimate of the fluorescence decay time may be obtained by simply plotting the natural logarithm of the intensity versus time for that part of the decay after $t=35\text{ns}$. This yields a fluorescence decay time of $7\pm 1\text{ns}$, which is typical for Chl a in pyridine. Interestingly there was also evidence of a longer lived decay in the fluorescence (See Chapter 7, section 7.2). Deconvolution of the fluorescence decay curve taking into account the distortion and broadening of the pulse by the detection system is necessary to obtain a true value of the fluorescence decay time.

Immediately after exposure of the PMT to the low intensity light pulses, the dark level of the tube was recorded again and found to be identical to that observed before the tube was exposed to light.

The PMT was then exposed to light 100 times more intense. In this case the PMT exhibited typical signs of saturation. Figure 6.4 shows that the modulation in the fluorescence decay curve no longer follows that in the dark level trace. Neither did the modulation in the dark level trace reproduce that of the tube before it was exposed to the higher light intensity. On continued exposure to the intense fluorescence the modulation of the fluorescence decay curves remained while the modulation in the dark level trace diminished and the trace became almost flat. The longer the tube was exposed to light, the flatter the baseline trace became. Figure 6.5 shows an expanded scan of the 50-100ns region which illustrates how the modulation in the fluorescence decay curve bears no relation to the dark level.

It was found that the PMT required at least 24 hours in the dark at 1500V to recover from the saturation effects. The modulation in the

Figure 6.4

Dark level and decay of the fluorescence signal at 678nm from Chl *a* in pyridine recorded with the RCA 7102 PMT saturated. Note the oscillations in the signal decay curve.

Figure 6.5

Part of the dark level and decay of the fluorescence signal shown in Figure 6.4 shown under higher gain.

baseline returned gradually from the flat baseline to the original pattern shown in Figure 6.1. The recovery time was shorter if the PMT was not completely saturated, that is, if there was still some modulation remaining in the dark level trace.

The same experiments were carried out with the RCA C31034, RCA 8850 and ITT FW130 PMTs and each displayed similar saturation behaviour.

The RCA C31034 was found to saturate at much lower light levels than the RCA 7102. In order for the signal to be displayed at the 100mV scale of the boxcar averager, it was necessary to use a pre-amplifier ($\times 100$) before the input of the boxcar. Unfortunately, the amplifiers further broaden the signal pulse and introduce additional noise. Thus although this tube has superior gain for use in Raman spectroscopy, its use in fluorescence spectroscopy or in cases when a Raman spectrum occurs with a large emission background appears to be limited by its low saturation threshold, unless time resolution is sacrificed by using a pre-amplifier.

The RCA 7102 can process peak signals of 250mV without saturation and in this case the time resolution is limited only by the response time of the PMT and the boxcar integrator. The RCA 8850, which is a blue sensitive tube, can effectively process signals on the 100mV scale, although its anode pulse decay time is slightly longer than that of the RCA 7102.

Since the input impedance of the boxcar integrator or pre-amplifiers is 50 ohms, the specified maximum anode current (see Table II) would suggest that the maximum input voltage to the boxcar amplifier from the PMT should be $\sim 0.5\text{mV}$ for the RCA 7102. In reality, effective saturation

is not observed in the pulsed mode for signals below 250mV. It thus appears that this PMT operates well in the pulsed mode without the necessity for changing the resistors in the interdynode chain to increase the maximum anode voltage. However, since the laser system was operated at only 25Hz, the PMT has 40 μ s to recover from each pulse and the average signal is low. Increasing the pulse rate may lower the saturation threshold.

The low value for the specified maximum average anode current of 100nA for the RCA C31034 is probably responsible for the fact that pre-amplifiers are required to operate this tube safely without saturation effects.

The RCA 8850 was predicted to saturate for cw radiation for a 10mV output signal, but it was found to cope with signals of $\sim$ 100mV without saturation problems. The same was true for the ITT FW 130, but its slow rise-time excludes its use for nanosecond time resolved spectroscopy.

The study of a low intensity long lived emission following a strong short lived decay may still pose problems with a conventional PMT system. In this case, either gating the PMT high voltage or using some type of optical shutter to cut the initial light pulse intensity can overcome this difficulty by amplifying only the latter part of the light pulse.

The tests performed showed that certain PMTs commonly used in fluorescence and Raman spectroscopy for cw work also perform well in processing strong pulsed light signals if certain conditions are met, without the need to modify the interdynode voltages. In addition the results emphasize the usefulness of recording the dark level in time both before and after running a fluorescence decay or time resolved spectra to check for non-linear effects and saturation.

CHAPTER 7FLUORESCENCE STUDIES. I:RED FLUORESCENCE OF DIMERIC CHLOROPHYLL A IN SOLUTION7.1 Characterisation of dimeric solutions of Chlorophyll a by analysis of the absorption spectra

It has previously been established that dry Chl a in dry benzene or carbon tetrachloride exists predominantly as a dimer over a wide range of concentrations ($10^{-6}\text{M} - 10^{-2}\text{M}$) (See Chapter 2). The absorption spectrum of these solutions provides a good indication of the degree of dimerization. In dry hexane the aggregation number of dry Chl a is highly concentration dependent and at higher concentrations ($\geq 10^{-3}\text{M}$) Chl a tends to oligomerize. Examination of the absorption spectrum of a solution of 10^{-4}M Chl a in dry hexane showed that the solution had almost identical spectral features to a 10^{-3}M solution of Chl a in carbon tetrachloride (Figure 7.1). After detailed analysis of the absorption and fluorescence spectra of Chl a in hexane over a range of concentrations ($10^{-6}\text{M} - 10^{-3}\text{M}$) and comparison with the absorption and fluorescence spectra of solutions of Chl a in benzene and carbon tetrachloride, it was concluded that the majority species in $10^{-4}\text{M} - 10^{-5}\text{M}$ solutions of Chl a in hexane was the dimer. The dimer concentrations in solutions of Chl a in benzene, carbon tetrachloride and hexane were determined by absorption difference spectroscopy as described below. The dimeric solutions in hexane proved to be particularly stable to prolonged irradiation during the fluorescence studies.

Figure 7.1

- (A) Absorption spectrum of 10^{-4} M Chl a in dry hexane (2mm path length cell);
 (B) calculated absorption spectrum of the Chl a dimer in hexane;
 (C) calculated absorption spectrum of the Chl a monomer in hexane. It is estimated that 19% of the Chl a in the dry solution is present as the monomer.

The equilibria between the monomer, dimer and larger oligomers in solution in non-polar solvents are dependent on the presence of water or other nucleophiles, L, and may be represented by the following:

Since the dimer exists only in the presence of monomer and/or oligomer the absorption spectrum of the dimer cannot be determined directly. In this situation analysis of the absorption difference spectra of solutions over a range of concentrations may be used to obtain the absorption spectrum of each species in solution.

In the case that the Chl a solution contains sufficient moisture or nucleophile that the equilibrium is shifted towards the left, it may be assumed that only the monomer and the dimer contribute to the absorption spectrum of the solution. The total absorbance of a solution containing a molar concentration c_{1m} of monomer and c_{1d} of dimer, with extinction coefficients $\epsilon_m(\lambda)$ and $\epsilon_d(\lambda)$ respectively at wavelength λ is given by:

$$A_1(\lambda) = \epsilon_m(\lambda)c_{1m}l_1 + \epsilon_d(\lambda)c_{1d}l_1$$

where l_1 is the optical path length of the sample cell. The total Chl a concentration as monomer is then $c_{1\text{total}} = c_{1m} + 2c_{1d}$. In a solution with a different total concentration of Chl a, $c_{2\text{total}} = c_{2m} + 2c_{2d}$, where c_{2m} and c_{2d} are the monomer and dimer concentrations respectively, the absorbance of the solution is given by:

$$A_2(\lambda) = \epsilon_m(\lambda)c_{2m}l_2 + \epsilon_d(\lambda)c_{2d}l_2$$

in a cell of optical path length l_2 . If either the path lengths of the cells or the concentrations can be adjusted so that $c_{1m}l_1 = c_{2m}l_2$ the difference spectrum recorded with one cell in the reference beam and one cell in the sample beam of the absorption spectrometer will represent the

absorption spectrum of the dimeric species only and the contribution of the monomer is subtracted out. Rather than actually adjusting the cell path lengths or sample concentrations, the subtractions may be accomplished mathematically by digitizing the absorption spectra of two solutions of different concentration, the spectra are then scaled numerically, to represent a change in the path length, and subtracted to obtain the difference spectrum.

The absorption spectrum of monomeric Chl a in a non-polar solvent may be obtained by titrating the dry solution with water or another nucleophilic ligand such as pyridine. Since the residual monomer in dry solutions arises from water or adventitious nucleophiles which are not removed, the concentration of the residual monomer in the dry solution was estimated for a first approximation using the absorption spectrum of Chl a pyridinate for the subtraction. The absorption spectra of the dry solution and the monomeric Chl a were digitized and the absorption spectrum of the dimer was calculated by subtraction of increasing amounts of monomer absorption from that of the dry solution until a smooth spectrum was obtained and the bandwidth of the Q_y band in the dimer spectrum conformed within experimental error with the corresponding fluorescence band (see section 7.3). For example, it was estimated from these calculations that the 10^{-4} M solution of Chl a in hexane contained about 19% monomer. The relative amounts of monomer and dimer in solutions of different concentrations were calculated in this way from the amount of monomeric absorption and the known total concentration of Chl a in the solutions. The absorption spectrum of the dimer was then obtained over the wavelength range from 800nm - 350nm by recording the

difference spectrum between two solutions of different Chl a concentrations in different path length cells so that the monomer absorbance cancels out and the difference spectrum represented purely dimeric absorption. The monomeric absorption spectrum was then obtained in a similar way. Using the new monomeric absorption spectrum, the procedure was repeated and the calculated values of the concentrations of the monomer and dimer were refined to give a good fit to the measured absorption spectra of the dry solutions over a range of concentrations 2×10^{-6} to 1×10^{-3} M. The resultant absorption spectra of monomeric and dimeric Chl a in hexane are shown in Figure 7.1B. Table III lists the main absorption maxima and calculated molar absorptivities of the monomer and dimer in dry hexane. The absorptivities are calculated on the assumption that the value of the molar absorptivity of Chl a.pyridinate at the red absorption maximum is a good approximation to the calculated absorption maximum of residual monomer in hexane. However, since the calculated absorption maximum of residual monomer in hexane is calculated to be 2nm blue shifted with respect to that in pure pyridine and the molar absorptivities⁹⁴ ϵ_{660} of monomeric Chl a in a number of different solvents lie in the range $6.6-8.5 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$, the values should be taken as relative rather than absolute. It was also assumed that there was a negligible contribution to the absorption spectra from oligomeric species of Chl a. This assumption seems justified because the solutions contain more than 10% monomer, which indicates that the equilibrium is not in favour of the larger oligomers.

Similar results were obtained from analysis of the absorption spectra of Chl a in carbon tetrachloride and in benzene. Several features of the

calculated dimer spectra should be noted. The main red absorption band of the Chl a dimer in hexane exhibits a single maximum at 669nm, a red shift of $\sim 230\text{cm}^{-1}$ from the red absorption maximum of the monomer in hexane at 659nm. The full width at half maximum (FWHM) of the dimeric absorption band ($\sim 630\text{cm}^{-1}$) is significantly broader than that of the monomer ($\sim 430\text{cm}^{-1}$). The corresponding maxima of the monomer and dimer absorption bands in dry carbon tetrachloride are 663nm and 672nm respectively (Table III). The molar absorptivities of the dimer at the maxima of the blue and red absorption bands are measured per mole of monomer in the dimer, and are significantly lower than those of the monomer, although the integrated absorption over the band is approximately the same for the monomer and the dimer.

The main red absorption band of the Chl a monomer in solution is due to the Q_y transition. The Q_x transition lies to the blue, underneath vibronic components of the Q_y band. If it is assumed that the bandwidths of the dimer transitions are similar to those of the monomer, the broad dimeric absorption band at 669nm in hexane (Figure 7.1B) could be interpreted as containing contributions from the two Q_y transitions of the dimer, split by $\sim 100\text{cm}^{-1}$. On the other hand, if it is assumed that the absorption band at 669nm arises from a single Q_y transition of the dimer, which is red shifted relative to that of the monomer Q_y transition, the band may be assigned to the transition terminating on the lowest lying excited singlet level of the dimer, which is red shifted with respect to that of the monomer. In this case, the second Q_y transition would probably be blue shifted relative to the monomer Q_y transition and would be expected to lie underneath vibronic sidebands

Table III

VISIBLE ABSORPTION DATA FOR MONOMERIC AND DIMERIC CHLOROPHYLL A IN HEXANE

	Red absorption band		Blue absorption band	
	$\lambda_{\text{max.}}$ (nm)	fwhm (cm^{-1})	$10^{-4} \epsilon_{\text{red}}^{\text{max}}$ ($\text{L mol}^{-1} \text{cm}^{-1}$)	$10^{-4} \epsilon_{\text{blue}}^{\text{max}}$ ($\text{L mol}^{-1} \text{cm}^{-1}$)
Chl <u>a</u> monomer	659	460	8.0	427
Chl <u>a</u> dimer	669	630	4.2	432
				5.8

The values in the table were obtained by difference absorption spectroscopy which showed that the 10^{-4}M solution of Chl a in hexane contained $\sim 19\%$ monomeric Chl a.

to shorter wavelengths ($\leq 650\text{nm}$). This would imply that the splitting of the dimer Q_y transitions would be $\sim 450\text{cm}^{-1}$.

The absorption spectra of several covalently linked chlorophyll dimers were described in Chapter 3 and it was shown that the optical properties of the dimers are strongly dependent on their structures. It is noted that the dimer spectrum of Figure 7.1B shows similar features to the absorption spectrum of the 'folded' singly linked dimer. Both spectra are characterised by a broad red absorption band which is shifted to longer wavelength.

The interpretation of the absorption spectra of Chl a in solution is complicated firstly by the existence of several species of Chl a in equilibrium in solution and secondly by lack of unambiguous experimental data to support the assignment of the transitions of the Chl a dimer which are predicted from simple theoretical models. It is clear, however, that the main red and blue absorption bands of the Chl a dimer are red shifted with respect to the corresponding monomeric absorption bands and this suggests that interpretation of the fluorescence spectra may be facilitated by wavelength selective excitation of fluorescence from each species of Chl a in solution (Figure 7.2).

Figure 7.2

Absorption spectrum of 10^{-4} M Chl *a* in dry hexane showing the relative contributions of the monomeric and dimeric absorption. The arrows indicate that excitation in the peak of the Soret absorption band of the mixture results in excitation of both monomer and dimer, whereas excitation to the red of the main absorption maximum may be used to selectively excite the dimer, because the monomeric absorption has fallen to a low value at this wavelength.

7.2 Fluorescence spectra of monomeric Chl a in pyridine

A typical spectrum of the well known red fluorescence of monomeric Chl a in solution is shown in Figure 7.3A. The emission is characterised by a strong band with a maximum at 670nm-680nm depending on the solvent, accompanied by vibronic sidebands towards 720nm. The fluorescence lifetime in pyridine is 7ns, and is typically 5 to 7ns in other solvents.¹⁵⁶ The fluorescence quantum yield is ~0.3. The strong red fluorescence is observed with excitation either in the Q absorption bands or the Soret absorption bands.

Although a detailed study of the fluorescence of Chl a in polar solvents, such as pyridine, was not pursued, it is interesting to note that the preliminary experiments which were done show that there may be more than one fluorescing species of Chl a in the solution. It is believed that Chl a is 99% hexacoordinated monomer in pyridine (i.e. Chl a.pyridine₂)¹¹³ and there is no doubt that the spectrum in Figure 7.3A is that of the majority species. However there is also a longer lived and broadened fluorescence spectrum observed in the time resolved spectrum recorded at $t_0 + 200\text{ns}$ (Figure 7.3B).

Several possible explanations for the origin of the longer lived component in the fluorescence spectrum have been considered, but none has been investigated thoroughly because work on the dimeric solutions took precedence. Firstly, it is known that in a Lewis base type of solvent such as pyridine, approximately 20% of the Chl a may be present as the C₁₀ epimer, Chl a'.⁴⁸ Although the absorption spectrum of the two isomers are almost identical, it is conceivable that the longer lived component could belong to the Chl a' isomer. Alternatively,

Figure 7.3.

Time resolved fluorescence spectra of 10^{-5} M Chl *a* in pyridine.

since the solutions were not thoroughly dried, there may be a small percentage of hydrated Chl a of some form in the solution. This explanation is unlikely because the lifetimes of species of Chl a with different ligands have not previously been reported to be very different.

Another suggestion was that photoexcitation may induce ligand release if the excited state geometry is unfavourable.¹⁷⁴ However, the lifetime of the pentacoordinated species is generally shorter than that of the hexacoordinated species, and an uncoordinated Chl a monomer would therefore be expected to have a shorter, rather than a longer fluorescence lifetime than the majority species in pyridine.^{106,167}

The possibility of there being a small amount of some type of dimer or aggregate in the pyridine solution of Chl a has previously been suggested to account for concentration quenching of fluorescence, but again most dimeric species have been reported to have shorter fluorescence lifetimes, and lower fluorescence yields, than the monomeric species which could not therefore account for the longer lived fluorescence component.

A more likely explanation for the other fluorescence component is that it is delayed fluorescence: this is radiative emission which occurs following indirect de-excitation of the singlet manifold via a longer lived intermediate state, which is usually a triplet state. This idea will be discussed further in reference to the fluorescence of dimeric Chl a and BChl a in Chapters 7 and 8.

It was also noted that the ratio of the intensities of the main band at 672nm to the vibronic sideband at 720nm, and the bandwidth of the fluorescence spectrum, as shown in Figure 7.4, was dependent on the

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Figure 7.4

Fluorescence spectra of 10^{-5} M Chl a in pyridine excited at 443.4nm;
(A) 0.5mW focussed with 28mm focal length lens; (B) 0.5mW with diffuse beam.

laser intensity. This can only be observed if there is either more than one fluorescing species of Chl a in the solution, or if there are dynamical processes which result in two different de-excitation pathways which produce emission of energy at the same wavelength but with a different time dependence. Clearly this system warrants further investigation.

7.3 Fluorescence of dimeric Chl a in dry non-polar solvents

The first indications of dimeric fluorescence were apparent on comparison of the fluorescence spectra of 10^{-5} M Chl a in dry benzene with the spectra of a similar solution in wet benzene (Figure 7.5A and C). In the dry solution the fluorescence band is broadened to the red of the main band at 668nm due to monomeric emission and an additional band at 625nm is present. In order to determine if the red shifted emission was due to dimeric species of Chl a or other species of Chl a in the solution, a series of solutions were prepared with higher dimer concentrations. This was accomplished either by further reducing the water content of the solution or by increasing the Chl a concentration. The optimum concentration was found to be 5×10^{-5} M to 10^{-4} M Chl a: at this concentration the majority species was found to be the dimer, with about 15-20% of the Chl a present as monomer and a negligible contribution from oligomers larger than the dimer. Increasing the concentration to shift the equilibrium towards the oligomers and to eliminate the monomeric species served no useful purpose since the monomeric absorption and fluorescence spectra are well characterised and their contribution to the fluorescence of the solution can more readily be accounted for than the contribution of oligomeric species. Solutions in benzene, carbon tetrachloride and hexane were investigated.

Wavelength selective excitation in the region of the Soret absorption bands.

The fluorescence spectra of Chl a in carbon tetrachloride and in hexane solutions are shown in Figures 7.6 to 7.8 for excitation in the Soret absorption bands of both the Chl a monomer and dimer.

Figure 7.5

Time resolved fluorescence spectra of 3×10^{-5} M Chl a in benzene solutions excited at 444.1 nm. Fluorescence spectra are shown for t_0 the time at which the fluorescence pulse reaches maximum intensity and for $t_0 + 40$ ns, for solutions in wet and dried benzene.

As noted in section 7.1, the dimeric absorption bands are red shifted relative to the monomeric absorption bands. This allows the fluorescence spectra of the two species to be selectively excited by tuning the excitation frequency into resonance with the absorption band of one species, or at least into the spectral region where the major part of the absorbance of the solution is due to one species.

When the 10^{-4} M solution of Chl a in dry carbon tetrachloride is excited with pulsed laser radiation at 430.5nm, which is in resonance with the maximum of the Soret absorption band, the fluorescence spectrum recorded (Figure 7.6A) is predominantly that of the monomer. The monomeric fluorescence is characterised by a main band at 668nm and a vibronic sideband at 720nm. By tuning the laser to longer wavelength away from the peak of the monomeric absorption band into a region in which the dimeric absorption is greater than that of the monomeric species (See Figure 7.2) a series of spectra were recorded as shown in Figure 7.6 A,B,C. Although the monomeric fluorescence dominates the spectra, additional emission bands at 627nm and 685nm are apparent in Figures 7.2 B and C. The effect of wavelength selective excitation is more clearly seen in the spectra obtained from a 10^{-4} M Chl a solution in dry hexane, which contains a greater proportion of the dimer (Figures 7.7 A,B,C and D). In this solution too, selective excitation in the region corresponding to predominately dimeric absorption results in strong fluorescence bands at 625nm and 688nm. Excitation at 434.5nm produces more intense emission at 625nm, while excitation at 450.5nm enhances the intensity of the 688nm band relative to the intensity of the monomeric fluorescence band at 668nm.

Figure 7.6

Fluorescence spectra of 10^{-4} M Chl a in dry carbon tetrachloride selectively excited at wavelengths in resonance with the blue (Soret) absorption bands of the monomer and the dimer. Excitation wavelength: (A) 430.5nm; (B) 444.5nm; (C) 450.5nm.

Figure 7.7

Fluorescence spectra of 10^{-4} M Chl a in hexane, selectively excited at wavelengths in resonance with the blue (Soret) absorption bands of the dimer and the monomer: (A) 430.5nm; (B) 438.5nm; (C) 444.5nm; (D) 450.5nm. The bands at 625nm and 688nm are assigned to the dimer.

Addition of water or pyridine to disaggregate the Chl a dimer in dry benzene, carbon tetrachloride or hexane solutions resulted in more intense monomeric emission and the bands at 625nm and 688nm diminished in intensity. Increasing the Chl a concentration of the dry solutions caused an increase in the intensity of the 625nm and 688nm fluorescence bands relative to the monomeric emission at 668nm. The bands at 625nm and 688nm were observed only for solutions which were known to contain the Chl a dimer, and were not detected for Chl a in polar solvents such as pyridine in which Chl a is not thought to exist as the (Chl a)₂ dimer. The analysis of the absorption spectra of the dry solutions suggested that wavelength selective excitation of the dimer in the spectral region 435nm - 450nm, to the red of the main monomeric Chl a absorption band, should be possible, and indeed it was observed that excitation in this region enhanced the intensities of the 625nm and 688nm fluorescence bands relative to the monomeric fluorescence. Since the analysis of the absorption spectra showed that the majority species in the solutions was the Chl a dimer, and HPLC analysis of the solutions before and after laser irradiation showed that apart from Chl a and a small percentage of the isomer Chl a' there were no other detectable impurities or degradation products in the solutions, the additional fluorescence bands at 625nm and 688nm for Chl a in hexane, and at 627nm and 685nm for Chl a in benzene or carbon tetrachloride solutions, are therefore assigned to the Chl a dimer.

Emission at ~720nm which is additional to the monomeric vibronic sideband may be assigned to i) vibronic sidebands of the dimeric fluorescence at 688nm and possibly to ii) emission from oligomeric species of Chl a. The latter assignment is based on the fact that in solutions

which are more thoroughly dried, and therefore more aggregated, the emission is further red shifted and the main band occurs at $\sim 700\text{nm}$.

Excitation at wavelengths in the region 450nm - 470nm also results in a more red shifted main band, and a much reduced contribution from the monomeric fluorescence. A weak broad emission band centred around 580nm was also observed with excitation in this wavelength range. The origin of the latter was not investigated.

Figure 7.8 shows a set of time resolved fluorescence spectra of the 10^{-4}M Chl a solution in hexane. Because the fluorescence spectrum is composed of a number of overlapping bands the decay of the fluorescence at most wavelengths is multiexponential. Rather than attempting to obtain reliable lifetimes from direct measurement of the fluorescence decay curves, an estimate of the lifetimes may be made by comparison of the time resolved spectra, which clearly show the differences in the lifetimes of the monomeric and dimeric fluorescence bands. The fluorescence lifetime of the Chl a monomer in solution is $6 \pm 1\text{ns}$. By recording the fluorescence spectra of the dimeric solution a few nanoseconds before and after the time at which the fluorescence pulse reaches maximum intensity, which is defined at t_0 , the differences in the lifetimes can be seen from the changes in the relative intensities of the three fluorescence bands. The behaviour in time of the intensity of the band at 625nm is similar to that of the monomeric emission at 668nm ($\tau_{f1} \approx 6\text{ns}$). The intensity of the dimeric fluorescence at 688nm decays significantly faster than the monomeric intensity and thus the fluorescence lifetime is less than 6ns .

Since the spectra in Figures 7.6 to 7.8 were first published in

Figure 7.8

Time resolved fluorescence spectra of 10^{-4} M Chl a in dry hexane. The intensities of the three spectra are approximately normalised for comparison. t_0 is the time at which the fluorescence intensity reaches a maximum. The lifetime of the monomeric emission at 668nm is ~ 6 ns. The intensity of the dimer band at 625nm shows approximately the same behaviour in time as the monomeric emission. The dimer band at 688nm decays significantly faster than the monomeric emission.

1983^{35,38} another group have investigated the time resolved fluorescence spectra of Chl a in dodecane and carbon tetrachloride and found similar results. Using a picosecond laser system they obtained lifetimes of 5.4ns for the monomeric component and 1.2ns for the 685nm fluorescence band.¹⁷⁵

Wavelength selective excitation in the region of the Q absorption bands

Typical spectra obtained by wavelength selective excitation in the red spectral region at ~600nm are shown in Figures 7.9 and 7.10. Excitation at 614.0nm results in strong monomeric fluorescence (Figure 7.9A) while excitation at 600-605nm causes a significant change in the relative intensities of the monomeric and dimeric bands in favour of the dimeric emission (Figure 7.9B and 7.10 A-D).

Since the fluorescence maximum of the dimeric band at 688nm is shifted by 410cm^{-1} from the corresponding absorption band it may be estimated that the wavelength of the absorption band corresponding to the emission band at 625nm should lie at ~610nm. The spectra in Figure 7.10 A-D are excited at wavelengths which are also in resonance with the vibronic sidebands of the monomeric absorption band. The presence of an absorption band of the dimer at $605\pm 5\text{nm}$ is therefore supported by the observation that excitation of fluorescence in this region results in strong fluorescence at 688nm (Figure 7.10). Excitation in this spectral region results in only very weak emission at 625nm.

In comparing the spectra in Figures 7.9 and 7.10 with the spectra obtained with blue excitation (Figure 7.6 and 7.8) it is noted

Figure 7.9

Fluorescence spectra of 10^{-4} M Chl a in hexane excited at wavelengths in resonance with the red absorption band of the monomer and dimer:
(A) 614.0nm; (B) 604.0nm; (C) 720nm.

Figure 7.10

Fluorescence spectra of 10^{-4} M Chl a in hexane excited at different wavelengths in the region of the Q_x absorption bands: (A) 605.3nm; (B) 603.3nm; (C) 601.3nm; (D) 599.3nm.

that the vibronic emission to the red of the 688nm band is more intense relative to the other bands with excitation in the red spectral region.

Excitation in the red indicates that there is coupling between the two dimer levels which absorb at 669nm and ~605nm. However the two fluorescence bands at 625nm and 688nm show a difference in their excitation profiles in the blue spectral region. The latter result suggests that energy relaxation to the two fluorescing levels following excitation in the blue at 430nm to 460nm may occur via two different channels, and/or there is more than one electronic origin of the dimer in this region, each of which is coupled differently with the two fluorescing levels of the dimer. It is known that the origins of both the B_x and B_y transitions in the Soret band of the monomer in solution are approximately degenerate.⁸⁸ The dimer absorption bands in the Soret region are therefore also expected to be due to both x and y polarised transitions which correspond to the monomeric transitions. The difference in the excitation profiles of the two dimeric fluorescence bands may reflect the difference in symmetry of the states of the dimer from which the fluorescence transitions originate.

Time resolved spectra with excitation at 603.5nm confirmed the result obtained with excitation in the blue that the dimeric fluorescence band at 688nm has a shorter lifetime than the monomeric fluorescence (Figure 7.11). Further information on the dynamics of energy conversion within the dimer was obtained by examining the dependence of the spectra on the laser intensity. The results are described in the following section.

Figure 7.11

Time resolved fluorescence spectra of 10^{-4} M Chl *a* in hexane excited at 603.5 nm. These spectra show that the band at 688 nm decays more quickly than the monomeric band at 668 nm, confirming the results found with excitation in the blue absorption bands.

Excitation at $\sim 630\text{nm}$ results in relatively strong emission at 625nm and strong monomeric fluorescence at 668nm , but only weak fluorescence in the region of the dimeric band at 688nm . The spectrum shown in Figure 7.12 was excited at 632.8nm with the He-Ne laser (cw). The spectrum recorded with pulsed laser excitation at the same wavelength was identical, but the laser dye required for excitation at $\sim 630\text{nm}$ produces superradiance in the region of the 625nm fluorescence band. Although this can be filtered out, the cw He-Ne laser avoids superradiance problems and confirms that the band at 625nm in Figure 7.12 is in fact due to fluorescence.

Figure 7.13 shows a series of spectra for solutions of Chl a in hexane of different concentrations excited at 444.5nm . As the concentration is increased, the equilibrium shifts in favour of dimeric and oligomeric species and the more red shifted fluorescence bands make up the major part of the spectra. Although the quantum yield of the dimeric fluorescence was not measured, an estimate of the relative quantum yields may be made by comparing the relative intensities of the monomeric and dimeric fluorescence bands, since the relative contributions of the two species to the absorbance of the solution are known. For excitation in the region of 440nm to 450nm , the dimeric fluorescence yield estimated in this way is $\sim 15\%$ of the monomeric fluorescence yield.

Figure 7.12

Fluorescence spectrum of 10^{-4} M Chl a in benzene excited with a 0.5mW He-Ne laser at 632.8nm. This spectrum is the same as that obtained with pulsed laser excitation at the same frequency. The cw spectrum does however confirm that the 625nm emission is fluorescence from Chl a and not due to superradiance from the laser dye close to the laser line.

Figure 7.13

Fluorescence spectra of Chl a in hexane excited at 444.5nm for solutions of different concentrations: (A) $5 \times 10^{-4} \text{M}$; (B) $5 \times 10^{-5} \text{M}$; (C) $5 \times 10^{-6} \text{M}$; (D) 5×10^{-6} with an excess of pyridine added.

7.4 Power dependence and photoquenching of fluorescence

The spectra shown in Figures 7.6 to 7.12 were excited with the laser beam unfocussed and an average power of $\sim 0.5\text{mW}$. This corresponds to a photon density of $\sim 10^{16}$ photons cm^{-3} so that for a 10^{-4}M Chl a solution the number of photons per pulse is approximately equal to the number of Chl a molecules in the irradiated volume. Significant population of excited states with nanosecond lifetimes therefore does not occur. However, if the photon density is increased by focussing the beam, with for example 10cm focal length spherical lens, the ratio of photons to irradiated molecules may be increased by a factor of 10^3 - 10^4 , and excited states with lifetimes of $\sim 100\text{ps}$ may then be populated.

Selective quenching of the monomeric fluorescence of Chl a in benzene was observed by excitation with focussed radiation at 440nm (Figure 7.5C and E). In this case fluorescence quenching is believed to be caused by population of the first excited singlet level which is followed by absorption of a second photon so that excited state absorption competes with relaxation by fluorescence. In the spectra shown in Figure 7.5, the quenching of the monomeric emission permitted observation of the weak dimeric and oligomeric fluorescence. In order to observe the spectrum in Figure 7.5E it was necessary to focus the laser beam with a 28mm focal length lens and place a spatial filter (slit) in front of the cell so that emission only from the focussed point was allowed to fall on the entrance slit of the monochromator. Under these conditions, there was no visible red fluorescence at the focussed point and the weak spectrum in Figure 7.5E was recorded.

The spectra in Figures 7.14, 7.15 and 7.16 show the effects of less intense focussing conditions. Figure 7.14 compares spectra of 10^{-4} M Chl a in hexane excited with focussed and unfocussed radiation at 444.5nm. It can be seen that at the higher photon flux density the dimeric emission bands are quenched relative to the monomeric band, and it appears that the 688nm band is quenched to a greater extent than the 625nm band. Figure 7.15 shows similar results for 10^{-4} M Chl a in carbon tetrachloride, which illustrate more clearly the quenching of the dimeric fluorescence under focussed irradiation at 450.5nm.

Spectra excited at 603.3nm showing quenching of the dimeric fluorescence of 10^{-4} M Chl a in hexane are shown in Figure 7.16. At this wavelength the relative contributions of the monomer and the dimer can be reversed by changing the focussing conditions.

Measurements of the power dependence of the transmission of the sample solutions at wavelengths in the red and blue spectral regions (discussed further in Chapters 9 and 10) show that population of the excited states of the dimer does occur when the laser radiation is focussed into the solution and the fluorescence quenching can therefore be explained by excited state absorption. It has been found that the first excited singlet state of the monomer absorbs in the region of 430nm¹⁷⁴ and the transmission measurements on the dimeric solution also indicate that this is true for the Chl a dimer.

Figure 7.14

Fluorescence spectra of 10^{-4} M Chl a in hexane excited at 444.5 nm (A) laser beam unfocussed, 0.5 mW average power; (B) laser beam focussed with a lens of focal length 80 mm, 0.5 mW average power. These spectra show that irradiation with a higher photon flux density quenches the two dimeric fluorescence bands relative to the monomeric fluorescence.

Figure 7.15

Fluorescence spectra of 10^{-4} M Chl *a* in carbon tetrachloride excited at 450.5 nm (A) laser beam focussed with lens of focal length 80 mm, 0.5 mW average power and (B) laser beam unfocussed. These spectra show more noticeably the photoquenching of the dimeric fluorescence bands under irradiation with a higher photon flux density as illustrated in Figure 7.14

Figure 7.16

Fluorescence spectra of 10^{-4} M Chl a in hexane excited at 603.3 nm at different photon flux densities, to illustrate photoquenching of the dimeric fluorescence bands. Laser power 0.5 mW average: (A) unfocussed; (B) partially focussed with 50 mm focal length lens; (C) focussed with 50 mm focal length lens.

7.5 Discussion of the red fluorescence spectra of dimeric Chl a in solution

From the study of the pulsed laser induced fluorescence spectra of the Chl a dimer in solution in hexane, benzene and carbon tetrachloride, it is concluded that the dimeric fluorescence is characterised by two bands at 625nm and 688nm. Time resolved fluorescence spectroscopy shows that the lifetime of the weaker fluorescence bands at 625nm is comparable to that of the Chl a monomer in solution (~ 6 ns) whereas that of the fluorescence at 688nm is shorter than 5ns. Analysis of the absorption spectra of the dimer together with the fluorescence data suggest that there are several possible assignments for the splitting of the lower lying singlet exciton levels of the Chl a dimer in solution.

The 2 electron model predicts that there should be one allowed and one forbidden dimer Q_y absorption transition. This suggests assignment of the strong absorption band of the dimer at 668nm to the allowed Q_y transition. The weaker absorption band which corresponds to the 625nm fluorescence band could then be assigned either to an allowed Q_x transition or to a weakly allowed (strictly forbidden) Q_y transition. The latter assignment would imply that the dimer Q_y exciton splitting was $\sim 1400\text{cm}^{-1}$. The former assignment would mean that the exciton splitting could not be determined from the absorption or fluorescence data (Figure 7.17)

The full width at half maximum (FWHM) of the fluorescence band of the dimer at 688nm is 550cm^{-1} and corresponds within experimental error to the FWHM of the red absorption band of the dimer at 668nm (FWHM 630cm^{-1}). If it is assumed that the band widths of each of the dimeric transitions are similar to those of the monomeric absorption

2 electron model:
one allowed and
one forbidden
dimer Q_y transition

DIMER Q_y EXCITON
SPLITTING

cannot be
determined
from absorption or
fluorescence data

4 orbital model:
2 allowed and
2 forbidden
dimer Q_y transitions

$\sim 1400\text{cm}^{-1}$

$\sim 100\text{cm}^{-1}$

Figure 7.17

The three possible assignments of the observed absorption and fluorescence bands in the Q_y and Q_x spectral regions for the Chl a dimer in solution, and interpretation of the exciton splittings (interaction energies) based on the available theoretical models.

bands (FWHM 460cm^{-1}) it is possible that the broad dimeric absorption bands at 669nm arises from the two Q_y exciton components of the dimer transition.²⁵ However, two bands of this width cannot be split by more than $\sim 100\text{cm}^{-1}$ to give a total bandwidth of $\sim 620\text{cm}^{-1}$. In this case, the narrower fluorescence band at 625nm (FWHM 300cm^{-1}) may be assigned to a Q_x transition of the dimer, although fluorescence from the Q_x level of the monomer has not been detected. The activity of the dimer Q_x transition indicates that symmetry considerations for the dimer transitions are different than for the monomer.

In the four orbital model of Haley and Koningstein¹⁰⁶ two absorption bands of approximately equal intensity are predicted for the Q_y dimer transitions, separated by the difference of the splittings of the two pairs of orbitals. This model would therefore be consistent with the assignment of the broad absorption band of the dimer at 669nm to the two Q_y dimer transitions. However to be consistent with the observed fluorescence band at 625nm (which would correspond to an absorption band at $\sim 605\text{nm}$) to a Q_x transition; the model must be extended to include orbitals from which the monomer Q_x transitions originate.

The experiments described in this chapter show that the Chl a dimer is fluorescent, but several alternative assignments are suggested for the origins of the two dimeric fluorescence bands. The work on BChl a described in the following chapter allows some of these assignments to be discarded and shows that the 625nm fluorescence band may in fact be assigned to a Q_x transition.

From the many earlier reports of red shifted emission from aggregated forms of Chl a in non-polar solvent,¹¹⁵⁻¹¹⁸ the work of Gurinovich et al is notable.¹¹⁶ In their work on the fluorescence of Chl a in dry petroleum ether, they observed purely monomeric fluorescence with excitation at 430nm and fluorescence at 680nm with excitation at 450nm, using a standard spectrofluorimeter. The band at 680nm was assigned to an unspecified aggregate of Chl a. This fragmented observation does however confirm that the key to observation of the dimeric or oligomeric fluorescence of Chl a in solution lies in wavelength selective excitation, whether this is with a laser or a conventional light source. Although excitation with a pulsed laser can also yield valuable information on the dynamics of energy conversion within the dimer, the photoquenching experiments also showed that the dimeric emission is easily quenched due to excited state absorption by the readily populated, lower lying excited singlet states with nanosecond lifetimes. The latter fact may lead the unwary laser spectroscopist to believe that the Chl a dimer is only weakly fluorescent.

CHAPTER 8

FLUORESCENCE STUDIES II:

FLUORESCENCE OF BACTERIOCHLOROPHYLL A IN SOLUTION

The Q_x absorption bands in Chl a lie underneath vibronic sidebands of the more intense Q_y absorption bands.⁸⁸ This hinders attempts to resolve the origin of the 625nm fluorescence transition by selective excitation of fluorescence at wavelengths in the region which would correspond to the predicted dimer absorption bands (see Chapter 7). In contrast, the Q_y absorption region of BChl a at ~780nm is separated by more than 4000cm^{-1} from the Q_x absorption bands at ~580nm.⁹³ Since an exciton splitting of the Q_y transitions of the BChl a dimer of sufficient magnitude to shift one or more of the Q_y dimer transitions into the 580nm region is improbable, any emission in the region of 580nm may be assigned to Q_x transitions of BChl a with more confidence than in the case of Chl a. The fluorescence study of BChl a in solution was therefore undertaken to determine whether there was any emission in the spectral region 580nm-600nm, and if so whether this emission could be assigned to monomeric or dimeric Q_x transitions, in order to clarify the assignment of the 625nm fluorescence band of the Chl a dimer.

8.1 Absorption spectra of BChl a in solution

The absorption spectrum of 10^{-5}M BChl a in dry hexane is shown in Figure 8.1A. The spectrum shows a broad Q_y absorption band at 770nm,

Figure 8.1

(A) Absorption spectrum of $10^{-5}M$ BChl a in dry hexane. The arrows indicate the laser wavelengths employed for excitation of fluorescence. [10mm path length cell] (B) Absorption spectrum of the same solution as for (A) after addition of pyridine ($2 \times 10^{-2}M$) to disaggregate the dimeric and oligomeric BChl a. The Q_y and Q_x absorption bands are indicated. The double peak in the Q_x absorption region indicates the presence of both 5-coordinate ($\lambda_{abs} = 580nm$) and 6-coordinate ($\lambda_{abs} = 600nm$) species of the monomer.

a weaker Q_x absorption band at 582nm and strong absorption bands in the Soret region with maxima at 360nm and 390nm. Titration of the dry solution with pyridine disaggregates dimeric or oligomeric species of BChl a in the solution forming the pyridinated monomer. The monomeric absorption spectrum is shown in Figure 8.1B. The absorption bands of the monomeric species are considerably narrower than those of the dimeric and oligomeric solution. It should be noted that the spectrum in Figure 8.1B shows a double Q_x absorption band indicating that both five- and six- coordinate BChl a pyridinate species are present in the solution.⁹³ BChl a appears to have a strong tendency towards five-coordination in the dry solvent even in the presence of a large molar excess of nucleophilic ligand.

The absorption maxima and the full width at half maximum (FWHM) of the Q bands of BChl a in several solvents are listed in table IV. For solutions in wet benzene, in which nuclear magnetic resonance studies have shown BChl a to be predominantly dimeric,⁸¹ the FWHM of the Q_y absorption band is broadened with respect to that of the monomeric absorption band, but not to as great an extent as observed for the bands of BChl a in dry hexane. This suggests that the hexane solution may contain a significant amount of oligomers larger than the dimer. Although the oligomeric solutions have considerably broadened absorption bands, there is no significant red shift as observed for oligomeric Chl a in solution. The monomeric absorption bandwidths are slightly narrower in hexane ($\sim 440\text{cm}^{-1}$) than in benzene or carbon tetrachloride ($\sim 480\text{cm}^{-1}$).

TABLE IV ABSORPTION AND FLUORESCENCE DATA FOR BACTERIOCHLOROPHYLL IN SOLUTION

solvent	absorption data					fluorescence data				
	absorption maxima					fluorescence maxima				
	concentration BChl a, M	pyridine, M	$\lambda_{\max}$, ± 1 nm	fwbm, ± 10 cm ⁻¹	Q_r , $\lambda_{\max}$, ± 1 nm	concentration BChl a, M	pyridine, M	$\lambda_{\max}$, nm	fwbm, ± 20 cm ⁻¹	Q_r , $\lambda_{\max}$, ± 2 nm
benzene (0.03% H ₂ O)	10 ⁻⁴		781	610	583			400	796	583
benzene	10 ⁻⁴	2 × 10 ⁻²	781	690	581			580	798	500
	10 ⁻⁴	2 × 10 ⁻²	781	500	~580 sh			580	795	460
(0.03% H ₂ O) + pyridine	10 ⁻⁴	4 × 10 ⁻²	781	480	~606					
	10 ⁻⁴	2 × 10 ⁻²	781	480	~586					
CCl ₄ (dry)	10 ⁻³		778	740	584			400	795	587
CCl ₄ (dry) + pyridine	10 ⁻³	2 × 10 ⁻²	780	480	~583					
hexane (dry)	10 ⁻³		769	830	582			385	778	585
	10 ⁻⁴		770	940	582			400	783	590
					~606 sh			400	785	583
					~606			400	785	583
					~606 sh			540	784/	850
					~606 sh			565	782	450
					~606 sh			580	783	590
					~606 sh			600	785	585
					~606 sh			620	785	690
					~606 sh			400	785	430
					~606 sh			580	785	430
					~606 sh			600	785	450
hexane (dry) + pyridine	10 ⁻³	2 × 10 ⁻²	775	430	~578			620	785	450
	10 ⁻⁴	2 × 10 ⁻²	769	450	~580			400	795	450
pyridine	10 ⁻³		780	430	609					

* Abbreviations: sh = shoulder; $\lambda_{\max}$ = laser excitation wavelength; vw = too weak to measure bandwidth reliably

8.2 Fluorescence spectra of BChl a in solution

The maxima and FWHM of the observed fluorescence bands of solutions of BChl a in benzene, carbon tetrachloride and hexane are summarized in Table IV together with the complementary absorption data. Fluorescence spectra for the solutions of BChl a in hexane are shown in Figure 8.2 for excitation at wavelengths in the region of the Soret absorption bands at 401.3nm, and in Figure 8.3 for excitation at five wavelengths in the region of the yellow Q_x absorption band.

In Figure 8.2 the fluorescence spectrum of $10^{-5}M$ BChl a in hexane (8.2A) is compared with the fluorescence spectrum from the same solution after titration with pyridine (8.2B). The intensity of the fluorescence from the dry solution is ~ 5 times weaker than that from the monomeric solution in hexane. While the main band at 783nm is not shifted significantly, the bandwidth in the dry solution is considerably broader ($\sim 590cm^{-1}$) than in the titrated solution (FWHM $\sim 430cm^{-1}$). In addition, a weaker band at 583nm is apparent in the aggregated solution, which diminishes in intensity when pyridine is added to the solution. The bandwidth of this latter band is relatively narrow ($\sim 300cm^{-1}$). After titration of the dry solution with sufficient pyridine to form monomeric BChl a.pyridine₂ completely, the spectrum resembles that of the six-coordinate monomeric species in pure pyridine (Figure 8.2C).

The fluorescence bands at 695nm in all spectra in Figure 8.2 are due to BVir, which is present as an impurity in each of the solutions. The intensity of this band increases on prolonged exposure of the BChl a solutions to the laser radiation if the samples are not thoroughly deoxygenated.

Figure 8.2

Fluorescence spectra of 10^{-5} M BChl a in (A) dry hexane; (B) dry hexane titrated with pyridine to disaggregate the dimeric and oligomeric species; (C) pyridine. The excitation wavelength was 401.3 nm. The relative amplification factors are given. The bands marked * are impurity fluorescence from the BChl a oxidation product, BVir.

In all solutions which have been titrated with pyridine to form the monomeric pyridinates of BChl a, the fluorescence is characterised by a relatively narrow band (FWHM $430\text{--}460\text{cm}^{-1}$) with a maximum between 783nm and 795nm depending on the solvent (Table IV). The bandwidths agree well with those found for the Q_y absorption bands of BChl a in the corresponding solvents.

Analysis of the absorption and fluorescence data for BChl a in the different solvents listed in Table IV shows that the fluorescence spectrum in Figure 8.2A originates from both dimeric and oligomeric species of BChl a in solution in dry hexane. This conclusion may be reached on the basis of the observed fluorescence spectra of monomeric BChl a and the knowledge that the wet benzene solutions have been found to contain predominantly the dimer⁸¹ with only minor amounts of the monomer and larger oligomers.

Excitation of fluorescence at different wavelengths in the region of the Q_x absorption bands results in selectivity of several different fluorescence bands (Figure 8.3). Excitation at $\sim 565\text{nm}$ results in a spectrum which is characteristic of the monomeric emission (Figure 8.3B, cf. Figure 8.2 B,C). Excitation near the peak of the Q_x absorption band of the solution at $\sim 580\text{nm}$ results in a spectrum consisting of a symmetrical band at 783nm accompanied by a weaker, narrower, band at 585nm (Figure 8.3C). The bandwidth of the main band at 783nm in the latter spectrum is 590cm^{-1} which is intermediate between that of the monomeric emission and the broad fluorescence band observed by excitation of the solution at 401.3nm (Figure 8.2A). Excitation at

Figure 8.3

Fluorescence spectra from 10^{-5} M BChl a in dry hexane excited in the region of the yellow (Q_x) absorption band at (A) 540.0nm; (B) 565.0nm; (C) 579.5nm; (D) 599.8nm; (E) 619.8nm.

wavelengths further to the red, at 600nm and at 620nm, resulted in broad fluorescence bands at 783nm (FWHM 590cm^{-1}) but it appeared that at these wavelengths there was insufficient energy to excite the 583nm emission band. The similarity of spectrum 8.3C to that obtained from solutions of BChl a in benzene (Table IV) suggests that the spectrum excited at 580nm (Figure 8.3C) is that of the BChl a dimer in hexane.

Selective excitation in the region of the Soret absorption bands was limited by the inability of the laser system to pump ultraviolet dyes efficiently. It was not therefore possible to study the excitation spectrum of the fluorescence excited in the region of the Soret absorption maximum at 360nm. However it was found that the spectra excited at 385nm were significantly different from those excited at 401.3nm. The fluorescence spectrum of BChl a in dry hexane excited at 385nm resembled that in Figure 8.3A which was excited at 540nm. The total Q_y fluorescence bandwidth of this spectrum is 800cm^{-1} , which agrees with the bandwidth of the absorption spectrum of BChl a in dry hexane (Figure 8.1A). The broad band in the fluorescence spectrum is therefore thought to arise from excitation of multiple species of dimeric and oligomeric BChl a in the dry solution, which are characterised by blue shifted absorption and fluorescence bands.

In summary, the spectrum in Figure 8.3C is characterised by a main band at 783nm (FWHM $\sim 590\text{cm}^{-1}$) and a weaker bands at 583nm (FWHM $\sim 300\text{cm}^{-1}$). Time resolved fluorescence spectra showed that the fluorescence lifetime of the 783nm bands was approximately equal to that of the monomeric

fluorescence ($\tau = 3 \pm 0.5 \text{ ns}$).¹⁶⁷ The fluorescence lifetime of the 583nm band is longer than that of the 783nm band. It is believed that this spectrum may be assigned to the dimer of BChl a in hexane for the following reasons:

- i) this spectrum is characteristic only of solutions which are known to contain a significant proportion of the BChl a as the dimer;
- ii) addition of pyridine to the dry solution to disaggregate the dimer results in disappearance of the 583nm fluorescence band and narrowing of the main fluorescence band at 783nm, yielding a fluorescence spectrum characteristic of the BChl a monomer in solution;
- iii) there were no detectable impurities in the solution which had a main absorption band at 580nm which would be present in sufficient concentration to account for the 583nm fluorescence intensity;
- iv) an impurity fluorescence would be expected to remain when pyridine was added to the solution, and the intensity of the fluorescence of any degradation product of BChl a produced by laser irradiation should increase with continued irradiation. This was not observed for the 583nm fluorescence band and the broadening of the 783nm band,
- v) assignment of the spectrum to the dimer rather than another oligomeric species may be made by comparison of the spectra in different solvents which are known to be oligomerized to different extents.

8.3 Discussion

Comparison of the fluorescence spectrum in Figure 8.3C which is assigned to the BChl a dimer in hexane with the spectra of the Chl a dimer in Figure 7.7 (page 125) shows that there is great similarity in the spectral features. Each spectrum is characterised by (i) a relatively broad fluorescence band to the red of the monomeric fluorescence band which corresponds to the region of the dimer Q_y transition and has a fluorescence lifetime which is of the same order of magnitude as, or slightly shorter than, that of the monomeric Q_y fluorescence band; (ii) a weaker fluorescence band in the region of the Q_x absorption band of the dimer which has a fluorescence lifetime which is similar to that of the monomeric fluorescence band. It is therefore believed that the origins of the fluorescence spectra of the BChl a dimer and the Chl a dimer are analogous.

It was not possible to make a definite assignment of the 625nm fluorescence band of the Chl a dimer from the results reported in Chapter 7, but it is clear that the 583nm fluorescence band of the BChl a dimer lies in the region of the Q_x absorption band. This band lies too far to the blue to originate in a Q_y transition of the BChl a dimer. For this reason it is concluded that the 783 nm fluorescence band corresponds to the Q_y absorption band of the BChl a dimer at 770nm and the 583nm fluorescence band corresponds to the Q_x absorption band of the dimer at 580nm.

The similarity of the form of the fluorescence bands of the Chl a dimer suggest that the assignment of the bands may be made by comparison with the results for BChl a. It is therefore believed that the 625nm and 688nm fluorescence bands of the Chl a dimer correspond to the Q_x and Q_y transitions respectively.

Assignment of the two fluorescence bands of each of the Chl a and BChl a dimers to the Q_x and Q_y transitions excludes the possibility suggested in Chapter 7, page 143, that the two fluorescence bands of the Chl a dimer may represent two dimer Q_y exciton components split by 1400cm^{-1} . However, the fluorescence spectra provide no further evidence of whether or not the Q_y and Q_x absorption and fluorescence bands are each due to a single allowed transition or to both exciton components of each transition. Clearly if the absorption and fluorescence bands do contain contributions from more than one component, the splitting in energies is $\leq 100\text{cm}^{-1}$ because the two peaks are not resolved, but examination of the fluorescence bands for evidence of two components of different lifetime was inconclusive.

While observation of fluorescence from the lowest excited singlet levels of the dimers of Chl a and BChl a is not unexpected since the corresponding monomers are highly fluorescent [with quantum yields of fluorescence of 0.3 and 0.2 respectively ¹⁰⁷] the observation of fluorescence from the Q_x levels is unusual for several reasons. Firstly, Q_x fluorescence has not been detected for the monomeric Chl a or BChl a species and secondly, the fluorescence lifetime of the higher energy Q_x band is in each case longer than that of the lower energy Q_y band, a situation which is not normally observed.

The fluorescence yields of the dimers are lower than those of the monomer, which may readily be explained by the fact that the association of two molecules in the dimer increases the number of internal degrees of freedom and therefore leads to a greater probability of non-radiative energy loss. The fluorescence quenching experiments

with excitation in the blue and red spectral regions suggested that there is efficient but selective coupling between some of the levels of the dimers and that non-radiative channels connecting the Q_x and Q_y levels are less effective than in the monomer. This could be a consequence of the symmetry of the electronic states of the dimer being different from those of the monomer because of the relative orientation of the x and y polarised transition moments in the two dimer molecules. Certainly, the activity of the Q_x dimer fluorescence transition suggests that selection rules are different for the dimer.

One explanation of the relatively long lifetime of the dimer Q_x fluorescence transitions could be that de-excitation of these levels takes place via a longer lived state, perhaps involving the triplet levels. In other words the Q_x fluorescence is in fact delayed fluorescence. More careful examination of the fluorescence decay in this region may also reveal a shorter lived component due to a direct de-excitation process. It was also noted that the fluorescence yield of the Chl a dimer Q_x band was greater for excitation in the Soret band than in the region of the Q_x absorption band, which suggests that indirect population of the Q_x levels from higher lying levels is particularly efficient.

Another interesting observation in comparing the spectra of the dimers of Chl a and BChl a is that the dimer and oligomers of Chl a exhibit marked red shifts in their absorption bands relative to the monomeric absorption bands, while the absorption bands of the dimer and oligomers of BChl a, if shifted at all, tend to be blue shifted relative to the monomeric bands. The sensitivity of the shift and bandwidth of the absorption bands of dimeric chlorophylls to the geometrical

structure was illustrated in the case of the synthetically linked chlorophyll pairs (Figure 3.8, page 48). This observation suggests that the detailed structures of the Chl a and BChl a dimers may not be identical. However, the fluorescence data indicate that the dynamics of energy transfer in the dimers of the two chlorophylls are very similar. The latter observation would be expected if chlorophyll-chlorophyll interactions are in fact important in determining the properties of the chlorophylls in vivo.

CHAPTER 9

FLUORESCENCE STUDIES III:

BLUE FLUORESCENCE SPECTRA OF DIMERIC CHLOROPHYLL A IN SOLUTION

Two photon induced blue fluorescence from the Chl a monomer in pyridine, excited in the region of the red (Q_y) absorption bands at 672nm was recently reported by Asano and Koningstein.²² This result was subsequently confirmed by Hunt et al.¹⁷⁶ Following the observation of red fluorescence from the supposedly non-fluorescent Chl a dimer in solution (Chapter 7) the two photon induced blue fluorescence spectrum of the Chl a dimer in solution was investigated to obtain further knowledge of the energy levels of this molecule in the region of the Soret absorption bands. This was important firstly to check the validity of the absorption spectrum of the Chl a dimer in solution which was obtained by absorption difference spectroscopy (section 7.1) and secondly to obtain an estimate of the positions of the electronic origins of the Soret transitions. The latter is of interest to studies of the excitation profiles of Raman active normal modes of the Chl a dimer in solution which are described in the following Chapter.

9.1 Experimental conditions

The sample solutions were 10^{-4} M Chl a in hexane, which were determined to be 80% dimeric Chl a. Solutions of monomeric Chl a in hexane were obtained by titrating the dry solutions with pyridine to

disaggregate the dimer. The two photon induced fluorescence spectra were obtained by excitation in the spectral region 660nm-690nm, in resonance with the Q_y absorption bands. The average laser power from the excimer-dye laser system was 1.5mW. This provided a peak photon flux of 10^{27} photons $\text{cm}^{-2} \text{s}^{-1}$ when the laser radiation was focussed into the sample cell with a 50mm focal length lens. The RCA 31034 photomultiplier tube was used in the detection system.

9.2 The blue fluorescence spectra

The two photon induced fluorescence spectra of dimeric and monomeric Chl a in hexane are shown in Figure 9.1. The dimeric fluorescence spectrum (Figure 9.1A) has a maximum at ~460nm and is noticeably broader than that of the monomer, which peaks at 438nm (Figure 9.1B). The intensity of the monomeric blue fluorescence was a factor of $\sim 2 \times 10^{-4}$ weaker than the normal strong red fluorescence. The intensity of the dimeric blue fluorescence was about half that of the monomeric blue fluorescence (Figure 9.1).

It was noted that excitation of the sample solutions with radiation at ~430nm, directly into the Soret absorption bands, also produces weak blue fluorescence in this region, which was observed as a background emission in the resonance Raman spectra of Chl a excited in the Soret region. Excitation further to the red, in the range 450nm-480nm, produced a broad weak emission band with a maximum at 510nm (Figure 9.2). A similar emission band at ~500nm was previously observed in the emission spectrum of monomeric Chl a in solution.²²

Figure 9.1

Blue fluorescence spectra from (a) dimeric and (b) monomeric Chl a in hexane (10^{-4} M) excited by consecutive two photon absorption at 672nm.

Figure 9.2

Typical fluorescence spectra of dimeric Chl a in hexane excited in the blue region of the spectrum. The spectrum excited at 434nm is similar to that in Figure 9.1 (a) excited by two photon absorption at 672nm.

9.3 Discussion of the blue fluorescence spectra

Monochromatic transmission measurements as a function of laser power in the spectral region from 660nm to 690nm indicate that even at average laser powers as low as 0.2mW the first excited singlet states of both the monomer and dimer of Chl a in solution may be effectively saturated. As an example, Figure 9.3 shows the change in transmission of the samples as a function of laser power at 672nm, the wavelength at which the spectra in Figure 9.1 were excited. Under the conditions where a significant population of the first excited state is created, consecutive absorption of a second photon is possible within the 10ns of the laser pulse.¹⁷⁶ The resulting highly excited state is then observed to relax via blue fluorescence in the region corresponding to the Soret absorption bands both in the monomer^{22,176} and in the dimer of Chl a in solution.

Figure 9.4 shows the absorption spectra of the monomer and dimer together with the red (Q_y) and blue fluorescence spectra of the two species. These spectra illustrate the approximate mirror symmetry of the corresponding absorption and fluorescence bands in both the Soret region and the Q_y region. It is interesting that the Stokes shift of the fluorescence bands relative to the absorption bands are approximately twice as large for the dimeric bands compared with the monomeric bands. This suggests that the potential surfaces of the excited states of the dimer are more different from those of the ground state than in the case for the monomer.

Figure 9.3
Laser power dependence of the transmission at 672nm of (a) monomeric Chl *a* and (b) dimeric Chl *a* in hexane. With increasing power the transmission increases rapidly due to increasing population of the first excited singlet state relative to the ground state, and levels off as the excited state becomes saturated. The continued increase in the transmission of the dimeric solution for powers greater than 1.0mW indicate that population of a second excited state is taking place.

Figure 9.4

Absorption spectra (solid lines) and fluorescence spectra (dashed lines) of (a) monomeric and (b) dimeric Chl *a* in hexane. The wavelengths of the absorption and fluorescence peaks are marked and the Stokes shift of each corresponding absorption and fluorescence band is indicated.

The calculation of the absorption spectrum of the dimer in solution involved fitting the subtracted spectra to match the band width of the observed red fluorescence. At that time the blue fluorescence data were not available and it was not known if the calculated spectrum also represented the blue (Soret) absorption bands correctly. However, the correspondence of the shape and position of the blue fluorescence band and the absorption band in the Soret region is similar to that observed in the red region of the two spectra (Figure 9.4). This is taken to support the validity of the assumptions used in obtaining the absorption spectrum of the dimer by absorption difference spectroscopy.

When the Stokes shift in the peak of a fluorescence band relative to the corresponding absorption band is small, the position of the (0-0) electronic transition can often be found from the wavelength at which the two spectra intersect. In the case of the Chl a monomer and dimer, this can be expected to give only a rough estimate of the position of the electronic origins in the Soret region because of the large Stokes shifts ($\sim 1500\text{cm}^{-1}$). The low signal-to-noise ratio of the very weak two photon induced fluorescence spectra also contributes to the uncertainty in the crossover point. Furthermore, it is known that the Soret bands of the monomer may contain contributions from at least the two B_x and B_y transitions.⁸⁹ In the dimeric spectra each of the corresponding monomeric transitions may be split by the interaction between the two chlorophyll molecules in the dimer, in which case there could be four or more electronic origins in this region. Accordingly the crossover points for the dimer at $\sim 440\text{nm}$ in the blue and at $\sim 678\text{nm}$

in the red should not be related to one particular transition. However further evidence that there are electronic origins of the dimer in this region is given in the following Chapter.

The emission band at 510nm also deserves further investigation. Asano and Koningstein²² observed a similar band in the fluorescence spectrum of monomeric Chl a in pyridine. It is not known which electronic state gives rise to this emission since there is no distinct absorption band in this region. The spectrum in Figure 9.2B^{*} with two maxima separated by 1200cm^{-1} - 1500cm^{-1} may possibly represent the (0-0) and (0-1) vibronic transitions from a new electronic state. It is interesting that the calculations of Petke et al.⁸⁸ (see also Figure 3.4, page 38) show a weak x polarized transition [$S_3(x)$] in this spectral region of the monomer.

CHAPTER 10

RESONANCE RAMAN SPECTROSCOPY OF DIMERIC CHLOROPHYLL A IN SOLUTION

10.1 Previous Raman studies of the chlorophylls in solution

The chlorophylls have strong optical absorptions in the Soret region which should lead to significant resonance enhancement of vibrational Raman scattering intensities. However the pulsed Raman spectra of the chlorophylls have proved to be elusive. One problem is the strong fluorescence, which creates a large background emission underneath the relatively weak Raman scattering intensity. Another problem arises because of the effectiveness of optical pumping of the 0.1-5ns lived excited states in the regions of the strong absorption bands. This depletes the ground state population and produces large populations of excited states which may not absorb strongly in the region of the pump radiation and therefore will not show resonance enhancement of Raman intensities.

Resonance Raman spectra of Chl a in solution using cw laser excitation were reported by Lutz.⁶⁵ Excitation profiles were measured for Chl a solutions in several solvents, at concentrations of 10^{-2} - 10^{-3} M, at the fixed frequencies of a helium-cadmium (He-Cd) laser and an argon ion (Ar^{+}) laser. These profiles show a marked increase in Raman intensities as the laser wavelength approaches the absorption maximum in the Soret region. However, the shortest wavelength available was 441.6nm, and the excitation profile close

to the peak of the absorption band was not measured. Although differences between the spectra of monomeric and aggregated Chl a in solution were noted, the state of aggregation of these solutions was not well defined and no attempt was made to probe specifically the dimeric species.

A similar cw Raman study by Cotton and Van Duyne^{66a} was undertaken to characterise interactions of BChl a in solution, but again, excitation in resonance with the Soret absorption band was not possible with the Ar⁺ laser system. In a later study, the same group employed stimulated Raman scattering from H₂ and D₂ as a source of pulsed radiation in the region of the Soret absorption band of BChl a. This was the first report of a pulsed resonance Raman spectrum of a chlorophyll.^{66b} Unfortunately, even with 10mW average laser power, the signal-to-noise ratios obtained with the PARC-OMA-2 detection system were poor. The authors concluded that the similarity of their pulsed and cw spectra indicated that irreversible photodamage was not significant, but also suggested that they had no method of determining whether "photoreversible intermediates" (i.e. presumably, excited states) were produced. In our experience, significant population of excited states would be created by optical pumping with 10mW average pulsed laser radiation in the region of the Soret absorption band and it is questionable whether the spectra observed were due to ground state or excited state scattering. This would have been clear if power dependent transmission measurements had been made.

The nanosecond pulsed tunable laser Raman system used for the studies reported in this Chapter allowed more detailed high resolution

Raman excitation profiles to be measured in the region of the Soret absorption maxima. Power dependent studies also showed that it is possible to investigate Raman scattering from excited states of Chl a in solution, the presence of which may be demonstrated by transmission studies at the excitation wavelength.

10.2 Experimental Results and Discussion

The Raman studies were carried out with 3×10^{-4} M Chl a solutions in hexane. This concentration is higher than that used for the fluorescence studies, but the Chl a is still predominantly dimeric and this concentration avoids significant contributions from solvent bands. Since samples of this concentration have a high optical density in the region of the Soret absorption bands, attempts to record a light scattering spectrum in the usual 90° scattering geometry were unsuccessful except at wavelengths longer than 450nm, where the absorbance of the solution is relatively low. The quality of the spectra was improved by using glancing incidence. The laser radiation was focussed into one face of a 10mm square quartz optical cell, using a 100mm focal length lens, at an angle of incidence of $\sim 60^\circ$ and the scattered radiation was collected at 90° to this face of the cell (Figure 5.2, page 81). The trace of the laser beam on the cell wall lay in the plane formed by the entrance slit of the monochromator and the optical axis of the collection system. It is estimated that in this geometry the spectra were recorded from a scattering volume which extended $\sim 200 \mu\text{m}$ into the solution. The irradiated volume was therefore $\sim 5 \times 10^{-6} \text{ cm}^3$. For a solution which is 3×10^{-4} M, this volume contains $\sim 10^{12}$ Chl a molecules, which would have been exposed to $\sim 10^{13}$ photons per laser pulse for an average power of 0.6mW.

Figure 10.1 shows part of the Raman spectrum of 3×10^{-4} M Chl a in hexane excited at 433.6nm. The maximum signal was ~50mV on an emission background of ~100mV. Figure 10.2 shows the effect of changing the excitation wavelength on a region of the Raman spectrum with shifts from 1000cm^{-1} to 1700cm^{-1} . As expected, the Raman intensity of the entire spectrum increased as the laser wavelength approached the peak of the Soret absorption band. There are no major frequency shifts, but there are a number of differences in the relative intensities of different bands in the spectra.

For comparison, Table V lists the frequencies found for pulsed excitation at 433.6nm with the frequencies found by Lutz for a more concentrated solution of Chl a in hexane excited at 441.6nm. The pulsed spectra unfortunately show much poorer signal-to-noise ratios which make it difficult to correlate spectral changes with either aggregation of the chlorophylls or possible excited state effects.

Because of the higher fluorescence quantum yield of monomeric Chl a in solution it was impossible to record a pulsed Raman spectrum of the monomer because of the very intense emission background.

Comparison of two spectra recorded at different power levels (Figure 10.3) were inconclusive due to poor signal-to-noise ratios. An average laser power of 0.6mW was found to give optimum Raman signals without excessive photodegradation of the samples which was observed on prolonged irradiation with focussed radiation. Also, increasing the power did not necessarily lead to increased Raman intensity because of the creation of excited state species which are not resonance enhanced at the exciting frequency (see page 183).

Figure 10.1
Pulsed laser induced resonance Raman spectrum of 3×10^{-4} M Chl a in hexane excited at 433.6nm.

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TABLE V

RAMAN SHIFTS FOR CHLOROPHYLL A IN HEXANE

CW Excitation * at 441.6nm $5 \times 10^{-2} M$ (aggregated) cm ⁻¹	Pulsed Excitation at 433.6nm $3 \times 10^{-4} M$ (dimeric) cm ⁻¹
575	572
695	
710	712
740	752
795	803
915	922
980	993
1045	1050
1115	(1115?)
1145	1158
1180	1189
1210	1214
1260	1253
1285	1295
1330	1332
1345	1357
1375	
1380	1387
1440	1443
1490	1493
1525	1533
1555	1565
1610	1615
1653	
1700	1710

*taken from the work of Lutz (ref. 65).

Figure 10.2

Comparison of the 1100cm^{-1} to 1700cm^{-1} region of the resonance Raman spectrum of dimeric Chl *a* in hexane excited at different laser wavelengths in the region of the Soret absorption bands.

Figure 10.3

Comparison of two Raman spectra of Chl a in hexane excited at different laser powers at 428.5nm: (A) 1.5mW; (B) 0.8mW.

Figure 10.4

Comparison of the resonance Raman spectra of $10^{-4} \text{ M Chl } a$ in dry hexane excited at 433.6 nm at average laser powers of (A) 1.5 mW and (B) 0.5 mW.

The Raman spectra shown in Figures 10.1 and 10.2 were recorded by scanning the monochromator at $\sim 10\text{cm}^{-1}\text{min}^{-1}$ and allowing the time constant of the boxcar averager to average the signal. However, the scan speed could not be decreased below about $2\text{cm}^{-1}\text{min}^{-1}$ without increasing the total scan time so much that sample degradation towards the end of a run became a problem. Even then, the signal-to-noise ratios were too low to allow detailed studies of the power dependence or wavelength dependence of the spectra to be made.

Towards the end of the project the computer controlled data acquisition system became functional and several runs were made to test the effect of digitization and signal averaging on the quality of the Raman spectra. As an example, Figure 10.4 shows part of the Raman spectrum of Chl a in dry hexane excited at 433.6nm recorded at two different laser power levels. This may be compared with the analogue run shown in Figure 10.1. The total scan times for both the analogue and the digitized spectra were approximately the same. The latter was run by averaging 400 data samples at 2cm^{-1} intervals.

The spectra in Figure 10.4 show some improvement in signal-to-noise ratios compared with the analogue spectra and in this case there are clearly some differences between spectrum 10.4A and spectrum 10.4B, which were recorded at laser powers of 1.5mW and 0.5mW respectively. Spectrum 10.4A presumably contains a higher proportion of excited states because the transmission of the solution increases with increasing laser power at this excitation wavelength. The most notable differences are in the bands with shifts in the region 1100cm^{-1} to 1200cm^{-1} . Examination of the spectrum in Figure 10.4A

shows a decrease in intensity at a shift of 1140cm^{-1} and an increase in intensity at 1150cm^{-1} compared with the run at lower power, which may possibly be attributable to excited state scattering.

Raman excitation profiles

A detailed study of the excitation profiles of the two strongest bands in the spectrum was undertaken in the spectral region from 427nm to 440nm, around the peak of the Soret absorption band of the Chl a dimer in solution. The power was normalised to 0.6mW and the Chl a Raman intensities were measured relative to the intensity of the strong Raman band of hexane at 2900cm^{-1} .

It is clear from Figure 10.5 that there are no significant intensity changes in the excitation profile at wavelengths around 428nm, which corresponds to the absorption maximum of the Chl a monomer in hexane. On the other hand intensity changes were recorded in the region from 430nm to 440nm where the absorption bands of the Chl a dimer are observed. The profiles of several Raman shifts in this region are identical which indicates that electronic origins of the dimer are located in the spectral region 430nm - 440nm. The Raman spectrum shown in Figure 10.1 was excited at 433.6nm, the wavelength at which maximum enhancement of the Raman intensities was observed for a laser power of 0.6mW. At this wavelength the greater part of the Raman intensity is due to ground state vibrational scattering from the Chl a dimer. The remaining intensity may contain contributions from pre-resonance scattering from other species of Chl a, including excited states.

Figure 10.5

The excitation profiles of the Raman shifts at 1189cm^{-1} and 1565cm^{-1} .

Figure 10.6

Plots to show the dependence of the Raman intensities of Chl a and the solvent, hexane, on laser power in the region of the excitation profiles shown in Figure 10.5. At 433.6nm and 433.3nm, near the peak of the excitation profile, the non-linear behaviour of the Raman intensities suggests that depletion of the ground state and population of an excited state occurs as the power is increased. However, in the region of one of the holes in the excitation profile; the Raman intensities increase linearly with increasing power. This suggests that in the region of the hole the excited state is saturated for laser powers between 0.22mW and 0.8mW. and therefore the holes in the excitation profile are caused by excited state effects rather than interference effects in the ground state scattering.

Figure 10.7

Power dependence of the transmission of (A) monomeric Chl *a* pyridinate at 434.1nm and (B) dimeric Chl *a* at 434.1nm (squares) and 435.3nm (circles). $[5 \times 10^{-4} \text{ M}$ solutions in hexane]. Whereas the transmission of the monomeric solution increases as the laser power is increased, the transmission of the dimeric solution initially decreases indicating that the first excited state of the dimer to be populated absorbs more strongly in this spectral region than the ground state. The Raman spectra were recorded in the region where the transmission begins to rise steeply. Expanded plots of the data indicate other plateaux at the positions indicated by the arrows.

The excitation profiles in Figure 10.5 show sharp minima at 434.1nm and 435.3nm. It was observed that the Raman intensities of both Chl a and the solvent depended non-linearly on the laser intensity for excitation wavelengths in the range 430nm to 436nm (Figure 10.6). This observation is very significant to the explanation of the minima, because it suggests that the 'holes' in the excitation profile are not due to antiresonance (interference effects) on the Raman intensities. Theory suggests that the latter may occur when there are two overlapping electronic transitions in the excitation region, which could quite possibly be the case for a dimeric system such as Chl a. However the results shown in Figure 10.6 and the information gained from transmission measurements and fluorescence studies about optical pumping of the excited states of the Chl a dimer lead to an alternative explanation of the holes in the excitation profile.

Laser dependent photoquenching of the red fluorescence of the Chl a dimer was observed with excitation at wavelengths in resonance with the Soret absorption bands (Section 7.3). This photoquenching was attributed to population of the singlet levels at $16,200\text{cm}^{-1}$ and $14,750\text{cm}^{-1}$ (which have lifetimes of $\sim 1\text{ns}$ and $\sim 5\text{ns}$ respectively) and the subsequent absorption of a second photon in the blue region of the spectrum, which competes with relaxation to the ground state via emission the red fluorescence. The experimental conditions required to record the Raman spectra are also favourable for population of these levels. The latter conclusion was made from power dependent transmission measurements at the excitation wavelengths (Figure 10.7).

Maximum population of the excited singlet levels should occur at the wavelength at which there is a maximum in the absorption band. If this maximum is due to a $(0-0)$ electronic transition then this wavelength should correspond to a maximum in the Raman excitation profile. Under these conditions, vibrational Raman scattering from the excited state may also contribute to the excitation spectrum in Figure 10.3. However it is highly unlikely that an accidental degeneracy occurs and that rigorous resonance conditions exist at the same wavelengths (433nm-436nm) for vibrational scattering from both the ground and excited states of the Chl a dimer. The sharp minima in the excitation profiles are therefore interpreted as an indication that at these wavelengths there is significant population of excited states, and the contribution from these states to the Raman intensities is smaller than that of the resonance enhanced scattering from the ground state. Some indication of the degree of population of the excited states can be obtained from the non-linear behaviour of the Raman intensities versus laser intensity at all wavelengths between 433nm and 437nm except those at the minima in the excitation profiles (433.6nm and 435.3nm) (Figure 10.5). Outside the holes in the profiles, the relative population numbers of ground and excited states are dependent of the laser intensity and thus cause non-linearities in the Raman intensities. In the hole, effective population saturation of the excited state is obtained at laser powers from 0.18mW (the minimum average power for which a Raman spectrum could be recorded) to 0.8mW, and the Raman intensity displays linear dependence on the laser intensity (Figure 10.5). Thus

the two holes in the Raman excitation profile appear to indicate the presence of two excited state electronic origins of the Chl a dimer at 23036cm^{-1} and 22972cm^{-1} . Whether the splitting of 60cm^{-1} is related to the exciton interaction between the two Chl a molecules in the dimer requires further study because it is known that even in the monomer, several electronic origins exist in the region of the Soret band.

A parallel may be drawn between the phenomenon described above and the holeburning in an inhomogeneously broadened absorption band of a solid, first observed by Szabo,¹⁷⁷ although there are differences in both the experimental techniques and the theoretical explanations of the Raman, absorption and related fluorescence line narrowing results.^{178,179}

The widths of the holes in the Raman excitation profiles are determined by the population density of the ground state $N_g(\lambda)$ and that of the excited state $N_e(\lambda)$ and the corresponding Raman cross sections $Q_g^q(\lambda)$ and $Q_e^q(\lambda)$, all of which are dependant on the excitation wavelength λ . The Raman intensity for the normal mode q is given by

$$I_{\text{Raman}}^q(\lambda) = N_g(\lambda)Q_g^q(\lambda)I_l + N_e(\lambda)Q_e^q(\lambda)I \quad (10.1)$$

where I_l is the laser intensity. Although $Q_g^q(\lambda)$ for the Chl a dimer in the wavelength interval 430nm-440nm is a rigorous resonance Raman cross section, it is not anticipated that this is also the case for $Q_e^q(\lambda)$. Thus it is expected that $Q_e^q(\lambda)$ varies gradually with wavelength in this region whereas $Q_g^q(\lambda)$ should show a sharp wavelength dependence. From the observed non-linear behaviour of the Raman intensities (of Chl a and hexane) as a function of laser power it can be concluded

that the population numbers $N_e(\lambda)$ and $N_g(\lambda)$ are also strongly dependent on wavelength. The shape of the excitation profiles as represented by equation 10.1 is therefore determined by three quantities which vary non-linearly with wavelength. In contrast to hole burning in an absorption band, the effect in the resonance Raman excitation profile is indirectly dependent on the absorption cross sections of the ground state and excited state transitions.

Some preliminary calculations¹⁸⁰ indicated that the sharp non-linearities in the populations of the ground and excited states could not be modelled with a three level system, and suggested that at least a three photon process is involved. This may possibly involve a photochemically unstable excited state, since some decomposition of the solution was observed with irradiation with focussed laser light, particularly in the region of the holes in the excitation profile. The proposed scheme is discussed in more detail, with the fluorescence data, in Chapter 11.

An improvement in signal-to-noise ratios of the Raman spectra by a factor of 2 or 3 would aid more detailed study of excited state scattering considerably. Clearly much further work remains to be done to obtain the full potential of resonance Raman scattering for the study of vibrational and electronic properties of the chlorophylls in solution, in particular for studying excited state properties. However the prospect of using non-linear Raman scattering techniques to resolve closely lying electronic origins appears promising, as illustrated by the hole burning observed in the resonance Raman excitation profiles of dimeric Chl a in solution.

CHAPTER 11CONCLUSIONS AND SUGGESTIONS FOR FURTHER DEVELOPMENTS

This concluding chapter discusses the more significant results of the pulsed laser induced fluorescence and resonance Raman scattering of the dimers of Chl a and BChl a in solution which are described in Chapters 7 to 10. Figure 11.1 and Table VI summarise the information gained on the energy levels, interaction energies and dynamics of energy conversion for the Chl a dimer in hexane since this was the system studied in most detail. These results represent in a qualitative way those obtained for the Chl a dimer in other solvents and for the BChl a dimer.

The first accomplishment of the time resolved fluorescence study was to show that the dimers of Chl a and BChl a formed in dry non-polar solvents are fluorescent. Analysis of the absorption spectra of the dimeric solutions showed that the absorption bands of the dimers are red shifted with respect to those of the corresponding monomers. Thus it was possible to selectively excite the dimer fluorescence by tuning the laser wavelength into resonance with the absorption bands of the dimer in the region of either the Soret transitions or the Q transitions. This overcame the problem of the relatively weak dimeric fluorescence being masked by the strong monomeric fluorescence which is observed if the solutions are excited in the peak of the blue absorption band where the monomer absorbs strongly. An estimate of the quantum yield of the fluorescence of the

dimer obtained from the ratio of the fluorescence intensities with excitation at 450nm yielded a value of ~15% of the monomeric quantum yield.

The red fluorescence spectra of the dimeric chlorophylls are characterised by two bands. One is red shifted with respect to the monomeric fluorescence and corresponds to the dimeric Q_y absorption band. The fluorescence lifetime is shorter ($\tau_{f1} \approx 1\text{ns}$) than that of the monomeric fluorescence. In addition to the expected fluorescence from the lowest excited singlet transition, there is a relatively weak fluorescence bands in the region of the Q_x absorption band. This emission band is characterised by an unusually long lifetime for a transition from a level of higher energy ($\tau_{f1} \approx 5\text{ns}$). Furthermore, blue emission from the Chl a dimer was observed in the region of the Soret absorption bands (S_4) (i) by direct pumping at 440nm and (ii) following two photon absorption with the laser wavelength at 672nm, in resonance with the Q_y absorption band. Excitation in the region 450nm-480nm produced emission at ~510nm, which is thought to arise from another electronic state (S_3). The latter was also observed for the Chl a monomer in solution. These radiative transitions are shown in the level schemes in Figure 11.1.

The fluorescence excitation profiles and the dependence of the fluorescence intensities on the incident laser power provided clues to the dynamics of radiative and non-radiative energy relaxation in the dimer. Some of these processes are represented in Figure 11.1. The thickness of the arrows is an indication of the relative cross sections of the various processes. For clarity the vibrational levels are not shown. However it should be remembered that the population of the lower vibrational levels at room temperature will contribute to

Figure 11.1

Schematic level diagram of radiative and non-radiative relaxation of dimeric Chl *a* under optical pumping in the region of the Soret absorption band with (A) low photon flux density; (B) high photon flux density. (A) represents the fluorescence experiments: emission from S_2 and S_3 , as well as strong S_1 emission is observed. (B) represents the conditions for the Raman experiments where the red emission is quenched by excited state absorption by S_1 and possibly S_4 . Population of S_1 and S_2 (and S_4 ?) depletes the ground state population and leads to the hole burning in the Raman excitation profile. See text for details. (The widths of the arrows are a very approximate indication of relative cross sections for the various processes).

the intramolecular energy relaxation. The double arrows on some of the internal conversion or intersystem crossing pathways indicating an upward energy transfer take this vibrational energy into account. Although the exciton splittings of the dimeric energy levels cannot be found from the absorption and fluorescence data, the dimer levels in Figure are shown as pairs, labelled S_n' , S_n'' to correspond to the states S_n of the monomer. The resonance Raman excitation profiles in the region of the Soret absorption band may be interpreted as showing that there are two electronic origins split by $\sim 60\text{cm}^{-1}$. However, further investigation is required to determine whether this separation represents an exciton splitting of dimer levels in the Soret region (for conjecture, included in Figure 11.1).

The shorter lifetime and lower fluorescence yield of the dimeric Q_y fluorescence band compared to the monomeric Q_y fluorescence may be explained by the larger number of internal degrees of freedom in the pair of molecules leading to an increased probability of non-radiative relaxation. Nevertheless it is significant that dimeric Q_x emission is observed because monomeric Q_x emission has not been detected. Since the Q_x fluorescence lifetime is longer than that of the Q_y fluorescence it could be that the Q_x emission is delayed fluorescence, involving indirect de-excitation via a longer lived triplet state. The mechanism may entail intersystem crossing in the region of the Soret absorption bands and relaxation within the triplet manifold or relaxation within the singlet manifold followed by interchange of energy between the lower lying singlet and triplet levels.

TABLE VI ABSORPTION AND FLUORESCENCE DATA FOR MONOMERIC AND DIMERIC CHLOROPHYLL A IN HEXANE

Transition	MONOMER			DIMER		
	$\lambda_{\max}$ (nm)	Absorption $10^{-4} \text{ l mol}^{-1} \text{ cm}^{-1}$	Fluorescence $\lambda_{\max}$ (nm) τ_{fl} (ns) relative intensity	$\lambda_{\max}$ (nm)	Absorption $10^{-4} \text{ l mol}^{-1} \text{ cm}^{-1}$	Fluorescence $\lambda_{\max}$ (nm) τ_{fl} (ns) relative intensity
S_1 Q_y	659	8.0	668 5 1	669	4.2	688 1 1.5×10^{-1}
S_2 Q_x	600	1.0	- - -	605	1.0	625 5 9×10^{-3}
S_3 ?	?	?	500 ? 10^{-4}	?	?	510 ? 10^{-5}
S_4 B	427	9.4	436 1ns 2×10^{-4}	432	5.8	460 ? 10^{-4}

The power dependence of the transmission of dimeric Chl a in solution shows that the lower lying singlet states (which have lifetimes of several nanoseconds) are easily populated by optical pumping at moderate laser photon flux densities. (Figures 9.3 and 10.7) Creation of excited state populations is followed by excited state absorption. The latter is expected to contribute to the hole burning phenomenon in the resonance Raman excitation profiles of the Chl a dimer, which may be explained by a three photon process resulting in a highly excited state S_n (Figure 11.18).

The transmission in the region of 430nm - 450nm shows an initial decrease before rising steeply as the laser power is increased (Figure 10.7). It is interesting that a similar intensity dependence of the transmission of Chl a in vivo has been reported.¹⁸¹ It is speculated that the initial decrease in transmission may be due to efficient intersystem crossing between the lower singlet states and a triplet state of a similar energy. Alternatively, the decrease in transmission could be explained by population of one of the singlet states which has a higher cross section for absorption at the pump wavelength than the ground state. Time resolved absorption studies would aid the identification of the excited states. Further investigation of the dynamics of energy relaxation would be aided by two photon experiments in which excited states are created by optical pumping at one wavelength followed by probing of the fluorescence with a second laser pulse of a different wavelength. Time resolved spectroscopy in the microsecond time range is required to study the triplet states.

Further work on the Raman scattering of the Chl a dimer in solution could be very productive. It was shown that the study of high resolution resonance Raman excitation profiles of the vibrational modes of the dimer in solution can be used to resolve closely lying electronic origins which may yield values for exciton interaction energies. The recent theoretical study¹⁰⁶ of the selection rules for electronic Raman scattering in the Chl a dimer also suggests that this technique may be able to provide a direct measure of the exciton splittings, if suitable experimental conditions can be found to obtain the Chl a dimer at low temperature. For more detailed studies of the vibrational Raman scattering it may be beneficial to use a non-linear Raman technique, such as coherent antiStokes Raman scattering, to eliminate the intense emission background which is a problem with ordinary resonance Raman scattering.

APPENDIX I Structures and visible absorption bands of some derivatives of Chlorophyll a

Derivative	Preparation	Structure			λ blue max	ϵ blue max	λ red max	$\frac{\epsilon \text{ blue}}{\epsilon \text{ red}}$
		R ₁	R ₂	R ₃				
Chlorophyll <u>a</u>	by chromatography	H	CO ₂ CH ₃	C ₂₀ H ₃₈	428	10.9x10 ⁴	661	1.29
Chlorophyll <u>a'</u>	heating in pyridine	same as Chl <u>a</u> with inversion at C ₁₀			428	10.9x10 ⁴	661	1.29
Chlorophyllide <u>a</u>	enzymatic hydrolysis	H	CO ₂ CH ₃	H	428	13.1x10 ⁴	660.5	1.49
Ethyl chlorophyllide- <u>a</u>	enzymatic hydrolysis in ethanol	H	CO ₂ CH ₃	CH ₂ CH ₃	427.5	11.9x10 ⁴	660	1.33
Methyl chlorophyllide <u>a</u>	enzymatic hydrolysis	H	CO ₂ CH ₃	CH ₃	427.5	10.8x10 ⁴	660.5	1.30
10-hydroxy-Chlorophyll <u>a</u>	allomerization or enzymatic oxidation	OH	CO ₂ CH ₃	C ₂₀ H ₃₈	428	-	660.5	1.29
10-methoxy-chlorophyll <u>a</u>	allomerization	OCH ₃	CO ₂ CH ₃	C ₂₀ H ₃₈	428.5	-	660.5	-
10-methoxy-actone-Chlorophyll <u>a</u>	allomerization	OCH ₃	CO ₂ CH ₃	C ₂₀ H ₃₈	416	-	656	1.82
Pyrochlorophyll <u>a</u>	heating in pyridine 100°C	H	H	C ₂₀ H ₃₈	429	11.9x10 ⁴	659	1.49
Methyl pyrochlorophyllide <u>a</u>	enzymatic hydrolysis and heating in pyridine	H	H	CH ₃	428	10.9x10 ⁴	659	1.52
9-desoxo-9hydroxy-Chl <u>a</u>	reduction with sodium borohydride	H	CO ₂ CH ₃	C ₂₀ H ₃₈ and H and OH in place of =O at C ₉	397	17.2x10 ⁴	655	3.25
2-devinyl-2-acetyl Chl <u>a</u>	oxidation of bacterio-chlorophylls	H	CO ₂ CH ₃	C ₂₀ H ₃₈	437	-	678	2.08
Protochlorophyll <u>a</u>	biosynthetic precursor	H	CO ₂ CH ₃	C ₂₀ H ₃₈ ring IV is reduced at C ₇ , C ₈	432	20 x10 ⁴	622	8
<u>Magnesium-free derivatives</u>								
Pheophytin <u>a</u>	aqueous mineral acid	H	CO ₂ CH ₃	C ₂₀ H ₃₈	409	15.6x10 ⁴	667	2.09
Pheophorbide <u>a</u>	refluxing in acidic acetone	H	CO ₂ CH ₃	H	408.5	14.1x10 ⁴	667	2.07

APPENDIX 2

PRISMS

A. Pellin-Broca constant deviation dispersing prism

If light is incident on face AD of the prism as shown, one wavelength will undergo minimum deviation, which is 90° . Rotation of the prism about an axis normal to the plane of the paper will result in a different wavelength emerging at minimum deviation. With a prism of this sort, one can set up a light source and detection system at a fixed angle of 90° and simply rotate the prism to select a particular wavelength.

B. Dove prism for rotation of image

This is a truncated version of a right angle prism, used with collimated light. It has the property of rotating the image twice as fast as it is rotated about the longitudinal axis. It is used to rotate scattered or fluorescent light into the plane of the entrance slit of a monochromator.

C. Glan Thompson polarizer

absorbing paint or glass

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