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CIRCULAR POLARIZATION OF FLUORESCENCE OF CHLOROPHYLL IN SOLUTION AND IN NATIVE STRUCTURES*

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SUMMARY

Chlorophyll dimers in solution, subchloroplast particles and chloroplasts were investigated by their circular dichroism and circular polarization of their fluorescence, which reflect their optical rotatory power in the ground state and electronically excited state, respectively. The chlorophyll dimers in fluid solution lose their optical activity upon electronic excitation, reflecting a marked concomitant change in the structure of the dimers. This change is arrested in a solution of very high viscosity. The pronounced difference between the circular polarization of the dimers in fluid media and that of subchloroplast particles and chloroplasts indicates that the former are not suitable models for associated chlorophyll in native structures in electronically excited states. Impairment of the photochemical activity of chloroplasts by heat treatment is accompanied by a reduction of the circular polarization of the fluorescence, which probably reflects a disorganization in structure. The same extent of circular polarization was observed in the fluorescence of chloroplasts regardless whether the reaction centers are open or closed; thus either the same molecules are emitting in the two cases or, if different molecules emit, they are packed in a similar way.

INTRODUCTION

The biological activity of the photosynthetic apparatus is dependent on the native organization of the chlorophyll molecules in the chloroplasts. A variety of optical methods, such as linear dichroism, optical rotatory dispersion and circular dichroism (CD) have been applied to the study of the arrangement of the chlorophyll molecules in chloroplasts and in molecular aggregates in vitro. These methods are capable of yielding interesting and important information about the systems studied; the data obtained are, however, strictly relevant, as a rule, to the structure of the molecules investigated in their electronic ground state. Due to the different electronic charge

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distributions in the molecules when in the ground state or in the electronically excited state, their conformation and interaction with neighbouring molecules may be markedly different in the different electronic states. By the Franck-Condon principle, light-absorption processes are related to the molecular structure in the ground state. In the case of photosynthesis, however, it is obviously the electronically excited state which is of prime interest. For the investigation of the excited state the properties of the luminescence processes and of the luminescence light emitted by the molecules studied are more directly relevant, since the molecules normally relax to the equilibrium structure and conformation of the excited state long before they emit the light quantum.

The optical-rotatory power of molecules in electronically excited states can be studied by the circular polarization of the light which they emit [1–5]. The ratio $\Delta f/f$ of the intensity of the emitted circularly polarized light, Δf, to the total fluorescence intensity, f, is related to the electric and magnetic transition dipole moments characteristic of the molecular conformation and environment in the excited state in the same way that the ratio $(\varepsilon_l - \varepsilon_r)/(\varepsilon_l + \varepsilon_r)$ is related to the above molecular properties in the ground-state conformation, ε_l and ε_r being the extinction coefficients for left-handed and right-handed circularly polarized light, respectively [2, 5]. In the following we describe the circular polarization of the fluorescence of dimers of chlorophyll *a* in solution, of subchloroplast particles and of intact chloroplasts, and discuss some implications of the results to the organization of the chlorophyll molecules in the first singlet excited state. It will be shown that the chlorophyll dimers in fluid solution undergo a pronounced change in structure upon electronic excitation and that changes occur in the mode of packing of the chlorophyll in chloroplasts upon heat inactivation.

EXPERIMENTAL

Materials

Chlorophyll *a* was extracted from spinach leaves and purified by the method of Anderson and Calvin [6]. Some of the experiments were carried out with chlorophyll *a* obtained from Sigma (Lot No. 103C-9750). Free chloroplasts were prepared from lettuce by the procedure described by Avron [7]; final suspending medium was 0.05 M Tricine, 0.15 M KCl, pH 7.8, buffer, except for some experiments where it was a 0.2 M KCl, 0.1 M sucrose solution. The chloroplasts were stored, when necessary, at liquid nitrogen temperature in a medium of 0.125 M KCl in water/glycerol (1 : 1, v/v). Subchloroplast particles were prepared from lettuce chloroplasts by forcing them through a French press cell at about 9000 lb/inch^2 [8]. A single green wide band was observed in sucrose-gradient centrifugation near the top of the gradient and was collected. The subchloroplast particles had the same photosynthetic activity per mg chlorophyll as intact chloroplasts.

Polystyrene, injection molding grade, crystal clear, was a product of Montecatini. Before use for the formation of gels with carbon tetrachloride as the swelling medium, the polystyrene was ground in a blender and the powder was sieved to obtain a powder of particle size less than 0.25 mm. Ferricyanide was a product of Analar, Analytic Grade. Organic solvents used were of spectroscopic grade (ethanol-free carbon tetrachloride: a Fluka product; toluene: a Fisher Co. product Sintanalyzed Grade). Water was double distilled.

Methods

Photosynthetic activity was determined by the Hill reaction, ferricyanide (10^{-3} M) serving as electron acceptor. The suspension of chloroplasts or subchloroplast particles was irradiated with red light of approx. 10^5 ergs $\cdot$ cm^{-2} $\cdot$ sec^{-1} and the change in absorption at 420 nm was followed on a Cary 1605 spectrophotometer.

Solutions of chlorophyll in rigid gels of polystyrene and carbon tetrachloride for the study of the circular polarization of the fluorescence were prepared as follows: the fluorescence cell, of 1.0 mm thickness, was about 3/4 filled with the polystyrene powder (0.25 mm mesh). A solution of chlorophyll in carbon tetrachloride was then introduced into the powder by a hypodermic needle equipped with a cleaning internal rod. The rod served to clear the needle from clogging by the forming gel. The polymer swells readily in the carbon tetrachloride, forming a clear viscous gel. The ratio by weight of polystyrene to carbon tetrachloride was 0.6 : 1.0; the desired final concentration of chlorophyll in the gel was thus obtained by using a proportionately higher concentration in the carbon tetrachloride solution injected into the polystyrene powder when forming the gel. The cell and its contents were checked between crossed polarizers to ascertain that they exhibited no perceptible birefringence.

The circular polarization of the fluorescence was measured with a sensitive instrument recently designed and built in our laboratory [1]. The fluorescence was excited by the 435-nm band of a 100-W high-pressure mercury arc (HBO 100w/2). To remove stray light, the excitation beam was passed through a saturated $CuSO_4$ solution, 1 cm path, which cuts off light above 600 nm, while the emitted light was passed through a 0.2 % potassium bichromate solution, 1 cm path, which removes all light below 520 nm. The spectral resolution of the excitation and emission monochromators was 30 nm and 15 nm, respectively. The optical-rotatory power associated with the ground-state or excited-state conformations is expressed by the anisotropy factors of absorption (g_{abs}) or emission (g_{em}), respectively, defined as $g_{abs} = 2(\varepsilon_l - \varepsilon_r)/(\varepsilon_l + \varepsilon_r)$ and $g_{em} = 2\Delta f/f$ [2,5]. If the conformation and environment of the chromophore are exactly the same in the ground state and excited state, g_{abs} at the long-wave-length absorption band and g_{em} should have the same sign and be of equal magnitude.

CD spectra were obtained with a Cary-60 spectropolarimeter equipped with a 6002 accessory. Cells of 0.1 mm optical path length were used for the chlorophyll dimers. For the chloroplasts and subchloroplast particles the optical path was 10 mm.

Absorption spectra were obtained with a Zeiss Model PMQ II spectrophotometer or with a Cary 1605 spectrophotometer. The light scattering from the chloroplast suspensions was approximately accounted for in the first instrument by subtraction, at each wavelength of the absorption spectrum, of the absorption value observed at 750 nm. Chlorophyll concentrations were determined spectrophotometrically [9].

Fluorescence spectra were obtained with a fluorimeter built by Professor E. Fischer, the Weizmann Institute of Science, and were not corrected for instrument response. The fluorescence spectrum of the chlorophyll dimers was measured using the front-face technique, in view of the relatively high concentrations, and hence high optical densities, of the solutions.

RESULTS AND DISCUSSION

The spectral characteristics of dimers of chlorophyll *a* in fluid solution are presented in Fig. 1. The solvent was ethanol-free carbon tetrachloride distilled over calcium hydride. Although the chlorophyll was dried in vacuum (by a mercury diffusion pump for 15 min at room temperature) it is not likely that it lost all traces of moisture, and the chlorophyll *a* dimers formed are probably hydrated dimers [10]. The chlorophyll concentration used was $8 \cdot 10^{-3}$ M. At this concentration the chlorophyll may be calculated to be 96 % in dimer form, using the dissociation constant of $(4.4 \pm 1.1) \cdot 10^4$ $\mathrm{l \cdot mole^{-1}}$ found by Trosper and Sauer for this system [11]. The presence of large amounts of dimer is also reflected by the peak of absorption at 668 nm [9]. Although the quantum yield of the dimer emission is 5- to 10-fold lower than that of the monomer emission [12, 13], the fluorescence observed under the conditions of the experiment is predominantly that of dimers due to the relatively small amounts of monomer present in non-polar solutions [10]. The CD spectrum is in good agreement with the data reported previously [14, 15], exhibiting the exciton splitting of the dimer molecules. In contrast to the CD data, no circular polarization could be detected in the emission, g_{em} being zero within experimental error (estimated to be $\pm 5 \cdot 10^{-5}$). The large difference between g_{abs} at the red edge of the absorption spectrum and g_{em} of the fluorescence band indicates that the structure of the chlorophyll dimers in the excited state is different from their structure in the ground state. Obviously, the forces which stabilize the dimer in the ground state change upon electronic excitation: they either stabilize a more symmetrical dimer conformation, or alternatively they weaken to such an extent that upon excitation the chlorophyll molecules comprising the ground-state dimer get apart far enough before light emission occurs to markedly weaken their mutual asymmetric perturbation.

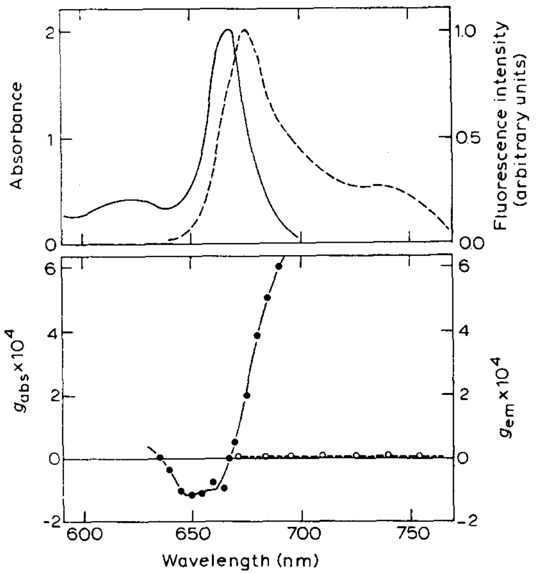

Fig. 1. Spectroscopic data for chlorophyll *a* dimers in carbon tetrachloride. Upper: absorption (—) and fluorescence (- - -) spectra. Lower: absorption anisotropy factor, g_{abs} (—), and emission anisotropy factor, g_{em} (- - -). Total chlorophyll concentration: $8 \cdot 10^{-3}$ M. Temperature: approx. 23 °C.

Further support to the conclusion drawn above that the disappearance of the optical-rotatory power in the excited state is due to a change in structure of chlorophyll dimers upon electronic excitation is obtained from experiments performed in highly viscous media. It was shown previously that changes in molecular conformation which take place in the time interval following light absorption and prior to fluorescence emission can be hindered by the resistance of the medium if the latter is a rigid gel [16]. Under such circumstances, the absorption and emission anisotropy factors were found to assume comparable values at the spectral range of overlap between absorption and fluorescence, although the corresponding values may diverge dramatically in fluid media [16]. Fig. 2 shows the results obtained for chlorophyll *a* in a gel composed of polystyrene and carbon tetrachloride (0.6 : 1, w/w). The values obtained for g_{em} are large indeed, reaching the magnitude of $8 \cdot 10^{-2}$ at 745 nm. The same results were obtained when different positions of the fluorescence cell were introduced into the light beam of the instrument, indicating that the gel is optically homogeneous. While small variations in magnitude were obtained in different experiments, due to difficulties in exact reproduction of the experimental conditions of gel rigidity, the shape of the spectra of g_{em} obtained was the same. For comparison, the spectrum of g_{em} for chlorophyll *a* in a solvent mixture consisting of toluene/carbon tetrachloride (0.6 : 1.0, w/w)

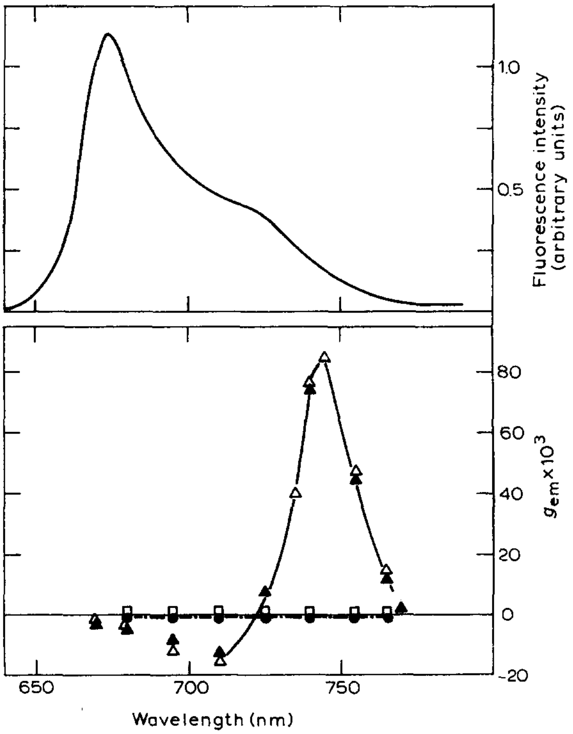

Fig. 2. Spectroscopic data for chlorophyll *a* in a gel polystyrene/carbon tetrachloride (0.6 : 1.0, w/w). Upper: fluorescence spectrum (chlorophyll concentration: $4.8 \cdot 10^{-4}$ M). Lower: emission anisotropy factor, g_{em}; △ and ▲, chlorophyll concentration $4.8 \cdot 10^{-3}$ M, measured at two different positions in the fluorescence cell; ●, chlorophyll concentration approx. $6.6 \cdot 10^{-5}$ M. For comparison: □, g_{em} of chlorophyll *a* ($4.8 \cdot 10^{-3}$ M) in a fluid solvent mixture of toluene/carbon tetrachloride (0.6 : 1.0 w/w). Temperature: approx. 23 °C.

is also presented in Fig. 2. The chemical composition of this solvent is very similar to that of the polystyrene/carbon tetrachloride gel, the principal difference between the two systems being their viscosity. As seen from Fig. 2, g_{em} in the fluid mixture is extremely low. Obviously, the ground-state conformation is frozen in the gel and exhibits high optical activity, while it changes dramatically in the fluid medium, practically losing all of the optical activity. These experiments dismiss the possibility that the effects observed are due to emission by small amounts of monomer present, since the monomer shows no circular polarization in its fluorescence either in rigid or fluid media, as measured at low chlorophyll concentrations (see Fig. 2).

Fig. 2 deserves some further comments. First the experimental data obtained below 700 nm are unreliable, because of the high optical density of the solution in this region. Thus, fluorescence light below 700 nm transversing the medium is partly absorbed and may acquire circular polarization because of preferential absorption of circularly polarized light of one sense or another. Second, g_{em} observed in Fig. 2, which we attribute to the chlorophyll dimers, is much higher than g_{abs} observed for the chlorophyll dimers in their absorption band. This is, however, only to be expected in the present case since the optical activity observed in absorption is the sum of two contributions of opposite sign of the two closely spaced exciton transitions of the dimers, with concomitant severe cancellation in the optical activity. In the fluorescence, only one transition is observed, i.e. from the lower excited state, with its abounding rotatory power.

Fig. 3 describes the spectral measurements carried out on subchloroplast particles. The absorption and CD spectra presented in Fig. 3 are in good agreement with those reported previously for subchloroplast particles prepared by other methods [14, 17, 18]. For allowed single electronic transitions g_{abs} or g_{em} is expected to be roughly constant across the corresponding spectral band [5, 19]. The variation of g_{em}

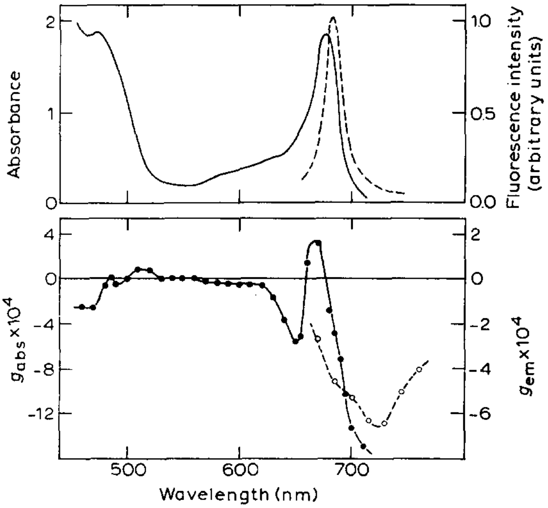

Fig. 3. Spectroscopic data for subchloroplast particles prepared from lettuce. Upper: absorption (—) and fluorescence (- - -) spectra. Lower: absorption anisotropy factor, g_{abs} (—) and emission anisotropy factor g_{em} (- - -). Medium: approx. 12.5 % sucrose solution. Temperature: approx. 23 °C.

across the emission band of the subchloroplast particles is possibly due to heterogeneity in the emitting chlorophyll molecules in vivo, or to heterogeneity in structure of the aggregates. The latter possibility is probably the cause for the similar behaviour of the chlorophyll *a* dimers (see Fig. 2). In marked contrast to chlorophyll dimers in fluid solution, subchloroplast particles exhibit pronounced circular polarization in their fluorescence. Obviously, any analogies drawn from CD data between the structure of the chromophores in subchloroplast particles (or chloroplasts, as seen below) and chlorophyll dimers in fluid solution [14, 15] do not apply to the molecular organization in the first excited singlet state. From this point of view chlorophyll dimers in fluid solution cannot serve as models for chlorophyll aggregates in native structures. Similarly, the comparison of the structure of dimers of different chlorophylls by CD measurements [14, 15] strictly applies to the ground-state conformation only.

The spectral data for intact chloroplasts are presented in Fig. 4. Since chloroplasts are birefringent [20–23], artefacts may occur in the measured optical activity both in absorption and emission due to the possible change in the state of polarization of the light traversing the medium. An upper limit could, however, be set to such artefacts by the following experiment: the cell in which the chloroplasts were suspended was placed between crossed polarizers. The extent of depolarization of the polarized light crossing the suspension was $3 \cdot 10^{-4}$, which was well below the measured signals in either CD or the circular polarization of the fluorescence. The values obtained for g_{abs} should, however, be taken with much reservation due to possible artefacts introduced by light scattering from the turbid suspensions of the chloroplasts [24]. It may be noted that g_{em} is not expected to be affected by light scattering to any appreciable

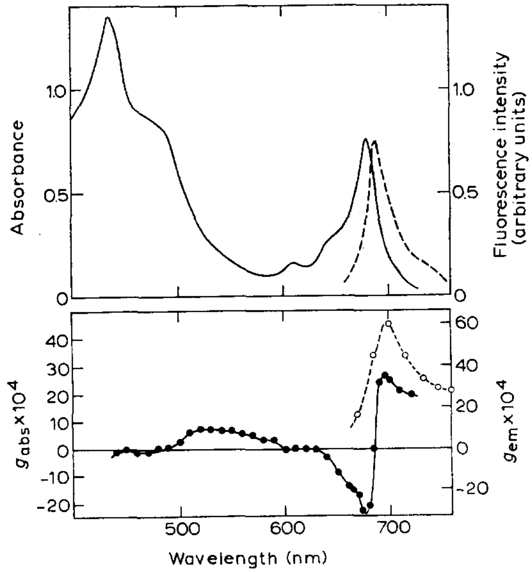

Fig. 4. Spectroscopic data for lettuce chloroplasts. Upper: absorption (—) and fluorescence (- - -) spectra. Lower: absorption anisotropy factor g_{abs} (—) and emission anisotropy factor g_{em} (- - -). Medium: 0.05 M Tricine, 0.15 M KCl, pH 7.8, buffer. Temperature: approx. 23 °C.

extent, since fluorescence is emitted over a very wide solid angle and effects of scattering of fluorescence travelling in various directions are mutually compensating. Similarly, the "sieve effect" [25], which tends to reduce g_{abs} [26, 27], has no effect on g_{em} [27].

It is of interest that upon partial deactivation of chloroplasts by heat treatment the changes in g_{em} correlated with the changes in photosynthetic activity as monitored by the Hill reaction. These results are exemplified in Table I. Apparently, the destruction by heat of the photochemical activity is associated with a parallel impairment of the organized structure of the chlorophyll in the chloroplasts as reflected by the drop in the circular polarization of the fluorescence.

TABLE I

EFFECT OF HEAT TREATMENT ON THE CIRCULAR POLARIZATION OF FLUORESCENCE AND ON THE HILL-REACTION ACTIVITY OF CHLOROPLASTS

Duration of heating (min at 55 °C)	Hill-reaction activity* (μmol e^-/mg chlorophyll *a* per hour)	$10^4 \times g_{em}$ (at 700 nm)
0	175	+64
2	64	+22
4	32	+17

* Hill-reaction activity of chloroplasts was determined by monitoring the changes in absorption at 420 nm of 10^{-3} M ferricyanide solution, upon irradiation of the suspension of chloroplasts with red light. Light intensity, approx. 10^5 ergs · cm^{-2} · sec^{-1}, after passing through a cut off ($\lambda > 665$ nm) filter.

The fluorescence quantum yield of chloroplasts depends on the state of their photochemical centers: it is appreciably lower when the photochemical centers are open than when they are closed. It has been suggested that the enhancement of the fluorescence upon closing of the centers is due to a class of chlorophyll, "live" chlorophyll, which is capable of transferring the excitation energy to the active center. The chlorophyll responsible for light emission when the centers are open presumably belongs to another class called "dead" chlorophyll [28]. The circular polarization of the fluorescence of chloroplasts in the above two states was compared. Due to the high intensity of the excitation light necessary for the measurements of the circular polarization of the fluorescence [1], the photochemical centers are normally in the closed state when tested; presence of Hill oxidants is known, however, to open the centers [28, 29]. Upon addition of ferricyanide (1 mM) to a suspension of chloroplasts, the quantum yield did indeed decrease by a factor of two; the emission anisotropy factor remained, however, unchanged. These results may either be due to the identity of the emitting molecules in the two experiments or, if two emitting classes exist [28], to a similar mode of packing of the "live" and "dead" chlorophyll.

The absorption anisotropy factor at the red edge of the absorption spectrum and the emission anisotropy factor are both positive for the chloroplasts and negative for the subchloroplast particles. Similarly, the respective emission spectra are not identical. While these differences reflect some dissimilarity in the environment of the emitting chlorophyll molecules in the two cases, the nature of this dissimilarity is not known. It is clear, however, that the electronically excited chlorophyll molecules are in an asymmetric environment both in the chloroplasts and the subchloroplast particles while the asymmetry vanishes in the excited chlorophyll dimers in fluid solution. Obviously, the matrix in which the chlorophyll molecules are embedded in vivo, similarly to the polystyrene gel, as shown in Fig. 2, exerts a stabilizing influence on the organization of the chromophores, which withstands deranging tendencies concomitant with electronic excitation of the chlorophyll.

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