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AGGREGATION OF CHLOROPHYLL I: SPECTROSCOPIC STUDY OF SOLVENT EFFECTS

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Summary

Careful purification of chlorophyll a and b (Chl a, Chl b) allowed the dilute solution absorption and fluorescence spectra in iso-octane (Chl a only), benzene and diethyl ether to be studied. The absorption spectrum in iso-octane shows the least perturbation by the solvent and can be taken as characteristic of molecularly isolated Chl molecules. The stronger solvents blur the vibronic structure of the Chl, and benzene introduces a specific vibronic coupling to the solvent. The addition of *n*-propanol causes the formation of small aggregates (judged to be dimers, trimers and tetramers) and so causes the first spectral changes characterizing the difference between isolated molecules *in vitro* and large stacks *in vivo*.

1. Introduction

During the last 4 or 5 years there has been a significant advance in the understanding of the overall process of photosynthesis [1]. Energy enters the photoactive system, is initially gathered by antenna chlorophyll (Chl) and other pigment molecules, and is then transferred to the reaction centre where it initiates the chemical electron transfer reaction.

The photophysical steps of this process depend critically upon the detailed changes which occur in the spectroscopic characteristics of Chl upon formation of an aggregate. Both antenna and reaction centre Chl are thought to be active in the form of aggregates. The light harvesting role of antenna Chl is believed to involve long stacks of Chl molecules, whereas reaction centre Chl forms rather shorter stacks usually associated with five or six

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peptide molecules [2, 3]. The energy harvesting efficiency of Chl *in vivo* has never been matched *in vitro*, one reason being the failure of a synthetic system to achieve the order represented by the stacks. Specially synthesized macromolecules [4] offer the possibility of complexing Chl into ordered arrays, and the research described in this and a subsequent paper is oriented towards the achievement of polymer–chlorophyll conjugates with energy migration characteristics resembling those of Chl in the natural chloroplast.

A recent study [5] of the effects of polar and non-polar solvents on the spectroscopic characteristics of Chl a and Chl b has indicated that when Chl is molecularly dispersed the energy of the lowest singlet absorption and fluorescence is a simple function of the polarizability of the medium in which it is placed. However, in hydrogen bonding solvents or systems which induce aggregation, deviations from this simple behaviour are observed. In this work we examine the addition of aggregating molecules to a "molecularly" dispersed Chl solution in order to characterize the effects of aggregation on the photophysical properties. This first part describes the effect of low molecular weight solvents, a necessary prerequisite to the study of the chlorophyll–polymer conjugates.

2. Experimental details

2.1. Chlorophyll

Much of the spectroscopic and photophysical work on Chl reported in the literature (even recently) is misleading because of inadequate separation of Chl a, Chl b and the pheophytins. Meaningful wavelength shifts and energy transfer characteristics can be obtained only after careful purification and fractionation in the dark and by removal of traces of water. For this reason we report the purification procedures in detail.

The Chl used in this study was obtained from fresh spinach. The spinach (200 g) was placed in boiling water (2 l) and heated for approximately 2 min. The liquid was then quickly cooled and decanted from the leaves. These were placed in methanol (500 ml) plus petroleum ether (125 ml) and the Chl was extracted. The deep green extract was filtered through a glass plug to remove residual cellulosic materials and was placed in a brown storage vessel. The liquor was passed through a chromatographic column of sucrose and a mixture of Chl a and Chl b was eluted with petroleum ether containing 0.5% *n*-propanol. The amounts of xanthophylls and pheophytins were monitored spectroscopically and fractions containing large amounts of of either were discarded. The crude Chl was then isolated by shaking the petroleum ether solution with water. The separation of Chl a and Chl b was achieved by further chromatography on similar columns. The solvent was completely removed from the final eluted fractions of Chl in a vacuum system. The solid was then redissolved in benzene which had itself been dried with $LiAlH_4$ on the vacuum line. The benzene was then removed on the vacuum system at 313 K. This process was repeated several times to remove

the last traces of water. The final solid was then partially dissolved in isooctane which had also been carefully dried. The remaining residue was dissolved in diethyl ether. This last treatment completed the separation of Chl a and Chl b. The final solutions were transferred under vacuum to a sealable cell which was used for recording the emission and absorption spectra.

2.2. Spectroscopic technique

The absorption spectra of the solutions were obtained using evacuated quartz cells and were recorded on a Beckman Acta IV spectrophotometer equipped with a derivative mode module. Fluorescence spectra were obtained using the same cell on a Perkin–Elmer MPF 44 fluorescence spectrometer calibrated using the 450.1 and 467 nm xenon lines. The emission spectra were recorded on ratio mode, thus automatically correcting for fluctuations in the lamp intensity. Rhodamine G and methylene blue were used as standards [6] to calibrate the wavelength dependence of the photomultiplier sensitivity, and a correction function was computed as follows:

$$E(\lambda)_{\mathrm{corr}} = E(\lambda)_{\mathrm{uncorr}}\, q(\lambda) \tag{1}$$

where $E(\lambda)_{\mathrm{corr}}$ and $E(\lambda)_{\mathrm{uncorr}}$ are the corrected and uncorrected normalized spectra respectively and $q(\lambda)$ is the correction function. Over the range of Chl a and Chl b emission $q(\lambda)$ can be approximated by the linear function

$$q(\lambda) = a + \lambda b \tag{2}$$

The long wavelength half of the emission spectrum is expressed as a gaussian function

$$E(\lambda)_{\mathrm{uncorr}} = \exp\left\{\left(-\frac{\lambda - \lambda_{\mathrm{max}}}{2^{1/2}\sigma}\right)^2\right\} \tag{3}$$

where λ_{max} is the wavelength of maximum emission in the uncorrected spectrum and σ is the standard deviation expressed in terms of the halfwidth $W_{1/2}$ at half-height:

$$\sigma = \frac{W_{1/2}}{(2 \ln 2)^{1/2}} \tag{4}$$

The solution of $\mathrm{d}E(\lambda)_{\mathrm{corr}}/\mathrm{d}\lambda = 0$ yields

$$(\lambda_{\mathrm{max}})_{\mathrm{corr}} = -\frac{b}{2a} + \frac{1}{2}\left\{\left(\frac{b}{a} - \lambda_{\mathrm{max}}\right)^2 + 4\left(\frac{b}{a}\lambda_{\mathrm{max}} + \sigma^2\right)\right\}^{1/2} \tag{5}$$

In these experiments, conversions of spectra to an energy scale [7] and the application of eqn. (5) resulted in a correction $(\lambda_{\mathrm{max}})_{\mathrm{corr}} - \lambda_{\mathrm{max}}$ of less than 0.2 nm, which is less than the precision of measuring the difference in the wavelength maxima at absorption and emission.

3. Results

Measurements were made on three solvent systems, iso-octane, benzene and diethyl ether; *n*-propanol was used as the aggregation-inducing additive in all cases.

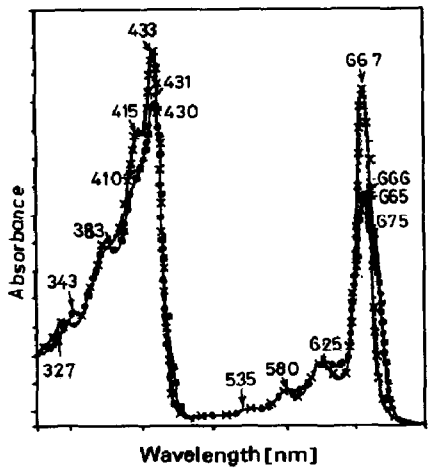
3.1. Iso-octane

Chl b does not dissolve in iso-octane. A Chl a solution with a concentration of 5×10^{-5} mol l^{-1} was examined. The absorption maxima occurred at 353 and 431 nm in agreement with the results of earlier studies [5]. On addition of *n*-propanol the red and Soret absorbances increased and the absorption at 675 nm disappeared. The absorptions at 415, 383 and 343 nm were observed to increase; in contrast, the intensities of the fluorescence maxima were shifted to longer wavelength (Table 1). The effects on the absorption spectrum are summarized in Fig. 1. The addition of *n*-propanol leads to changes in the ratio of the intensities of the 431 and 653 nm bands as a consequence of aggregation (Fig. 2). This ratio has a value of approximately 1.39 in pure iso-octane where the Chl is molecularly dispersed and decreases to 1.04 in 14 M *n*-propanol (Fig. 3). The addition of *n*-propanol has the effect of shifting the position of the absorption and emission bands (Fig. 4). The shift reaches a maximum at 1.02 M *n*-propanol. These data indicate that the shifts are a consequence of direction interaction between the *n*-propanol and the Chl rather than a local field dielectric effect. If the latter were operative, it would be expected that the shift would vary continuously with changes in the concentration of the mixture.

TABLE 1

Spectral shifts produced following the addition of *n*-propanol (5×10^{-5} mol l^{-1}) to chlorophyll

Chlorophyll	*Solvent*	*Absorption maximum* (nm)	*Excitation* (nm)	*Emission maximum* (nm)
Chl a	Iso-octane	653	657	663
Chl a	Iso-octane + *n*-propanol	667	666	671
Chl a	Benzene	665	665	672
Chl a	Benzene + *n*-propanol	666	667	673
Chl b	Benzene	648	648	648
Chl b	Benzene + *n*-propanol	652	646	653
Chl a	Diethyl ether	660	659	668
Chl a	Diethyl ether + *n*-propanol	663	667	672
Chl b	Diethyl ether	644	640	644
Chl b	Diethyl ether + *n*-propanol	650	642	657



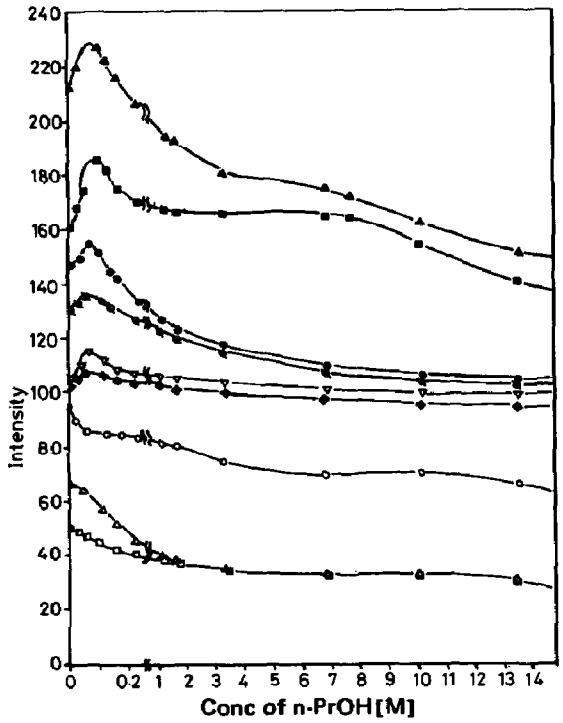


Fig. 1. Absorption spectra of Chl a in iso-octane: ■, dried Chl a in iso-octane; ●, dried Chl a in iso-octane + 2% *n*-propanol; ×, dried Chl a in iso-octane + 4% *n*-propanol.

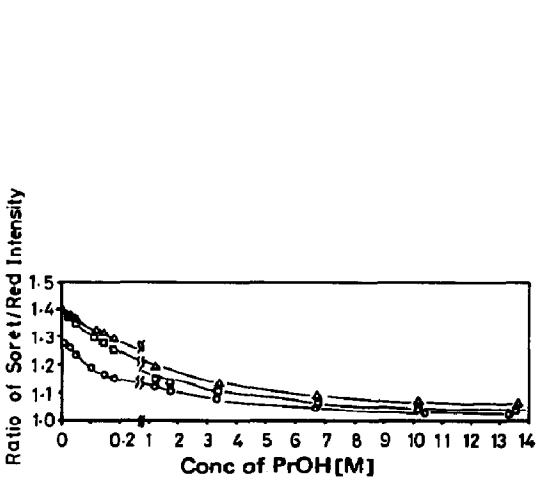
Fig. 2. Emission and absorbance intensities of Chl a in the Soret and red bands *vs.* the concentration of *n*-propanol in the solvent.

Solvent	*Soret band*	*Red band*	*Emission*
Ethyl ether	▲	■	○
Benzene	●	▽	△
Iso-octane	⊘	◆	□

The halfwidths for the band at 653 nm increased from 9.9 to 10.2 nm and those for the 431 nm band increased from 18.5 to 23.5 nm. A close examination revealed that two types of change occurred. A band at 410 nm increased in intensity relative to a general decrease in intensity of the features at 329, 343, 383 and 433 nm. The addition of *n*-propanol also caused a doublet at 665 and 675 nm to change to a single peak with a corresponding change in intensity.

3.2. Benzene

Both Chl a and Chl b are soluble in benzene, and solutions of concentration 5×10^{-5} mol l^{-1} and 3.9×10^{-6} mol l^{-1} respectively were examined. The spectral shifts are summarized in Table 1 and are commensurate with those observed in iso-octane. The intensity of the absorption again increases, reaching a maximum at 0.1 mol l^{-1} *n*-propanol (Fig. 2). In contrast with iso-



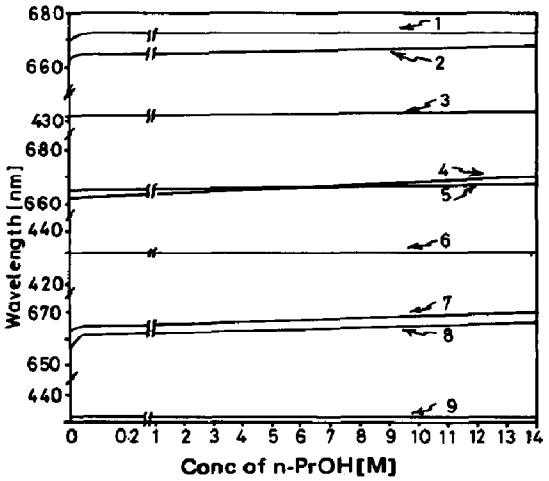


Fig. 3. Ratios of Soret to red band intensities of Chl a *vs.* the concentration of *n*-propanol in the organic solvent: ○, ethyl ether; □, benzene; △, iso-octane.

Fig. 4. Absorbance and emission wavelengths of Chl a *vs.* the concentration of *n*-propanol in the organic solvent.

Solvent	*Emission*	*Absorption*	
		Red band	*Soret band*
Benzene	Curve 1	Curve 2	Curve 3
Diethyl ether	Curve 4	Curve 5	Curve 6
Iso-octane	Curve 7	Curve 8	Curve 9

octane, the fluorescence intensity first increases and then decreases on the addition of *n*-propanol. As in the case of iso-octane, the ratio of the intensity of the absorption of the Soret and red bands changes with the variation in concentration of the *n*-propanol (Fig. 3). In dry benzene the Chl a peak at 665 nm is a singlet and the ratio of the intensities at 432 and 665 nm varies little with concentration. In Chl b a new peak at 415 nm in dry benzene replaces the 430 nm absorption. Further, the distribution of intensities in the low wavelength excitation spectrum differs noticeably from that of Chl a, with the 665 nm peak becoming a multiplet in 14 M *n*-propanol (Fig. 5).

The variation in the excitation and absorption intensities as a function of the Chl concentration is shown in Fig. 6. The observation of a peak in the concentration variation is indicative of the occurrence of molecular interactions in the solution. The changes in the spectral profile with changes in the concentration of pure Chl b are shown in Fig. 7. The variation in the emission and absorption intensities with the concentration of *n*-propanol is summarized in Fig. 8. The variation in the peak position with the concentration of *n*-propanol is shown in Fig. 9. In contrast with the observations on iso-octane, there is a steady shift with the change in concentration of *n*-propanol which is indicative of a solvent effect in addition to a specific solvent–chlorophyll interaction effect.

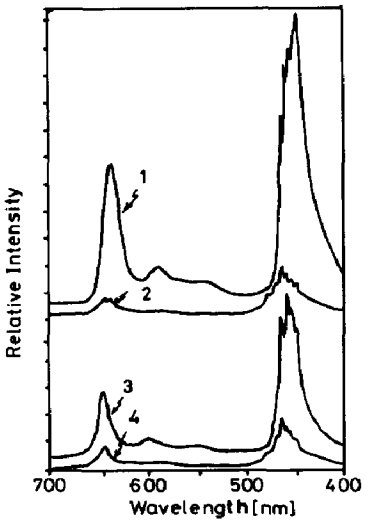
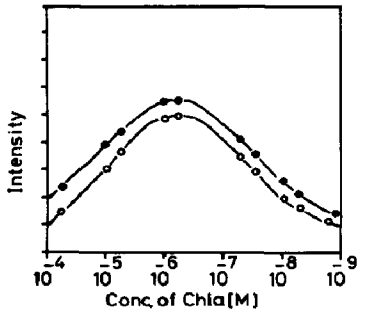
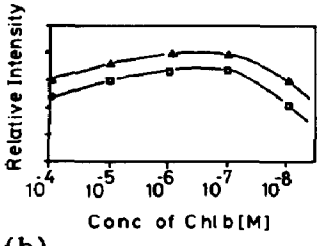


Fig. 5. Excitation spectra of Chl b in pure solvents and in solvents containing *n*-propanol: curve 1, pure ethyl ether; curve 2, 14 M *n*-propanol in ethyl ether; curve 3, pure benzene; curve 4, 14 M *n*-propanol in benzene.



(a)



(b)

Fig. 6. Emission (○, □) and excitation (●, △) intensities *vs.* the concentration of (a) Chl a in ethyl ether and (b) Chl b in benzene.

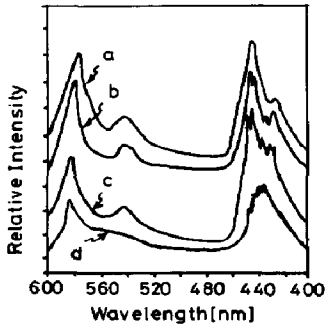


Fig. 7. Excitation intensity *vs.* the concentration of Chl b in ethyl ether: curve a, 3.7×10^{-6} M; curve b, 2.5×10^{-5} M; curve c, 1.6×10^{-4} M; curve d, 3.7×10^{-3} M.

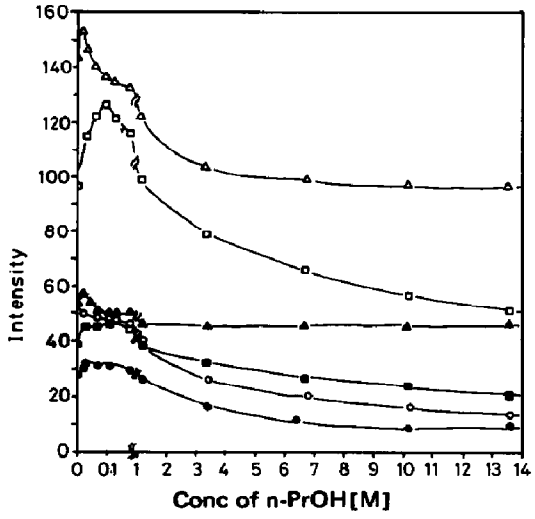


Fig. 8. Emission and absorbance intensities of the Chl b Soret and red bands *vs.* the concentration of *n*-propanol in organic solvents.

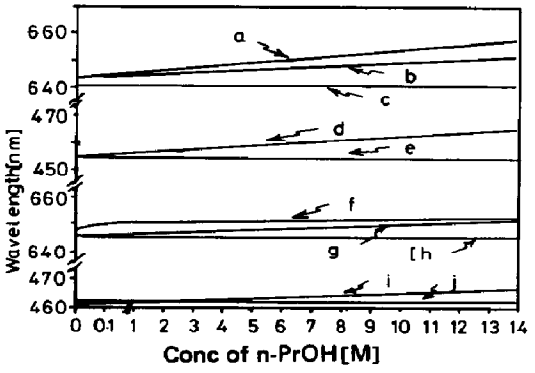


Fig. 9. Wavelength shift of the absorbance, emission and excitation of Chl b in ethyl ether and benzene.

Solvent	*Absorbance*		*Excitation*		*Emission*
	Soret band	*Red band*	*Soret band*	*Red band*	
Ethyl ether	Curve d	Curve b	Curve e	Curve c	Curve a
Benzene	Curve i	Curve g	Curve j	Curve h	Curve f

3.3. Diethyl ether

Both Chl a and Chl b are soluble in diethyl ether, and solutions of concentrations 5×10^{-6} mol l^{-1} and 3.9×10^{-6} mol l^{-1} respectively were examined. In contrast with the previous solvents, changes in the concentration led to quite distinct changes in the spectral profile (Fig. 10). It was also observed that the addition of *n*-propanol to the dry diethyl ether solution of Chl a led to a decrease in the absorption intensity (Fig. 2), although the fluorescence intensity exhibited a maximum at approximately 0.03 M. For Chl b

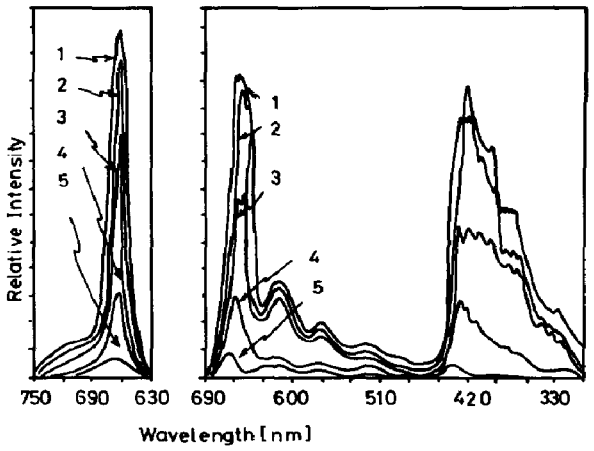


Fig. 10. (a) Emission and (b) excitation intensities *vs.* the concentration of Chl b in benzene: curve 1, 3.1×10^{-2} M; curve 2, 2.5×10^{-3} M; curve 3, 5.2×10^{-4} M; curve 4, 3.3×10^{-6} M; curve 5, 7.8×10^{-8} M.

the addition of *n*-propanol led to a maximum in the absorption intensity and a corresponding monotonic decrease in the fluorescence intensity. Changes in the spectra similar to those observed in benzene were also observed (Fig. 6).

4. Discussion

Chl a is only weakly fluorescent in dry hydrocarbon solvents. The differences in the initial absorption spectra cannot be only a consequence of the solvent permittivity (Tables 2 and 3). It is probable that in a solvent such as benzene polarization interactions (specific solvation effects) also perturb the electronic transition. However, there is evidence for a general trend in the variation in the band intensities with dielectric constant (Table 4).

Studies by Perkins and Roberts [8] have indicated that the ratio of the intensity of the Soret band to the intensity of the red band is 1.3 in ether and 1.1 - 1.2 in ether containing methanol, and their observations are in

TABLE 2

Shifts in the chlorophyll a absorbance, excitation and emission wavelengths produced by the addition of n-propanol (up to 14 M) to pure solvents

Solvent	*Soret band* (nm)		*Red band* (nm)		*Emission* (nm)
	Absorbance	*Excitation*	*Absorbance*	*Excitation*	
Ethyl ether	432 433(1)	431 433(2)	665 668(3)	664 667(3)	682 623(1)
Benzene	431 433(2)	429 435(6)	653 667(4)	657 666(8)	662 671(9)
Iso-octane	427 432(5)	426 433(7)	658 663(5)	659 668(9)	664 682(18)

The numbers in brackets indicate the net change produced in λ_{max} by the addition of up to 14 M *n*-propanol to the pure solvent.

TABLE 3

Shifts in the chlorophyll b absorbance, excitation and emission wavelengths produced by the addition of *n*-propanol (up to 14 M) to pure solvents

Solvent	*Dielectric constant ϵ at 20 °C*	*Absorbance* (nm)		*Emission* (nm)
		Soret band	*Red band*	
Benzene	2.284	461 467(6)	646 652(6)	648 635(5)
Ethyl ether	4.335	455 465(10)	644 651(7)	644 657(13)

The numbers in brackets indicate the net change produced in λ_{max} by the addition of up to 14 M *n*-propanol to the pure solvent.

TABLE 4

Absorbance and emission intensities of chlorophyll a in pure solvents

Solvent	*Dielectric constant ϵ at 20 °C*	*Absorbance (Soret band)*	*Emission*
n-hexane	1.890	11.1	52.5
Iso-octane	1.940	12.2	46.5
Benzene	2.284	13.8	45.0
Ethyl ether	4.335	15.1	43.5
o-dichlorobenzene	9.93[a]	17.4	27.7

[a]Value at 25 °C.

accord with the data obtained in this study. Further, rather similar changes in the structure of the absorption spectrum have been reported by other workers [9].

The spectral characteristics of the more concentrated Chl solutions differ markedly from those obtained *in vivo*. For example, colloidal bacteriochlorophyll exhibits absorptions at 848, 785, 590 and 375 nm. This must be considered as the ultimate result of the aggregation which in this study is being observed in its early stages.

With reference to the detailed spectral changes, we propose that *n*-propanol forms a solvation sheath around the metal atom which remains complexed in the porphyrin ring. The metal atoms will have virtually no interaction with iso-octane and only weak interactions in solutions of benzene and diethyl ether, these being replaced by *n*-propanol in the mixed solvent systems. It is therefore probable that the tail at shorter wavelengths and the doublet at 665, 675 nm are characteristic of the vibrational structure of truly isolated Chl a. The splitting of the excitation spectrum in benzene is probably the effect of vibronic coupling of the excited state with benzene, and this strong interaction persists even in the presence of *n*-propanol (Fig. 10).

The increase in the intensity of absorption with the addition of *n*-propanol is a consequence of the formation of a dimer or similar oligomeric

structure. Further addition of *n*-propanol is unlikely to generate extended structures since the solvation blocks the perfection of the stacking arrangement. Whilst the shifts and changes are clearly in the general direction of those expected from the *in vivo* observations, the extent of the changes is small. Therefore it is probable that the Chl exists as dimers or at most tetramers in these solutions.

Acknowledgments

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