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PHOTOSELECTION STUDIES ON THE ORIENTATION OF CHLOROPHYLL a_I IN THE FUNCTIONAL MEMBRANE OF PHOTOSYNTHESIS

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SUMMARY

The orientation of chlorophyll a_I in the functional membrane of photosynthesis in green plants is studied by a photoselection technique. On excitation of an isotropic suspension of isolated spinach chloroplasts with a linearly polarized flash of light linear dichroism of the absorption changes of chlorophyll a_I (wavelengths 705 and 430 nm) is observed. The dichroism is maximum for excitation at wavelengths greater than 690 nm, medium at excitation into the blue band of the chloroplast absorption spectrum, and it is small if excitation goes into all red transition moments above 600 nm. This reflects the degree of order between the transition moments of the antennae system around Photosystem I. We conclude as to a higher order between the transition moments at the long-wavelength end of the spectrum in comparison with a lower degree of order between the transition moments belonging to the intervall from 600 to 680 nm. This confirms the results of other authors which were obtained with oriented chloroplasts. However, the photoselection approach avoids characteristic artifacts which may affect linear-dichroism studies with oriented membranes.

A quantitative interpretation of the observed photoinduced dichroism of chlorophyll a_I to yield the orientation of the respective porphyrin rings in the membrane is not feasible yet due to the absence of specific information on the symmetry properties of the antennae system and on the geometry of the chlorophyll a_I aggregate. Under the assumption of a circular degenerate antennae system a rather flat inclination of chlorophyll a_I has to be expected.

INTRODUCTION

Photosynthetic electron transport in green plants is driven by two photochemical reaction centers. Light-induced oxidation of reaction center I is characterized by negative-going absorption changes at 700 [1] and 438 nm [2] and a minor component at 682 nm [3]. These absorption changes have been attributed to the oxidation of a special chlorophyll *a*: chlorophyll a_I [2].

Three independent lines of evidence have led to the conclusion that chlorophyll

a_I is a dimer of chlorophyll *a* molecules or even a higher oligomer. The appearance of a double band at 682 and 700 nm can be interpreted by exciton interaction in an oblique dimer [3]. The circular-dichroism spectrum of the absorption change at 700 nm has features characteristic for an oligomer in exciton interaction [4]. The line width of the ESR spectrum of the oxidized species corresponds to the line width expected for an electron delocalized over two chlorophyll *a* molecules [5]. So we use the term chlorophyll a_I to denote the aggregate of chlorophyll *a* molecules, which produces the above absorption changes on photooxidation.

The photochemical reaction center I contributes to the generation of an electric potential difference across the functional membrane [6]. There is evidence that the electric potential difference is generated by vectorial electron transport from the inner to the outer side of the functional membrane [7]. Any molecular description of the role of chlorophyll a_I in the vectorial electron transport requires information on its structure, its location and its orientation in the membrane.

In this paper we report on linear-dichroism studies on the orientation of chlorophyll a_I in the inner chloroplast membrane. A straightforward way to obtain information on the orientation of pigments in membranes is to study their linear dichroism in macroscopically aligned membranes. Because of difficulties with the alignment of the inner membranes of functionally intact chloroplasts of the broken type we relied on the photoselection technique which is applicable to isotropic suspensions of chloroplasts without need for orienting forces. In a previous paper we reported that excitation of a chloroplast suspension with a linearly polarized flash of light resulted in linear dichroism of the transient absorption changes of chlorophyll a_I at 705 and 430 nm [8]. The observed photoinduced dichroism led us to conclude that the porphyrin ring of chlorophyll a_I lies almost planar in the functional membrane. In this article we extend our earlier studies. Emphasis lies on an evaluation of the influence of possible artifacts inherent in the applied technique and on a quantitative estimate for the angular range of possible orientations of chlorophyll a_I in the functional membrane.

DIGRESSION ON THE APPLICABILITY OF PHOTOSELECTION TO CHLOROPLASTS

Photoselection techniques include fluorescence polarization and polarized photochemistry. Their application in studies on the anisotropy of electronic transitions in organic molecules has been reviewed in ref. 9. When an isotropic system of dye molecules is excited with linearly polarized light, an oriented ensemble will be selected. The absorption probability is proportional to the squared orientation cosine between the transition moment and the *E*-vector of the exciting light. If excitation causes photochemical bleaching of the dye, absorption changes become observable, the extent of which depends on the relative orientation between the exciting *E*-vector and the *E*-vector of the measuring beam. The dichroic ratio of these absorption changes $\Delta A_{\parallel}/\Delta A_{\perp}$, for parallel and perpendicular orientation of the two *E*-vectors, varies between 3/1 and 1/2. The higher value is expected if the transition moments interacting with the exciting and the measuring light, respectively, are parallel to each other in the molecule, the lower value refers to perpendicular transition moments. For molecules with a higher than 2-fold axis of symmetry the dichroic ratio will be in between these values [9]. However, the ideal dichroic ratios will

be rarely met. Imperfect polarization of the two light beams, mixed polarizations of the vibronic subtransitions within an absorption band, molecular rotation and resonant-energy transfer between molecules decrease the difference between $\Delta A_{\parallel}$ and $\Delta A_{\perp}$.

Most important for photoselection studies on chloroplasts is the fact that a quantum of light absorbed by one of the antennae chlorophylls undergoes about 100 (Photosystem I) resonant-energy transfers before being trapped by the photochemical reaction center [10]. If the resonant antennae chlorophylls were oriented at random in the membrane, information on the polarization of the exciting beam is lost with increasing number of resonant-energy transfer steps. In fact, very small fluorescence polarization has been observed on isotropic suspensions of isolated chloroplasts [11].

It was concluded that the antennae chlorophylls have a very low degree of orientation in the membrane. If so, photoselection studies on isotropic suspensions of chloroplasts will not be very promising. However, linear-dichroism studies on oriented chloroplast membranes have revealed that some of the antennae transition moments have a higher degree of orientation than others. This opens up the possibility to obtain higher dichroic ratios by selective excitation of the oriented transitions.

Starting from the pioneering work of Menke [12], using a polarization microscope, various techniques have been applied for linear-dichroism studies. More recent studies were carried out on oriented chloroplasts. Orientation was achieved

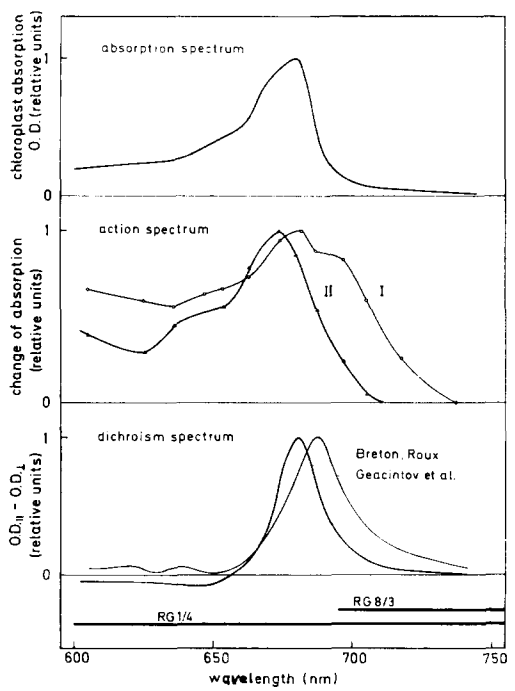

Fig. 1. Comparison of the absorption spectrum (above, our measurements), the action spectra (middle [36]), and linear-dichroism spectra [14, 18] of spinach chloroplasts. The bars at the bottom indicate the range of transmission (10 %) of the absorption filters we used for the exciting light.

by exposure to high electric- [13] or magnetic-field strength [14, 15], hydrodynamic shear [16], and the forces at metallic [17] or dielectric [18, 19] surfaces. There is general agreement that some of the antennae transition moments at the long-wavelength end of the chloroplast absorption spectrum are oriented in the plane of the membrane. Discrepancies exist as to the percentage of oriented transition moments in the red ranging between 2 % [17] and about 50 % [18]. While imperfect orientation of chloroplasts may cause too low a dichroism, there are specific artifacts which might be responsible for too high values (for a careful discussion, see ref. 19).

The dichroism spectra obtained by two different orientation methods are depicted in Fig. 1, together with the absorption spectrum and the action spectra of chloroplasts. From their spectrum Breton and Roux [18] concluded that the transition moments at the long-wavelength half of the red absorption band of chloroplasts are oriented almost in plane of the membrane. Since resonant-energy transfer proceeds toward longer wavelength, a quantum of light once absorbed by one of the oriented oscillators will not leave the oriented ensemble until being trapped. The original polarization of the excitation will be smeared out in plane of the membrane, but not isotropically. Thus, when exciting chloroplasts into the oriented oscillators (e.g. via the filter RG 8/3, see bottom of Fig. 1) one selects those membrane orientations with their plane parallel to the *E*-vector of the exciting light. The dichroic ratio of absorption changes then depends on the orientation of the reacting pigment to the plane of the membrane. This opens the possibility to study the orientation of the various electron-transport components in the functional membrane of photosynthesis by photoselection.

EXPERIMENTAL

Broken chloroplasts were prepared from market spinach according to the method in ref. 20. The preparation was stored under liquid N_2 until use. A typical value for the rate of non-cyclic phosphorylation after thawing: 45 $nM \cdot \mu M^{-1} \cdot s^{-1}$. Chloroplasts were suspended at an average chlorophyll concentration of 10 μM in the following reaction medium: 3 mM Tricine (pH 8), 20 mM KCl, 3 mM NH_4Cl, 1 mM $MgCl_2$, 3 μM benzyl viologen, 0.1 μM valinomycin. The temperature was at 21 °C. Valinomycin was added at a relatively high concentration to accelerate that component of the absorption changes at 430 and 705 nm which is sensitive to changes in the ionic conductivity of the membrane [26, 27], so that it became unresolved at the time scale of our experiments (125 ms).

The 2-cm absorption cell was mounted into a rapid kinetic spectrophotometer [21]. Photosynthetic electron transport was stimulated by a short Xenon flash (half time: 15 μs). Three types of absorption filters were used to select the wavelength range of the exciting light (Schott and Gen.): RG 1/4 ($\lambda > 600$ nm, see Fig. 1), RG 8/3 ($\lambda > 690$ nm, see Fig. 1) and BG 28/4 (380 nm $> \lambda >$ 490 nm). The wavelength of the measuring beam was selected by small-band interference filters (Schott and Gen., Dr. Anders KG) centered at 430 nm (11 nm,) 524 nm (12 nm) and 705 nm (4 nm), respectively.

Changes in transmission on excitation were recorded. The signals were excited repetitively by periodical flashes and averaged in a CAT 1000 on line computer for improvement of the signal to noise ratio [21]. The electrical band width

of the detection system was limited by the dwell time per address of the averaging computer, which was chosen to 125 μs.

Experiments at a measuring wavelength of 705 nm were complicated by flash-burst artifacts due to scattering of the turbid suspension and to the spectral overlap of two excitation filters (RG 1/4, RG 8/3) with the guard filter placed on the cathode of the photomultiplier. Contrary to the experimental conditions in our prior communication on this subject [8], we eliminated flash-burst artifacts by small aperture between the cuvette and the photocathode and by a rapid relaxation of the detection system. However, the measuring light intensity was kept much lower at below 500 erg $\cdot$ cm^{-2} $\cdot$ s^{-1}. This was to reduce the influence of preselection by the linearly polarized measuring light (see following section). The absence of flash-burst artifacts at a 125-ms time scale is demonstrated experimentally in the following section.

The exciting and the measuring beam impinged on the sample perpendicular to each other with both propagation vectors in the horizontal. The *E*-vector polarization of the exciting beam was in the vertical. The polarization of the *E*-vector of the measuring light was either in parallel or perpendicular to the polarization of the exciting light (for an illustration, see Fig. 1 in ref. 8). Both light beams were polarized by polaroid sheets placed between the sample and the respective light source. Depolarization of the light ($\lambda = 430$ nm) by the turbid chloroplast suspension was below 5 %. The degree of polarization was limited by the beam divergence which was about 10^{-2} for the measuring beam and 10^{-1} for the exciting one. For photoselection the output energy of the flash was attenuated by neutral gray filters. The designation of say 20 % saturation in the graphs and the tables means that the absorption changes had 20 % of their extent under saturating excitation.

To avoid apparent dichroism resulting from polarized fluorescence and scattered flash light we did not place any polarizer between the absorption cell and the photomultiplier.

Check-up for artifacts

In our prior communication we reported on photoinduced dichroism of chlorophyll a_{I} at 430 and 705 nm, respectively [8]. The dichroic ratio of the absorption changes was about 1.2. It was observed at non-saturating excitation energy, say at 20 % saturation. This implies that the dichroic difference of the absorption changes ($\Delta A_{\parallel} - \Delta A_{\perp}$) amounts to only 4 % of the total extent of the absorption change under saturating flash energy. Under our conditions the dichroic difference was in the order of 10^{-5} ($\Delta A = -1/2.3 \times \Delta I/I$). To make experiments on linear dichroism by photoselection relevant, this rather small difference between absorption changes has to be resolved from stochastical noise and from artifacts inherent in the experimental technique. While the resolution from noise was solved by the sampling and averaging technique (see above) the role of possible artifacts deserves some further discussion.

Scattered flash light and fluorescence. Light scattering and fluorescence are polarization dependent. Since the *E*-vector polarization of the exciting light was unchanged throughout the experiments and since there was no polarizer placed between the cuvette and the photomultiplier, flash-burst or fluorescence artifacts would not have contributed to the dichroic difference. This is experimentally demonstrated in Fig. 2. Moreover, it is demonstrated that even the contribution to the extent of the absorption changes is negligible.

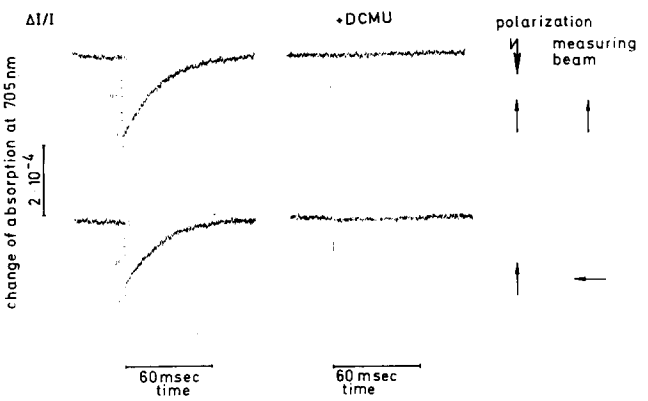

Fig. 2. Flash-induced absorption changes of chlorophyll a_I at 705 nm. Above, exciting and measuring beam polarized in parallel to each other; below, exciting and measuring beam polarized perpendicular to each other; right side, plus 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (1 μM)+ ferricyanide (3 mM). Excitation at wavelengths greater than 690 nm (filter RG8/3), repetition rate 4 cps, Preparation No. 1136.

The two traces in the left of Fig. 2 show the absorption changes of chlorophyll a_I at 705 nm for two different polarizations of the measuring beam. The two traces in the right were obtained under the same polarization conditions; however, electron transport was blocked by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea plus ferricyanide. This treatment leaves the scattering properties of chloroplasts under flash excitation practically unaffected, while the fluorescence yield is even increased. It is obvious that scattering and fluorescence artifacts are negligible.

Preselection by the polarized measuring light. The continuous measuring light stimulates photosynthetic electron flow at a low rate. Because of its linear polarization in our experiments this breaks the isotropy of the chloroplast suspension. The question arises whether preselection by the measuring light increases or decreases the dichroic ratio of the flash-induced absorption changes. The following argument shows that preselection by the measuring light tends to decrease the dichroic ratio at least at a wavelength of 705 nm. Our studies revealed a photoinduced dichroism greater than 1 at 705 nm on excitation at wavelengths greater than 690 nm. Grossly speaking this indicates that the transition moments for excitation and interrogation are more or less parallel to each other. Now, measuring light at 705 nm excites by preference Photosystem I thus oxidizing chlorophyll a_I and decreasing the extent of the negative-going absorption changes at 705 nm. If the measuring light is polarized in parallel to the exciting light it selects for the same ensemble as the exciting flash. Pre-excitation by the measuring light then tends to decrease the extent of the flash-induced absorption change. This would cause apparent dichroic ratios which are more close to one. Thus preselection is no source of artifactous dichroism in our experiments. We have checked the absence of preselection effects experimentally by varying the intensity of the measuring light between 100 and 500 erg $\cdot$ cm^{-2} $\cdot$ s^{-1}.

Photoselection due to sieve effect. The question arises whether the observed dichroic ratios are due to photoselection according to the orientation of the antennae in the membrane or to sieving [22]. Chlorophyll molecules in a thylakoid stack are

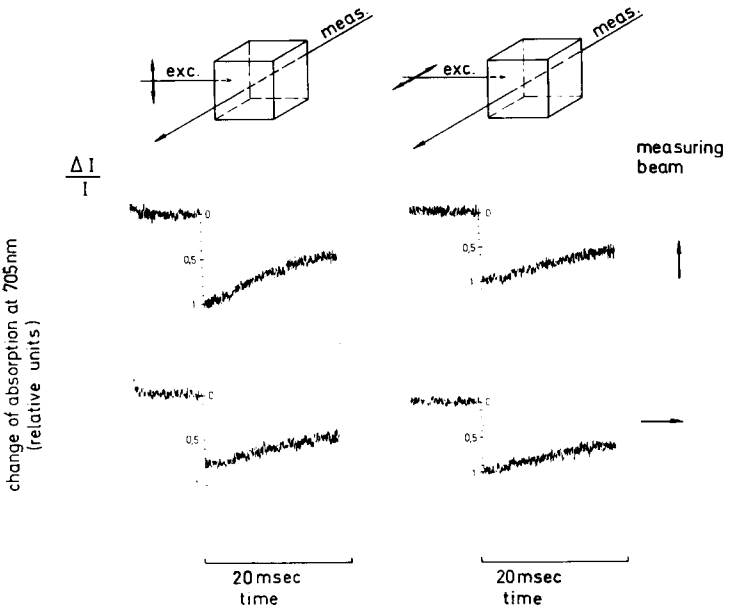

Fig. 3. Flash-induced absorption changes of chlorophyll a_I at 705 nm. Left side, polarization as in Fig. 2; right side, polarization of the exciting beam changed into horizontal position. Excitation at wavelength greater than 690 nm (filter RG8/3), repetition rate 7.7 cps, Preparation No. 1136.

tightly packed. Sieving occurs if some chlorophylls which precede others in the path of the exciting beam got "all" available quanta so that the latter get none. It is obvious that the extent of sieving depends on the orientation of a thylakoid stack with respect to the propagation vector of the exciting light. Sieving selects for those membrane orientations which "expose most chlorophyll molecules" to the exciting light. We have tested the degree of photoselection by sieving experimentally. The result is depicted in Fig. 3. The two traces in the right were obtained for horizontal polarization of the exciting beam. As the *E*-vector of excitation is perpendicular to both directions for the *E*-vector of the measuring beam no "true" photoselection can be expected but only selection due the to sieve effect. As obvious from a comparison of the two traces in the right of Fig. 3 no dichroism was observed, in contrast to the result for vertical polarization of the exciting beam (Fig. 3, left traces) This demonstrates that the influence of the sieve effect on our results is very small.

Brownian rotation. Rotational relaxation of photoselected systems decreases linear dichroism. In our experiments there were two possible sources for this: Brownian rotation of whole membranes in the suspension and rotation of chlorophylls in the membrane. We excluded in our earlier paper [8], that Brownian rotation of broken chloroplasts in the suspension interferes with our experiments at an 125-ms time range. However, as shown for rhodopsin [23] and for the cytochrome oxidase [24] even large proteins which are tightly bound to biological membranes may carry out rapid Brownian rotation. The appearance of photoinduced dichroism of chlorophyll a_I in the absence of cross-linking agents [8] demonstrates, that chlorophyll a_I does not rotate isotropically, if at all. Moreover, rapid rotation is improbable, since the line

width of the ESR spectrum of chlorophyll a_I^+ is practically independent of temperature [25].

RESULTS

Dependence of the dichroic ratio on the flash energy

Photoselection is expected only at non-saturating flash energies. The higher the flash energy the lower the relative dichroism will be. Since a quantitative estimate of the saturation curves for the two polarizations of the measuring beam involves assumptions on the relative geometries of chlorophyll a_I and the antennae, we relied on an experimental test. The result is given in Table I. While no dichroism (dichroic ratio zero) was observed at saturating flash energy, the dichroic ratio of the absorption changes of chlorophyll a_I at 705 nm varied between 1.16 at 11 % saturation and 1.13 at 28 %, only. Thus we regard the dichroic ratios obtained at say 20 % saturation as rather fair approximations to the limiting value at zero flash energy.

TABLE I

DEPENDENCE OF THE DICHROIC RATIO ON THE RELATIVE SATURATION

Measuring wavelength 705 nm, repetition rate 4 cps, Preparation No. 1393, excitation at wavelengths greater than 690 nm (filter RG8/3), percentage saturation defined as: $\Delta A_{\parallel}/\Delta A_{\parallel}^{\text{satur.}} \times 100$.

Saturation (%)	$\Delta A_{\parallel}/\Delta A_{\perp}$
11	1.16
16	1.15
21	1.14
28	1.13

Dependence of the dichroic ratio on the chloroplast preparation

In our studies we used broken chloroplasts which were all prepared by the same method. The only difference between the preparations used was their origin from different batches of market spinach.

Considerable variation of the dichroic ratio was observed. The ratio obtained for the absorption changes at 430 nm on excitation at wavelength greater than 690 nm (filter RG 8/3, see Fig. 1) varied between 1.35 and 1.15 (Table II). This exceeds the standard deviation of the mean in each series of about ten experiments with a given preparation. The factors affecting the degree of order between the antennae and chlorophyll a_I (growing conditions, aging by storage under liquid N_2, preparation procedure) are under study.

The upper two rows in Table II refer to the same chloroplast preparation. However, the dichroic ratio was measured at different viscosities of the suspending medium (ficoll was added and the temperature lowered, see ref. 8). The equal extent of the dichroic ratios within statistical scatter, under conditions where the minimum relaxation time for Brownian tumbling of whole membranes varied between 8 ms and 125 ms, demonstrates that the effect of tumbling on our experimental results is negligible [8].

TABLE II

DEPENDENCE OF THE DICHROIC RATIO ON THE CHLOROPLAST PREPARATION

Viscosity of 15 cP was obtained by adding ficoll to the reaction medium ($t = 2$ °C), excitation at wavelengths greater than 690 nm (filter RG8/3), repetition rate 4 respectively 7.7 cps, the standard deviation of the mean from five to ten pairs of experiments is indicated, a new suspension was used for each measurement, measuring wavelength 430 nm.

Preparation No.	Viscosity (%)	$\Delta A_{\parallel}/\Delta A_{\perp}$	Saturation (%)
1136	15	1.34±0.04	12
1136	1	1.35±0.07	10
1269	1	1.30±0.09	13
1205	1	1.15±0.05	17
1233	1	1.16±0.02	17
1193	1	1.17±0.05	17

Dependence of the dichroic ratio on the wavelength of observation

At excitation via the filter RG 8/3 ($\lambda > 690$ nm) we studied the dichroic ratios of the two main bands of chlorophyll a_I at 430 and 705 nm, respectively, and for the carotenoid band around 520 nm. The latter band of absorption changes we attributed earlier to the response of chloroplast bulk pigments [28], mainly carotenoids [27], to a light-induced electric field across the inner chloroplast membrane. The result is shown in Table III. The dichroic ratio of the two bands of chlorophyll a_I was almost the same for the three preparations under study, around 1.16. However, we found no significant dichroism at 520 nm, the band of the electrochroic absorption changes. The absence of dichroism at 520 nm will be interpreted in a subsequent paper. Here we would like to mention, that it does not necessarily point to a random orientation of the carotenes (or a "magic angle" orientation) within the membrane. One has to take into account that this absorption change does not reflect an event restricted to a small section of the membrane (e.g. the area covered by the 300 Photosystem I chlorophylls) but the electric field which has been shown to exist at a functional unit at least as big as one thylakoid [28]. If this functional unit is highly swollen, as in our experiments, so that its shape is approximately spherical, no dichroism can

TABLE III

DEPENDENCE OF THE DICHROIC RATIO ON THE MEASURING WAVELENGTH

Excitation at wavelengths greater than 690 nm (filter RG8/3), repetition rate 4 respectively 7.7 cps.

$\lambda_{absorption}$	$\Delta A_{\parallel}/\Delta A_{\perp}$	Saturation (%)	Preparation no.
430	1.15±0.05	17	1205
	1.16±0.02	17	1233
	1.17±0.05	17	1193
705	1.15±0.03	23	1205
	1.16±0.03	27	1233
	1.16±0.04	25	1193
520	1.01±0.04	22	1136

be expected via photoselection, no matter whether the responding pigments are oriented or not. Studies on this subject will be reported in a subsequent paper.

Dependence of the dichroic ratio on the wavelength of excitation

We studied the dichroic ratios of the absorption changes of chlorophyll a_I at 430 and 705 nm in dependence of the excitation filters BG28/4, RG8/3 and RG1/4 (see Experimental). The result is shown in Tables IV and V, respectively.

The highest values for the dichroic ratio ($\Delta A_{\parallel}/\Delta A_{\perp} = 1.16$) were observed at an excitation by the filter RG8/3 ($\lambda > 690$ nm). Medium dichroism was observed if the excitation was into the Soret band of the antennae chlorophyll *a* molecules and to a less extent into chlorophyll *b* and the carotenoids via the filter BG28/4 (380 nm $< \lambda <$ 490 nm). The smallest dichroism was observed for excitation into the Q-bands of all chlorophylls at wavelengths greater than 600 nm.

TABLE IV

DEPENDENCE OF THE DICHROIC RATIO ON THE EXCITATION WAVELENGTH

Measuring wavelength 430 nm, repetition rate 7.7 cps.

$\lambda_{excitation}$	$\Delta A_{\parallel}/\Delta A_{\perp}$	Saturation (%)	Preparation no.
> 600	1.08±0.04	27	1205
	1.06±0.04	30	1233
	1.05±0.04	29	1193
> 690	1.15±0.05	17	1205
	1.16±0.02	17	1233
	1.17±0.05	17	1193

TABLE V

DEPENDENCE OF THE DICHROIC RATIO ON THE EXCITATION WAVELENGTH

Measuring wavelength 705 nm, repetition rate 4 cps.

$\lambda_{excitation}$	$\Delta A_{\parallel}/\Delta A_{\perp}$	Saturation (%)	Preparation no.
> 690	1.15±0.03	23	1205
	1.16±0.03	27	1233
	1.16±0.04	25	1193
380–490	1.13±0.03	20	1205
	1.12±0.05	20	1233
	1.13±0.02	19	1193

DISCUSSION

We divide the discussion into two sections, a qualitative first one and a quantitative second one. This is to separate arguments which involve as yet open assumptions from those, in the first section, which do not.

The degree of order in the antennae system

As cited above, linear dichroism studies on oriented photosynthetic membranes

yielded information on the orientation of the antennae transition moments in the membrane. The applied techniques may bring forth specific artifacts thus causing misinterpretation. If we accept that at least some of the orientation techniques (magnetic field and hydrodynamic shear) do not distort the membrane structure, artifactual dichroism may still arise from textural properties of the system and selective light scattering. However, Breton et al. [19] demonstrated that artifactual dichroism from these two sources is negligible in their experiments. On the other hand, they could not fully exclude a contribution from another source which they named selective polarized reflection. This effect can be expected for membranes sandwiched out of pigmented and unpigmented layers. Reflection occurs at the interface of two such layers which differ in their index of refraction. According to Kramer-Kronig's relation the refractive index undergoes characteristic spectral changes around the wavelength of an absorption band of the pigmented layer. This may cause polarization-dependent reflection with spectral characteristics similar to the dispersion law. The selective reflection at the oriented interfaces may mimic linear dichroism even under conditions where the antennae transition moments are oriented perfectly at random in the membrane.

This differs from the situation in photoselection experiments with an isotropic suspension. If the antennae transition moments were oriented at random in the membrane the very many resonant-energy transfer steps (see above) between them would prevent photoselection. Thus the isotropy of the system was not broken and because of this symmetry no selective reflection comes into play. So any dichroism observed in photoselection experiments with chloroplasts reflects the degree of order in the antennae system.

The experimental results which are shown in Table IV reveal a higher dichroic ratio ($\Delta A_{\parallel}/\Delta A_{\perp} = 1.16$) for excitation at wavelengths greater than 690 nm than for excitation at greater than 600 nm ($\Delta A_{\parallel}/\Delta A_{\perp} = 1.06$). We conclude as to a high degree of order among the transition moments of the antennae chlorophylls of Photosystem I at wavelengths above 690 nm. Comparatively lower order exists between the transition moments between 600 and 690 nm.

This confirms the results of Breton and Roux [18] and Geacintov et al. [15] which were obtained for oriented membranes. As illustrated in Fig. 1 these authors reported a higher degree of orientation with respect to the membrane plane of the transition moments at the long-wavelength end of the absorption spectrum of chloroplasts.

Breton and Roux [18] in their studies on oriented membranes observed a negative linear dichroism in the blue spectral region which points to an inclination of the Soret transition moments of the antennae chlorophylls to the membrane at more than 35° ("magic angle"), in contrast to a less than 35° inclination of the Q-band transition moments. We observed positive dichroism at the 705 nm band of chlorophyll a_I no matter whether the excitation was into the Soret or the Q-bands of the antennae chlorophylls (see Table V). On first sight, this might appear incompatible with the findings of Breton and Roux [18]. However, a quantitative evaluation under the assumption of a circularly degenerate antennae system (see following section) shows that it is compatible, if the transition moment of chlorophyll a_I at 705 nm is inclined at less than 35° to the cone formed by the antennae transition moments. These cones according to the results of Breton and Roux [18] have to be visualized

as inclined at $\alpha = 0°$ (red transitions) and $\alpha > 35°$ (blue transitions) to the membrane. The above condition for compatibility is easily fulfilled, if the transition moment of chlorophyll a_I is inclined at say 25° to the membrane.

The orientation of chlorophyll a_I in the membrane

A quantitative interpretation of the above experiments to yield the orientation of chlorophyll a_I in the functional membrane of photosynthesis can be based on the above-cited experimental evidence for an in plane orientation of the transition moments of the antennae chlorophylls at the long-wavelength end of the chloroplast absorption spectrum [14, 15, 18, 19]. However, any rigorous interpretation requires information on two further items: (1) the angular distribution of the original polarization of the exciting light via resonant-energy transfer among the antennae; (2) the orientation of the transition moments of chlorophyll a_I at 430 and 705 nm, respectively, within the coordinate system of the chlorophyll *a* aggregate which makes up chlorophyll a_I.

Unfortunately there is only very limited information on the symmetry properties of the antennae system and on the geometry of chlorophyll a_I. However, it seems worthwhile to specify the type of information required and to review briefly our present state of knowledge.

The angular distribution of the original polarization of the exciting light within the antennae system is affected by two factors: the average number of transfer steps between absorption and the final trapping of a quantum and the topology of the antennae system. Borisov and Ilina [10] estimated that there are more than 100 transfer steps in Photosystem I. Thus an average quantum visits a considerable fraction of chlorophyll molecules belonging to a reaction center I. Then the depolarisation depends mainly on the relative orientations of these chlorophyll molecules to each other. At the extremes there will be no depolarisation if the transition moments of all chlorophylls (Q-bands) are in parallel to each other: on the other hand there will be an almost circular distribution if the directions of those transition moments which lie in plane of the membrane (see above) make up a two-dimensional asterisk with an at least 3-fold symmetry axis. As yet we cannot even discriminate between these two extreme possibilities (whether the antennae behave as a linear or as a circular absorbing dipole). Electron microscopy and small-angle X-ray scattering are of little help as to the symmetry of the membrane's plane structure since they yielded conflicting results on whether it is irregular or rectangular [32–36]. Clarification can be expected from fluorescence depolarisation or photoselection studies on oriented membranes.

The orientations of the respective transition moments of chlorophyll a_I at 430 and 705 nm in the coordinate system of the aggregate are unknown either. As cited above there are three types of evidence that chlorophyll a_I is at least a dimer concerning excitation interaction [3,4] and that it is a dimer in rapid charge-transfer interaction [5]. The directions of the resulting transitions moments of a dimer may differ considerably from the directions of the respective monomer components [31, 37] . The situation is furthermore complicated by the fact that we observed the dichroism of absorption changes which relates our results to differences between transition moments rather than to a single transition moment. Oxidation of monomeric chlorophyll in organic solvents causes almost complete disappearence of

any absorption in the spectral region of the red band. However, it is unknown, whether oxidation of chlorophyll a_I bleaches the dimer absorption (double band at 682 and 700 nm, see ref. 3) totally or whether it is equivalent to the bleaching of one chlorophyll, only, as in reaction centers of bacterium chromatophores [38].

Despite the defizit in the necessary background information let us evaluate our data under some simplifying assumptions as an illustration. If we assume that multiple resonant-energy transfer among those antennae transition moments which lie in the plane of the membrane lead to a perfect circular distribution of the original linear polarization of the exciting light we might treat the antennae as a single circularly degenerate absorption dipole. The theory for photoselection predicts the following limiting values (zero flash energy, no rotation, perfect polarization) for the absorption changes obtained for parallel and for perpendicular polarization of the measuring beam with respect to the exciting one (see ref. 9, Eqn (8):

$$\Delta A_{\parallel} \approx a_x q_x + a_y q_y + a_z q_z + 2(q_x(a_y + a_z) + q_y(a_x + a_z) + q_z(a_x + a_y))$$

$$\Delta A_{\perp} \approx 3(a_x q_x + a_y q_y + a_z q_z) + q_x(a_y + a_z) + q_y(a_x + a_z) + q_z(a_x + a_y)$$

wherein x, y, z are the coordinates of the molecular system under study. We take x, y as lying in the plane of the circular antennae system which coincides with the plane of the membrane. $a_{x,y,z}$ denote the probabilities for the absorption of light which is linearly polarized in parallel to the respective axis of the system. $q_{x,y,z}$ stand for the corresponding probabilities for absorption of the measuring light. For a circularly degenerate antennae system $a_x = a_y = 1/2$, while $a_z = 0$. The absorption probabilities of an oscillator inclined at the angle φ to the plane of the membrane then are: $q_x = \cos^2 \varphi$, $q_y = 0$, $q_z = \sin^2 \varphi$. The dependence of the expected dichroic ratio

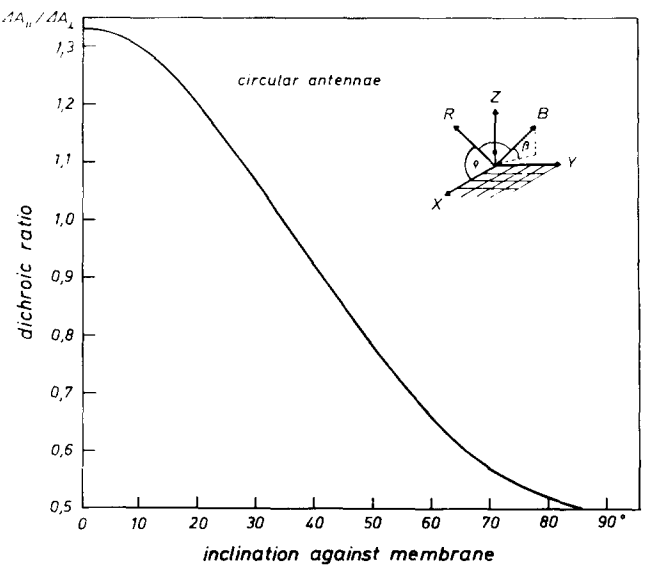

Fig. 4. Dependence of the dichroic ratio of the absorption change of chlorophyll a_I on the inclination (φ, β) of its transition moments to the membrane. A circular degenerate antennae system was assumed.

on the inclination of the interrogated oscillator to the membrane is depicted in Fig. 4. It varies between 4/3 for an oscillator oriented in plane to 1/2 for an out of plane oriented oscillator. A dichroic ratio of 1.16 which we observed for both major peaks of chlorophyll a_I implies an inclination of about 25° of the transition moments to the plane of the membrane. Since experimental imperfections except for flash-burst artifacts, which we excluded above, tend to decrease the apparent dichroic ratios the true values will be even higher implying an even flatter orientation of the respective transition moments in the membrane. In monomeric chlorophylls the Soret and the Q-band transition moments are expected to lie roughly perpendicular to each other in the plane of the porphyrin ring [29, 30]. If the absorption changes of chlorophyll a_I at 430 and 705 nm represented the bleaching of a single chlorophyll, then the rather flat orientation of the respective transition moments implies a rather flat orientation of the prophyrin ring in the plane of the membrane.

However, this interpretation cannot be taken too seriously, yet, due to the above described uncertainties as to the symmetry properties of the antennae and to the geometry of the chlorophyll a_I complex.

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