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ABSORPTION DIFFERENCE SPECTROSCOPY OF CHLOROPHYLL *a* IN ETHANOL SOLUTION

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SUMMARY

Absorption difference spectra between highly concentrated and dilute solutions of chlorophyll *a* have been measured in ethanol, maintaining the product of concentration and path length constant. A log–log plot of dimer *versus* monomer concentration, derived from the data, was linear with a slope of 2.0, which is consistent with the interpretation that dimers are present in this solvent. The absorption spectrum of the dimer in ethanol was calculated, and a value of $4.5 \pm 0.8\ M^{-1}$ was obtained for the equilibrium constant for dimerization.

INTRODUCTION

It has previously been inferred by BRODY AND BRODY, on the basis of absorption[1] and fluorescence[2] spectroscopy that dimers of chlorophyll *a* exist in ethanol solutions at concentrations greater than 0.01 M at room temperature. We have now measured a series of absorption difference spectra of chlorophyll *a* in this solvent, maintaining the product of concentration and path length equal to each other in the sample and reference solutions, in order to obtain accurately the equilibrium constant for dimerization and the dimer absorption spectra in ethanol. Our results were treated mathematically with the method described by SAUER, SMITH AND SCHULTZ[3] in their study of chlorophyll absorption difference spectra in CCl_4.

EXPERIMENTAL

Absorption difference spectra were measured over a chlorophyll concentration range of 6.6 to 31 mM. Pure chlorophyll *a* was prepared by a combination of methods[4,5] as described previously[6]. Samples were weighed out, then dissolved in 0.3 ml of absolute ethanol, with the aid of a 100-μl Hamilton precision syringe which was used to draw up and expel the solution 50 times. The solutions were prepared in subdued light, in a glove bag, which was 3 times alternately evacuated and filled with prepurified nitrogen saturated with ethanol. These precautions were necessary to prevent allomerization of the chlorophyll and evaporation of the solvent. The concentrated sample was contained in a 1-cm path length rectangular cuvette, equipped with a 9.95-mm spacer. Calibration of the cuvette–spacer combination showed a deviation of less than 1 % in the path length from the nominal value of 0.05 mm; the latter

value was therefore used in our calculations. The reference, prepared by quantitative 200-fold dilution of an aliquot of the concentrated sample, was contained in a 1-cm path length cuvette. Difference spectra could be obtained at rather high absorbances with the Cary Model 14R recording spectrophotometer, equipped with a high intensity quartz-iodide lamp and operated at maximum sensitivity. Resolution at the red maximum in the difference spectrum (677 nm) was better than 3 nm, in the densest sample.

RESULTS

Fig. 1 (lowest curve) shows a typical difference spectrum. The inset in the figure shows the log–log plot of dimer *vs.* monomer concentration. The dimer and monomer

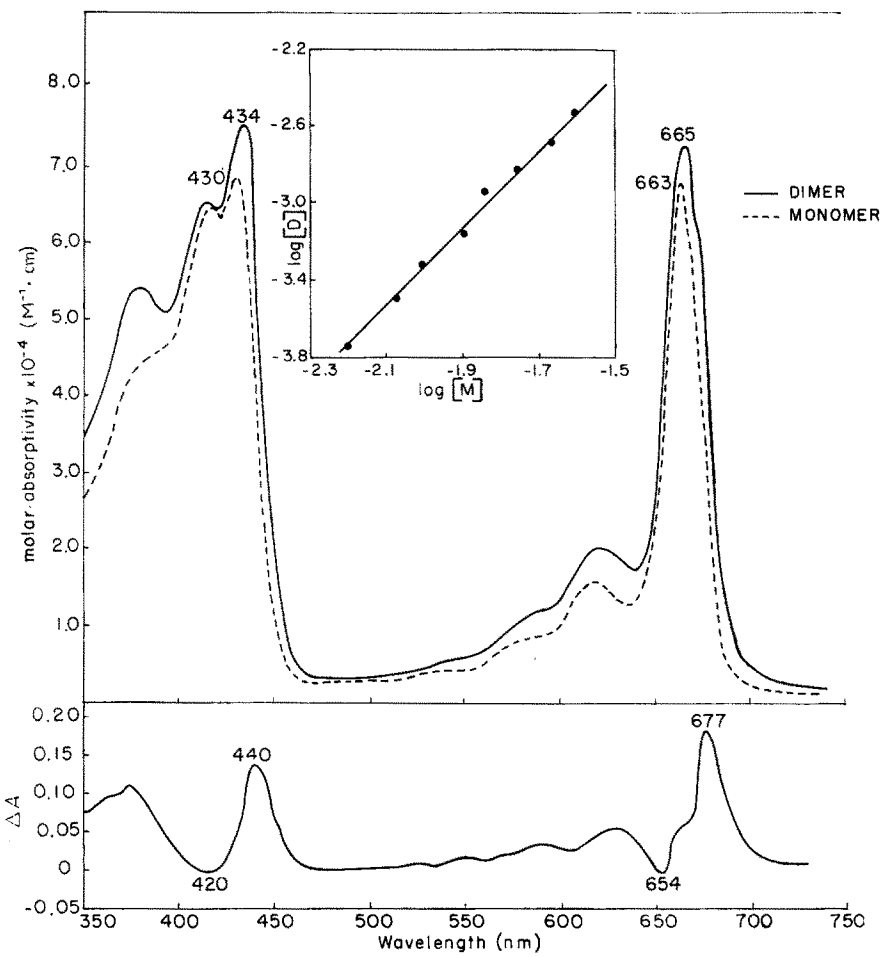

Fig. 1. Lower curve: Absorption difference spectrum for chlorophyll *a* in ethanol. Total chlorophyll concn. 17 mM in sample and 85 μM in reference. Path length of cuvettes: 0.05 mm for sample, 1 cm for reference. Upper curve: ———, absorption spectrum of chlorophyll *a* dimer in ethanol, calculated from difference spectrum shown in lower curve (molar absorptivity is per monomer unit = $\varepsilon''/2$). – – – –, absorption spectrum of chlorophyll *a* monomer, from a 5.0 μM solution. Inset: log–log plot of dimer *versus* monomer concentration, at concentrations of total chlorophyll given in Table I.

concentrations in the sample ([D] and [M], respectively) were calculated from ΔA, the absorbance difference at 677 nm, and the total chlorophyll concentration (see Table I), and the monomer and dimer absorption coefficients at 677 nm. The dimer absorption coefficient was determined from a least squares analysis, which fitted the absorption difference data at 677 nm to the line $\log[D] = 2.000 \log [M] + \log K$, the absorption coefficient of the dimer and the equilibrium constant, K, being adjustable parameters. For a slope of 2.000 ± 0.002, the dimer absorption coefficient

TABLE I

ABSORBANCE DIFFERENCE AT 677 nm AS A FUNCTION OF TOTAL CHLOROPHYLL CONCENTRATION

Total chlorophyll (mM)	$\Delta A_{677\ nm}$
6.6	0.028
9.1	0.050
11	0.073
14	0.106
17	0.177
21	0.230
26	0.320
31	0.460

is $(4.38 \pm 0.40) \cdot 10^4\ M^{-1}\ cm^{-1}$ per monomer unit ($= \varepsilon''/2$) at 677 nm, and the equilibrium constant is $4.5 \pm 0.8\ M^{-1}$. With these parameters a very good linear fit of the data to the line of slope 2.0 is obtained, as shown in the figure. A plot of $\Delta A_{677\ nm}$ as a function of square of chlorophyll concentration is also linear in the lower concentration range. Plots against other powers of concentration are not linear.

A difference spectrum employing dilute solutions of high absorbance (5.0 at 663 nm; path length 1 cm) in both sample and reference beam was also measured. ΔA was found to be 0 between 350 and 750 nm. Difference spectra for these solutions were also measured with a Corning 2-68 blue filter positioned between the solutions and the photomultiplier, so that the red fluorescence of chlorophyll was filtered out, in both sample and reference. Subsequently, the blue filter was placed between the incident beam and the solution in the reference only, thereby permitting fluorescence from the reference to enter the phototube. In both cases ΔA was 0 throughout the wavelength range examined (400 to 500 nm).

Also given in Fig. 1 is the absorption spectrum of the monomer in ethanol, as measured in our laboratory, and the dimer spectrum calculated from the equation $\varepsilon''(\lambda) = [\Delta A(\lambda) - \varepsilon'(\lambda)\ ([M]\delta - C)]/[D]d$ where $\Delta A(\lambda)$ is the absorbance in the difference spectrum (Fig. 1) at wavelength λ, $\varepsilon''(\lambda)$ and $\varepsilon'(\lambda)$ are the dimer and monomer molar absorptivities at wavelength λ, d is 0.05 mm (the optical path length of the concentrated sample), and C is the chlorophyll concentration in the dilute 1.0-cm path length reference. The dimer spectrum calculated at other concentrations is virtually identical. Both red and blue bands (maxima at 434 and 665 nm) are broadened with respect to the monomer bands, particularly on the long-wavelength side (there appears to be an unresolved band at about 675 nm and a shoulder at about 700 nm). Similar broadening of the dimer bands is observed in CCl_4 solution[3].

DISCUSSION

As pointed out by SAUER, SMITH AND SCHULTZ[3], the fit of the absorption difference data to a linear relationship between log [D] and log [M] is consistent with the existence of a monomer–dimer equilibrium; when species other than dimers are present, data treated in this manner leads to curvature in the log–log plots. The linear relationship between ΔA and square of chlorophyll concentration is also in agreement with the interpretation that a low concentration of dimer is in equilibrium with monomer. Furthermore, this is not subject to the insensitivity of a log–log plot. The difference spectrum between concentrated and dilute solutions of chlorophyll *a* in acetone given by SAUER[7] also indicates the presence to a small extent of another species besides the monomer in his concentrated sample.

The value of 4.5 M^{-1} for K in ethanol may be compared with the equilibrium constant in CCl_4[3] which is 10^4 M^{-1}. At a total chlorophyll concentration of 31 mM, the dimer concentration in ethanol is 2.9 mM or 9.3 %, and at 6.6 mM, the dimer concentration is 0.18 mM, or 2.7 %. Consequently, attempts to observe dimers in polar solvents at concentrations below 5 mM would meet with experimental difficulties. The value for K reported here is considerably lower than the earlier estimate[1] of 30 M^{-1}.

STENSBY AND ROSENBERG[8] have questioned the solubility of chlorophyll *a* in ethanol at concentrations greater than 8 mM. However, we find no difficulty in preparing highly concentrated solutions by the technique described, with chlorophyll which is sufficiently pure. In particular, colorless impurities, such as quinones must be removed by adequate washing[6]. The solutions we obtain are optically clear, with no evidence of scattering, when viewed in a variable path length cell where the absorbance is less than 1.0. Furthermore, there is no evidence of absorption bands due to chlorophyll microcrystals[9] in our difference spectra when the chlorophyll is in solution, whereas such bands are observed when suspended particles remain. And finally, the systematic variation of absorbance in the difference spectra with total chlorophyll concentration (Table I), from which the linear plot given in Fig. 1 is derived, would not have occurred if scattering had produced the difference spectra.

The difference spectra also do not result from artifacts produced by stray light, since no absorbance differences were obtained when optically dense dilute solutions served as both sample and reference. Fluorescence, acting as 'stray light' also does not contribute, since we found that, in the extreme case where fluorescence by the reference only was completely filtered out, no absorbance differences occurred in dense dilute solutions.

Aggregation in polar solvents like ethanol and acetone is probably not *via* interaction between magnesium and cyclopentanone carbonyl, as is the case in CCl_4, since the large excess of solvent competes successfully for coordination with the magnesium[10,11]. On the other hand, dimerization could take place by mechanisms not involving competition with the solvent. One possibility, as already suggested[12]. is π–π interaction, which has been proposed[13] to account for the dimerization of pheophytin, the magnesium-free chlorophyll derivative.

While the mechanism of dimerization in ethanol apparently is different from that in CCl_4, the relative orientations of the long-wavelength electric dipole transition

moments appear to be similar, as evidenced by the similar splitting of the long-wavelength absorption band[3]. In CCl_4 dimerization causes the absorptivity per monomer unit to decrease, for example from 10.2 to $8.4 \cdot 10^4$ M^{-1} cm^{-1} at the blue maximum[3]. In ethanol, on the other hand, as a consequence of the lack of negative bands in the difference spectrum, there is an increase in the calculated absorptivities on dimerization, for example from 6.9 to $7.5 \cdot 10^4$ M^{-1} cm^{-1} at the blue maximum. As a result, the maximum absorptivities of dimers in ethanol and CCl_4 are more similar to each other than the corresponding absorptivities of the monomers. Apparently the effect of solvent on the transition from the ground state to the excited singlets of chlorophyll is less for the dimer than the monomer.

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