ORIENTATION OF CHLOROPHYLLS WITHIN CHLOROPLASTS AS SHOWN BY OPTICAL AND ELECTROCHROMIC PROPERTIES OF THE PHOTOSYNTHETIC MEMBRANE

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ABSTRACT   The effects on the optical properties of photosynthetic membranes caused by several types of chlorophyll differing in resonance frequency and in spatial disposition are theoretically analyzed. Using a method of moments and the linear dichroism spectrum of the lamellae, we evaluated the mean angle (Φ) between the transition moment of each chlorophyll and the normal to the lamellae. We have confirmed that at about 695 nm the transition moment is in the plane of the lamellae, and outside it for chlorophyll $b(\Phi \approx 48.6°)$.

By integrating over frequency the absorption variations affected by ionophores, we show that they may be ascribed to a Stark effect, and we analyze the dependence of this effect on the orientation of the chlorophylls. From this dependence and the degree of polarization of the Stark effect, we calculate the spatial fluctuations of the angle Φ. The calculation shows that a definite value of Φ corresponds to each resonance frequency of chlorophylls a found in vivo. This proves that the chlorophylls a are not oriented partly at random. For chlorophylls b, on the other hand, Φ may fluctuate by some 10° about its mean value. The structural consequences of these results are discussed.

INTRODUCTION

A knowledge of the spatial arrangement of pigments within the chloroplast membrane is important in discussing the transport of electronic excitation energy in photosynthetic units (Seely, 1973). It also has much intrinsic significance, since it provides information on the structure of the photosynthetic membrane. Lastly, it is needed in the interpretation of various experiments involving electronic properties of the pigments.

Measurements of absorption with linearly polarized light (linear dichroism) (Breton and Roux, 1971) and of the polarization of fluorescence emission (Geacintov et al., 1974) on suspensions of oriented chloroplasts have shown that the photosynthetic pigments were not randomly oriented in the photosynthetic membrane. Such experiments show that the transition moments corresponding to absorption at long wavelengths (>680 nm) are oriented mainly parallel to the plane of the photosynthetic membrane, whereas those of chlorophyll b are in the red mainly outside this plane (Breton, et al., 1973).

Optical measurements, however, do not give directly the true orientations of the various transition moments, for two reasons: (*a*) Several types of chlorophyll exist in vivo (French, 1971) that absorb in the same wavelength range, especially in the red. The orientation of the transition moments of these different chlorophylls is not the same, and a measurement of linear dichroism therefore gives a superposition of contributions that is difficult to analyze. (*b*) The transition moments for chlorophylls of a given type may a priori have various orientations. Again, the contributions of these moments are averaged in a measurement of the absorption of light.

We can therefore say that the degree of order shown by optical measurements is the result of averaging both in space and over a range of resonance frequencies. The purpose of the present paper is to find the true orientation of the transition moments of chlorophylls in the chloroplast membrane.

For this purpose we determined the mean direction of the transition moments of each type of chlorophyll that contributes to the absorption in the red, and then the spatial fluctuations of the orientation of these transition moments about their mean position. To do so, we need the results of experiments other than measurements of the linear dichroism. These must clearly involve physical processes in which spatial arrangement is concerned, as is the case for the optical effects caused by the action of electrostatic fields on the pigments (Stark effect). Some changes in the chlorophyll absorption spectra after illumination have been attributed to such a Stark effect (Junge and Witt, 1968). We shall show by means of recent experimental results (Breton and Paillotin, 1976) that this hypothesis is valid and that we can thus specify the orientation of the transition moments of the chlorophylls.

We shall first set up the theoretical basis.

LINEAR DICHROISM AND ELECTROCHROMISM OF CHLOROPHYLLS IN VIVO

Let us consider initially the very simple case where all the chlorophylls in the chloroplast lamellae are the same, their resonance frequency in the red being ω_0. The absorption spectrum of such lamellae is Gaussian about this frequency (Paillotin, 1974). More precisely, the extinction coefficient $Q(\omega)$ at the frequency ω is

$$Q(\omega) = (\omega/c) G(\omega - \omega_0), \tag{1}$$

where c is the velocity of light and $G(\omega - \omega_0)$ is a Gaussian distribution centered at the frequency ω_0.

Let us now suppose that the directions μ of the transition moments of the chlorophylls are oriented about the normal ν to the lamellae in such a way that only the angle Φ between μ and ν is not random (Fig. 1). With a suspension of parallel lamellae, two extinction coefficients $Q_\parallel(\omega)$ and $Q_\perp(\omega)$ can be measured, with light polarized parallel or perpendicular to the plane of the lamellae, respectively. The linear dichroism $D(\omega)$ is given by

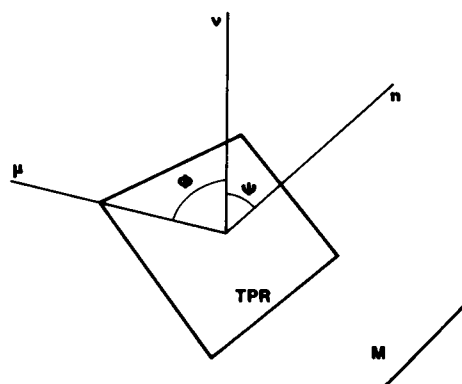$$D(\omega) = Q_\parallel(\omega) - Q_\perp(\omega). \tag{2}$$

FIGURE 1 Notations used to define the orientation of a chlorophyll in the plane of a lamella: TPR, tetrapyrrole ring; ν, normal to the plane M of the membrane; n, normal to the TPR; μ, direction of the transition moment; Φ, angle between ν and μ; ψ, angle between ν and n.

The contribution to this dichroism from a molecule whose transition moment is at an angle Φ to ν is (Breton et al., 1973) $s(\Phi) Q(\omega)$, where

$$s(\Phi) = \tfrac{1}{2}(1 - 3\cos^2\Phi). \tag{3}$$

For the assembly of molecules, then,

$$D(\omega) = <s(\Phi)> Q(\omega), \tag{4}$$

where $<s(\Phi)>$, the mean value of $s(\Phi)$ over the angles Φ, is called the order parameter. In this simple case it is found directly by comparing the linear dichroism and the absorption, but it is only an angular average of $s(\Phi)$, and from it alone we therefore cannot specify the orientation of the transition moments. For example, the dichroism $D(\omega)$ and $<s(\Phi)>$ are zero both when the pigments are disordered and when every moment μ is at an angle Φ to $\nu = 54.7°$, so that $\cos^2\Phi = 1/3$.

This ambiguity can be eliminated only by knowing the spatial fluctuations of the transition moment orientations.

Let us now consider the information available from the electrochromism of the chlorophylls, again supposed identical. Pigments subjected to an electrostatic field in general undergo a change in optical properties. The most marked effect is on the resonance frequencies. When a static field is present, the frequency ω_0 will be changed by an amount $\delta\omega_0$. $\delta\omega_0$ may be written (in units such that $\hbar = 1$)

$$\delta\omega_0 = \mathbf{F}\,\mathbf{P} \tag{5a}$$

where $\mathbf{P}$ is the difference between the polarization in the ground state and in the considered excited state. From the present hypothesis (Schmidt et al., 1971) we have:

$$\mathbf{P} = \mathbf{P}_0 + \overline{\overline{\alpha}}\,\mathbf{E} \tag{5b}$$

$\mathbf{P}_0$ is the value of $\mathbf{P}$ in the vacuum and $\overline{\overline{\alpha}}\mathbf{E}$ the extra polarization created by the

medium. In these notations $\bar{\bar{\alpha}}$ is a static polarizability difference between the ground and the excited state and **E** the permanent local electric field within which the membrane exerts its action on each chlorophyll. Since the surfaces of the chloroplast membrane are equipotentials, **F** is almost independent of the position of the pigment on which it acts.

This is not so for **P**: such a polarization varies according to the orientation of the chlorophylls and the value of **E**. **E** may have quite different values along a lamella. Thus $\delta\omega_0$ has different values for different chlorophylls: let $\delta\omega_0^{(i)}$ be its value for the ith chlorophyll molecule.

The extinction coefficient $Q(\omega)$ is altered through the action of the field, by an amount $\delta Q(\omega)$, which, from Eq. 1, is

$$\delta Q(\omega) = \frac{1}{N}\sum_i (\omega/c)[G(\omega - \omega_0 - \delta\omega_0^{(i)}) - G(\omega - \omega_0)], \qquad (6)$$

where N is the number of chlorophylls per lamella. Since $\delta\omega_0^{(i)}$ is small, we have

$$\delta Q(\omega) \simeq \frac{1}{N}\sum_i (\omega/c) \frac{\partial}{\partial\omega} G(\omega - \omega_0)\delta\omega_0^{(i)} \qquad (7)$$

If we put

$$Q_1(\omega) = \omega \int d\omega' [\delta Q(\omega')/\omega'], \qquad (8)$$

then

$$Q_1(\omega) = Q(\omega)\frac{1}{N}\sum_i \delta\omega_0^{(i)}. \qquad (9)$$

The spectrum $Q_1(\omega)$ obtained by integration of the changes of absorption due to an electrostatic field thus has the same form as the spectrum $Q(\omega)$. Its amplitude is proportional to the main value of $\delta\omega_0^{(i)}$. From symmetry we may write:

$$\frac{1}{N}\sum_i \delta\omega_0^{(i)} = F <P_0\nu> + EF<\alpha\nu\nu> \qquad (10)$$

where $<P_0\nu>$ and $<\alpha\nu\nu>$ are the mean of $\nu \cdot \mathbf{P}_0$ and $\nu \bar{\bar{\alpha}} \nu$ over all possible orientations of the chlorophylls, ν being the normal to the membrane. On account of the symmetry of the chlorophylls (Reich et al., 1976) we have:

$$\nu \cdot \mathbf{P}_0 = P_0\cos\Phi \text{ and } \alpha\nu\nu = \alpha_0\cos^2\Phi + \alpha_1\sin^2\psi \qquad (11)$$

where Φ is again the angle between the transition moment μ and ν, and ψ the angle between the normal to the tetrapyrrole ring and ν (Fig. 1). If we put:

$$c(\Phi, \psi) = P_0 F\cos\Phi + EF(\alpha_0\cos^2\Phi + \alpha_1\sin^2\psi) \qquad (12)$$

we have from Eqs. 9 and 10:

$$Q_1(\omega) = <c(\Phi, \psi)> Q(\omega). \tag{13}$$

Thus, with the linear dichroism (Eq. 4) and the electrochromism $Q_1(\omega)$, we have two quantities that depend on the mean orientation of the pigments. A third can also be found. With suspensions of oriented lamellae, we can measure two variations of the extinction coefficient under the action of a field: $\delta Q_{|}$ and $\delta Q_{\perp}$, depending on whether the absorbed light is polarized parallel or perpendicular to the plane of the lamellae. The quantity $D_1(\omega)$, defined by

$$D_1(\omega) = \omega \int d\omega' [\delta Q_{|}(\omega') - \delta Q_{\perp}(\omega')]/\omega', \tag{14}$$

may also be written

$$D_1(\omega) = <s(\Phi)c(\Phi, \psi)> Q(\omega). \tag{15}$$

A knowledge of the four spectra, $Q(\omega)$ (absorption), $D(\omega)$ (dichroism), $Q_1(\omega)$, and $D_1(\omega)$ (electrochromism), leads to the spatial fluctuations of the transition moment orientations: from Eqs. 4, 13, and 15, we can write

$$Q(\omega)D_1(\omega) - Q_1(\omega)D(\omega) = Q^2(\omega) <(s(\Phi) - <s(\Phi)>)(c(\Phi, \psi) - <c(\Phi, \psi)>)>. \tag{16}$$

The right-hand side evaluates the fluctuations of orientation. In particular, it is zero if the transition moments are all at the same angle Φ to the normal to the lamellae.

Hitherto we have assumed there to be only one type of chlorophyll in the photosynthetic membrane. The actual situation is more complex, since in fact there are several chlorophylls in the lamellae (French, 1971), and each type of pigment contributes to the various spectra. The extinction coefficient is therefore

$$Q(\omega) = (\omega/c) \sum_n p_n G_n(\omega - \omega_n), \tag{17}$$

where p_n is the fraction of chlorophyll type n and ω_n its resonance frequency. $G_n(\omega - \omega_n)$ is again a Gaussian curve centered at ω_n. The linear dichroism $D(\omega)$ is similarly written

$$D(\omega) = (\omega/c) \sum_n p_n s_n G_n(\omega - \omega_n), \tag{18}$$

where s_n is the order parameter of chlorophyll type n, $s_n = <s_n(\Phi)>$ (Eq. 4).

It is still possible to define an apparent order parameter $S(\omega)$ such that

$$S(\omega) = D(\omega)/Q(\omega). \tag{19}$$

However, this parameter depends on ω and is not easily related to the various values of s_n. We can nevertheless distinguish two situations illustrated by the simple example of a mixture of two types of chlorophyll (with resonance frequencies ω_1 and ω_2, order parameters s_1 and s_2) whose absorption bands have the same shape. The two situations are as follows.

(a) If the difference $|\omega_1 - \omega_2|$ exceeds the band width, two Gaussian curves are clearly distinguishable in the spectra of both absorption and linear dichroism (Fig. 2a). The parameter $S(\omega)$ depends on ω, but the values of $S(\omega_1)$ and $S(\omega_2)$ differ only slightly from s_1 and s_2 (Fig. 2a). This situation is close to what occurs in vivo for chlorophylls a and b. In this case we can retrieve the values s_1 and s_2 by resolving the absorption and linear dichroism spectra into Gaussian curves.

(b) If the difference $|\omega_1 - \omega_2|$ is less than the band width, the absorption and dichroism spectra are almost Gaussian. The order parameter $S(\omega)$ still depends on ω, but here $S(\omega_1)$ and $S(\omega_2)$ are quite different from s_1 and s_2 (Fig. 2b). Fig. 2b shows that the dichroism spectrum has a lower amplitude and a frequency shift compared to the absorption spectrum.

This latter property follows from a more general result, which will be proved for linear dichroism. Let us consider the case where several resonance frequencies have very similar values. They may be regarded as belonging to the same type of chlorophyll, say type n, but depending slightly on a parameter, in this case Φ. Thus ω_n is a function of Φ, $\omega_n(\Phi)$. To simplify the notation, we shall say that ω_n is the average of $\omega_n(\Phi)$ over the angle Φ:

$$\omega_n = \langle \omega_n(\Phi) \rangle, \tag{20}$$

and similarly

$$s_n = \langle s_n(\Phi) \rangle. \tag{21}$$

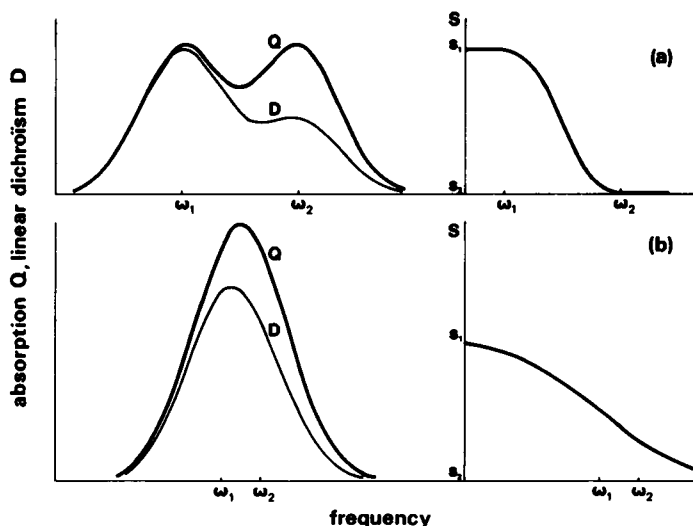The contributions to the absorption and dichroism spectra from pigment type n are

FIGURE 2 Frequency dependence of the absorption Q, of the linear dichroism D, and of the apparent order parameter S for a mixture of two pigments with resonance frequencies ω_1 and ω_2 and order parameters s_1 and s_2. (a). $|\omega_1 - \omega_2|$ greater than band width; (b). $|\omega_1 - \omega_2|$ less than band width.

then

$$Q^{(n)}(\omega) = (\omega/c) < G_n(\omega - \omega_n(\Phi)) >, \tag{22}$$

$$D^{(n)}(\omega) = (\omega/c) < s_n(\Phi) G_n(\omega - \omega_n(\Phi)) >. \tag{23}$$

Since $|\omega_n(\Phi) - \omega_n|$ is small, we can expand these two contributions as far as the first order in $\omega_n(\Phi) - \omega_n$:

$$Q^{(n)}(\omega) = (\omega/c) G_n(\omega - \omega_n), \tag{24}$$

$$D^{(n)}(\omega) = (\omega/c)\left[s_n G_n(\omega - \omega_n) - <\Delta s_n \Delta \omega_n> \frac{\partial G_n(\omega - \omega_n)}{\partial \omega} \right], \tag{25}$$

where

$$<\Delta s_n \Delta \omega_n> = <(s_n(\Phi) - s_n)(\omega_n(\Phi) - \omega_n)> \tag{26}$$

is a measure of the correlation of the angular fluctuations of $s_n(\Phi)$ and $\omega_n(\Phi)$.

Thus the existence of small fluctuations of the eigenfrequencies does not influence the absorption spectra, in the first order. They affect the band width only in the second order. But if the order parameter s_n (Eq. 21) is not zero, these fluctuations cause a band shift in the linear dichroism: from Eqs. 25 and 24 we have

$$D^{(n)}(\omega) = s_n Q^{(n)}(\omega - <\Delta s_n \Delta \omega_n >/s_n). \tag{27}$$

The shift depends on the correlation of the eigenfrequency fluctuations and the fluctuations of the transition moment directions.

Lastly, if s_n is zero, the dichroism spectrum is, by Eq. 25, the derivative of an absorption spectrum.

Thus, omitting pigments for which s_n is zero, we have for the actual chloroplast lamellae.

$$Q(\omega) = (\omega/c) \sum_n p_n G_n(\omega - \omega_n) \tag{28}$$

and

$$D(\omega) = (\omega/c) \sum_n p_n s_n G_n[\omega - \omega_n - \Delta_n(s)], \tag{29}$$

where

$$\Delta_n(s) = <\Delta s_n \Delta \omega_n >/s_n. \tag{30}$$

The argument given above for dichroism is also valid for electrochromism, so that

$$Q_1(\omega) = (\omega/c) \sum_n p_n c_n G_n[\omega - \omega_n - \Delta_n(c)], \tag{31}$$

$$D_1(\omega) = (\omega/c) \sum_n p_n(cs)_n G_n[\omega - \omega_n - \Delta_n(cs)], \tag{32}$$

where $c_n = \langle c_n(\Phi, \psi) \rangle$ (see Eq. 12),

$$(cs)_n = \langle s_n(\Phi) c_n(\Phi, \psi) \rangle, \tag{33}$$

$$\Delta_n(c) = \langle \Delta c_n \Delta \omega_n \rangle / c_n, \tag{34}$$

$$\Delta_n(cs) = \langle \Delta(cs)_n \Delta \omega_n \rangle / (cs)_n. \tag{35}$$

In these latter expressions, however, we must take account of the fact that the eigen-frequencies may vary not only with Φ but also with the permanent electric field $\mathbf{E}$ acting on the pigments. Thus the correlations in Eqs. 34 and 35 involve these field fluctuations, which are a priori expected to predominate, since they may be much greater than the angle fluctuations. The field of a dipole decreases by a factor of almost 30 when the distance from the dipole increases from 5 to 15 Å, less than the size of the chlorophylls.

Our analysis shows that, for a mixture of chlorophylls of different types, the spectra under consideration consist of Gaussian curves whose peak positions depend on the spectrum in question.

We can now make use of these theoretical results, as follows. We first find the values of the order parameter s_n for the various values of the resonance. We then calculate $Q_1(\omega)$ and $D_1(\omega)$ from Eqs. 8 and 14. We shall show that they correctly represent a Stark effect, and we shall analyze the angle dependence of the parameter $c(\Phi, \psi)$ (Eq. 12). Finally, we calculate the spatial fluctuations of the transition moment directions from Eq. 16, with a correction for the band shifts described above.

ORDER PARAMETER

Fig. 3 shows the results of linear dichroism measurements $D(\omega)$ for suspensions of oriented chloroplasts (Breton et al., 1973). The same diagram includes the wavelength dependence of the apparent order parameter $S(\omega)$:

$$S(\omega) = D(\omega)/Q(\omega) \quad \text{(cf. Eq. 19)}.$$

This dependence indicates that a wide variety of chlorophyll types contributes to the dichroism, since there is no clear plateau. Note also the shift of the dichroism spectrum peak relative to the absorption peak, representing a correlation between the frequency fluctuations and the order parameter.

Our purpose in this section is to find the value s_n of the order parameter corresponding to the resonance frequency ω_n. This is equivalent to evaluating the function $s_n = s(\omega_n)$, possible in principle by resolving the absorption spectrum $Q(\omega)$ and the dichroism spectrum $D(\omega)$ into Gaussian curves. Such a method is in fact difficult, and also inexact in that the Gaussian curves forming $D(\omega)$ may be shifted in frequency relative to those forming $Q(\omega)$ (see Linear Dichroism section). We have chosen a simpler method whose accuracy is adequate for our purpose. It is based on the physical hy-
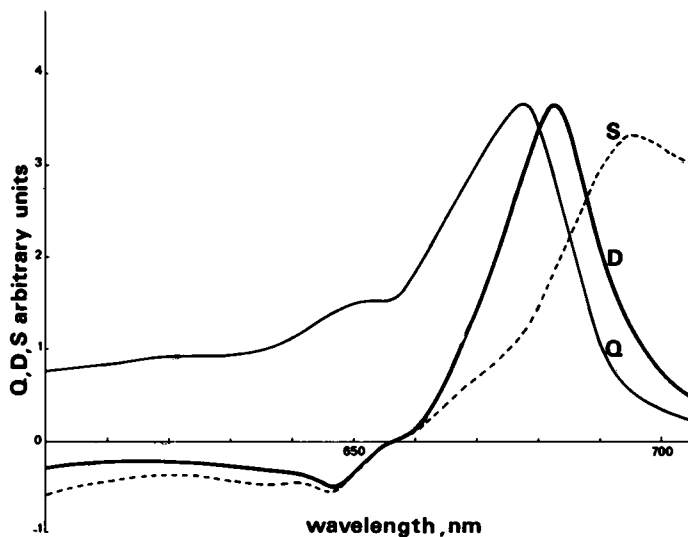
FIGURE 3 Wavelength dependence of the absorption Q, the linear dichroism D, and the apparent order parameter S for a suspension of oriented lamellae.

pothesis that the band widths of the various chlorophylls are comparable. This is supported by the results of Gaussian resolutions (French, 1971; Rabinowitch et al., 1967) and represents the fact that the band width results from the coupling of the electronic and nuclear motions within the chlorophyll molecule; such coupling does not involve the peripheral nuclei and hence depends only slightly on external perturbations (Paillotin, 1974). We have used two methods of calculation, described in the Appendix. One is based on the rule of moments and is very general. The other uses the derivative spectra of $S(\omega)$ and $Q(\omega)$.

The two methods give comparable results. Fig. 4 shows the function $s(\omega_n)$ thus obtained. The accuracy of the calculation itself is such that the error is below 10% for wavelengths less than 690 nm. Above 690 nm, both methods are inexact and give only qualitative results. The decrease of s observed in this range is not significant. Another source of error is the treatment of the frequencies ω_n as being continuously distributed. The curve shown in Fig. 4 is therefore only the envelope of the actual values of the parameter s. For this reason it cannot show any sudden changes that may occur in its frequency dependence.

In any event, Fig. 4 shows that the order parameter s exceeds the apparent parameter S above 677 nm, and is less than or equal to S below this wavelength. The transition moment of chlorophyll b is effectively oriented (Breton and Paillotin, 1976) out of the plane of the membrane. If we take $s_{max} \approx 0.5$, which is compatible with the highest experimental values observed for $S(\approx 44\%$ at 695 nm), $s = -15.5\%$ at 647.5 nm, corresponding to $<\cos^2\Phi> = 0.437 = \cos^2 48.6°$. A plateau is observed near 658 nm, indicating the existence of chlorophyll a with $<\cos^2\Phi> = 1/3$. The presence of this chlorophyll, which is compatible with the results of French (1971), accounts for the
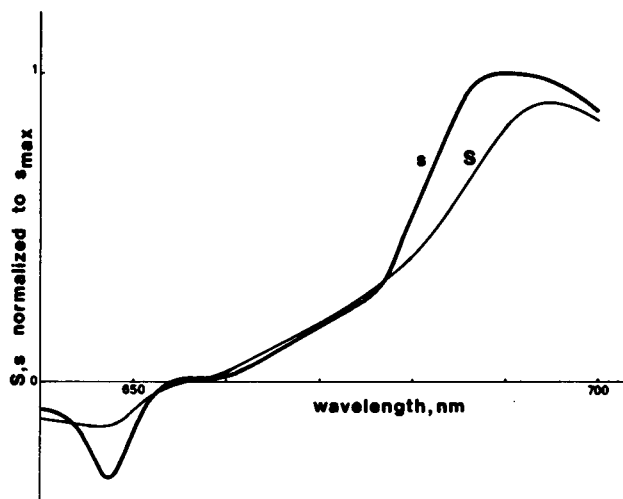
FIGURE 4   Wavelength dependence of the apparent order parameter S and the order parameter s.

form of the dichroism spectrum at this wavelength, which has three components: one negative due to chlorophyll b, one positive due to chlorophylls a absorbing at longer wavelengths, and one that is a derivative (see under Linear Dichroism).   These three contributions appear more clearly in linear dichroism spectra obtained at low temperatures (Breton et al., 1973; Vermeglio et al., 1976).   Near 670 nm, s is about 8.8%, corresponding to $<\cos^2 \Phi> = 0.275 = \cos^2 58.4°$.   Near 680 nm, there is a highly significant observed variation of the parameter s: its mean value at 682.5 nm is 38%, or $<\cos^2 \Phi> = 0.083 = \cos^2 73.22°$.   Thus the chlorophylls a that absorb at long wavelengths are almost in the plane of the membrane.   The large variation of s near 680 nm indicates that several pigments are concerned in the linear dichroism over the wavelength range from 677 to 690 nm.   In this way, we have been able to evaluate the order parameter of the various chlorophylls in the photosynthetic membrane.   The values thus found are in some cases (chlorophyll b and the chlorophylls that absorb at long wavelengths) very different from what would result in the apparent order parameter.   All the values thus calculated are spatial averages of $\cos^2 \Phi$.

ELECTROCHROMISM OF CHLOROPHYLLS

On the basis of our experimental results (Breton and Paillotin, 1976) for chlorophyll absorption variations attributable to a Stark effect, we have calculated $Q_1(\omega)$ (Eq. 8) by integrating the spectra of these variations.   Fig. 5 shows the results.   For an actual Stark effect (see Linear Dichroism section 1), $Q_1(\omega)$ must be a superposition of chlorophyll absorption bands modulated by a factor $<c(\Phi,\psi)>$ (Eq. 13) that depends on the orientation of the pigment concerned.

  It is seen from Fig. 5 that the form of Q_1 agrees with the hypothesis of Stark effect and shows a contribution from several chlorophylls.   Chlorophyll b is represented in
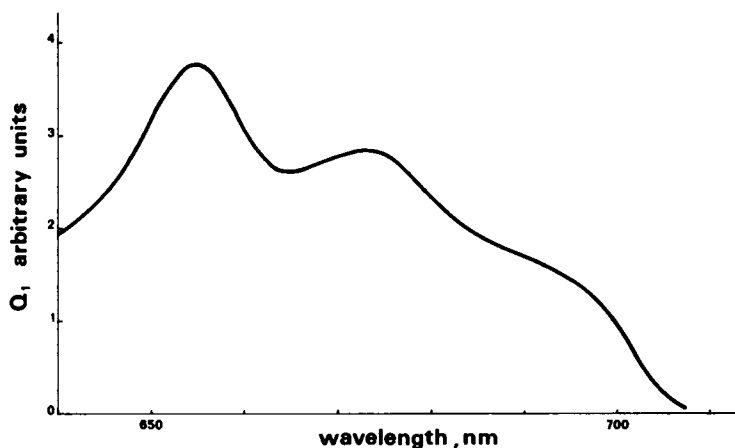
FIGURE 5   Wavelength dependence of the quantity Q_1 obtained by integration of the electrochromic effect (see text).

this spectrum by a peak between 635.5 and 655.5 nm, and there are also various chlorophylls a at about 673, 685, and 695 nm.

The intensity of the bands in $Q_1(\omega)$ seems to vary oppositely to that of the bands in the linear dichroism spectrum (Fig. 3). This is not surprising, in that $<c(\Phi,\psi)>$ varies with Φ as a linear combination of $<\cos^2\Phi>$ and $<\cos\Phi>$ (Eq. 11). Thus we were led to test the hypothesis:

$$<c(\Phi,\psi)> \sim <\cos^2\Phi> + \text{constant} \qquad (36)$$
$$\sim \text{constant} - <s(\Phi)>.$$

If there is such a linear relation between $<c(\Phi,\psi)>$ and the order parameter, there must be a linear combination of the dichroism spectrum $D(\omega)$ and the spectrum $Q_1(\omega)$ which gives the absorption spectrum apart from a shift due to the fluctuation correlations of the frequencies and the constant field E, discussed under Linear Dichroism, Eqs. 31 and 34.

We have sought and found such a linear combination; the result is shown in Fig. 6. From D and Q_1, the absorption spectrum can be reproduced (with a shift to the red of about 5 nm) very accurately between 655 nm and 685 nm, i.e. the major part of the chlorophyll a spectrum. Apart from the large value of the red shift, explained in the following, two anomalies occur as is seen from the difference between the combination shifted 5 nm to the blue and the absorption spectrum (Fig. 6).

One relates to chlorophyll b and is easily explained. In the Stark effect theory, it would suggest that chlorophyll b has a greater electrochromism than chlorophyll a. This is so: the experiments of Schmidt et al. (1971) show in fact that, for equal concentrations, chlorophyll b has an electrochromism in the red this is 75% of the value for chlorophyll a. For equal absorptions, this gives an electrochromic effect for chlorophyll b that is about 20% greater than for chlorophyll a.
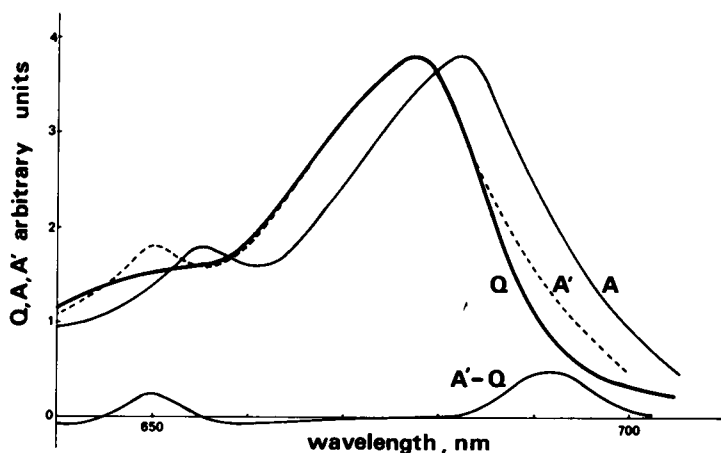
FIGURE 6 Fitting the absorption spectrum Q by a linear combination A of Q_1 and the dichroism D. A' corresponds to A shifted 5 nm to the blue. The difference between A' and Q indicates bands at 650 nm and 692 nm.

The other anomaly corresponds to an absorption band with a peak at about 692 nm. This might be interpreted as corresponding to a group of chlorophylls with electronic properties differing from those of the monomers. Note that, as well as photo-induced spectrum changes that depend on the ionic permeability of the membranes, there are always other changes not dependent on this permeability and attributed to the existence of chlorophyll cations (Breton, 1976). The latter changes have of course been subtracted from those which yield $Q_1(\omega)$. However, the existence of such cations is accompanied by that of local fields, which may increase the electrochromism of pigments subjected to them. It seems that the band observed at 692 nm may be explained by assigning it to a Stark effect for chlorophylls subjected to the additional permanent field due to the chlorophyll cations. But whatever the reason called upon to explain this 692 nm band, it is of little importance in our discussion, since the absorption at 692 nm is weak.

Thus, apart from the 692 nm band, the spectrum $Q_1(\omega)$ can be derived from the dichroism and absorption spectra only. This results greatly favors the existence of an intramembrane field (Junge and Witt, 1968). It implies that the effect represented by the spectrum $Q_1(\omega)$ is global and depends only on the optical and geometrical properties of the ensemble of pigments, like the Stark effect. The red shift of 5 nm (~ 100 cm^{-1}) observed in our fit is incompatible with the band shifts due to a static field (Emrich et al., 1969), but is entirely explicable by the fluctuation of the constant field (Linear Dichroism section; see also Conjeaud, 1976).

Lastly, the linear relation (Eq. 36) observed between $<c(\Phi,\psi)>$ and $<s(\Phi)>$ implies that $<\sin^2\psi>$ varies only slightly with Φ or that it varies in proportion to $<\cos^2\Phi>$. A further analysis of this relationship would yield relations between the orientation of the relevant transition moment in the red and that of the normal to the tetrapyrrole plane. For the present work we note that $<c(\Phi,\psi)>$ is found to

vary oppositely to $<s(\phi)>$ except in the 692 nm band. This property now enables us to evaluate the spatial fluctuations of the chlorophyll transition moment orientations.

SPATIAL FLUCTUATIONS OF TRANSITION MOMENT ORIENTATIONS

We have seen in Eq. 16 that these fluctuations can be evaluated from the spectra $Q(\omega)$, $D(\omega)$, $Q_1(\omega)$, and $D_1(\omega)$. The linear dichroism $D(\omega)$ is shown in Fig. 3, the electrochromic effect $Q_1(\omega)$ in Fig. 5, and the polarization of this effect $D_1(\omega)$ calculated from Eq. 14 is shown in Fig. 7.

Eq. 16 is $Q(\omega)D_1(\omega) - Q_1(\omega)D(\omega) = Q^2(\omega) <(s(\Phi) - <s(\Phi)>) (c(\Phi,\psi) - <c(\phi,\psi)>)>$. In the previous section we saw that $<c(\Phi,\psi)>$ varies oppositely to $<s(\Phi)>$. If this is assumed to be true for $c(\Phi,\psi)$ and $s(\Phi)$, the right-hand side of the equation is always negative. The two products $Q(\omega)D_1(\omega)$ and $Q_1(\omega)D(\omega)$ are plotted in Fig. 8. We can see that this property holds; the deviations observed between 670 and 655 nm are less than the uncertainty in $D_1(\omega)$. The results of the previous section are thereby confirmed. A further confirmation comes from the similarity of form of the two products, according to Eq. 16. The shift between peaks observed near 680 nm has already been analyzed under Linear Dichroism and shows (Eqs. 34 and 35) that $\Delta_n(c) \sim -100\,cm^{-1}$, $\Delta_n(cs) \sim 0$. The first result follows from the discussion in the previous section. It is easily verified that the second follows from the opposite variation of $c(\Phi,\psi)$ and $s(\Phi)$.

Let us now consider in detail the difference between Q_1D and QD_1. In the chlorophyll b absorption range, QD_1 is negative and about twice Q_1D. Thus the transition moments of chlorophyll b are not all oriented in the same way relative to the normal ν to the plane of the lamellae. From the values of $<s(\Phi)>$ and $<c(\Phi,\psi)>$ and from Eq. 16 we can say that the fluctuations of Φ are about 10°. Thus, from the Order Pa-
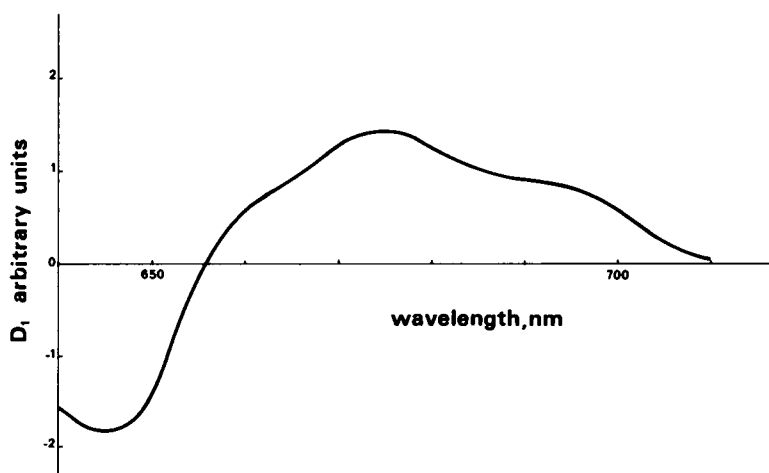

FIGURE 7   Wavelength dependence of the polarization D_1 of the electrochromic effect.
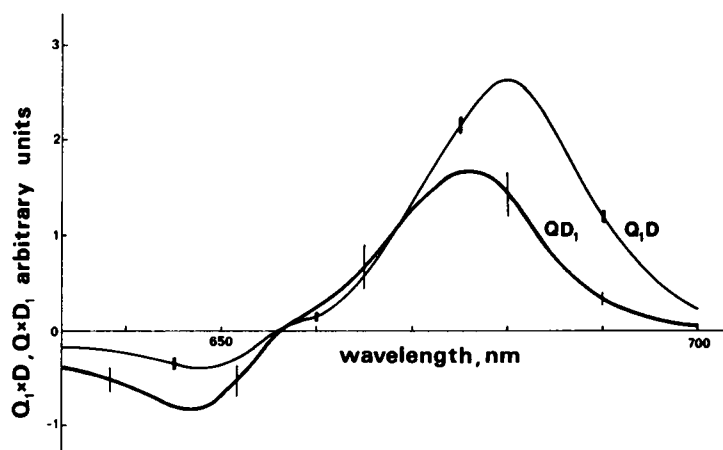
FIGURE 8 Wavelength dependence of the products QD_1 and Q_1D. The vertical lines show the error ranges.

rameter section, the angle of the chlorophyll b transition moment (in the red) with ν is $48.5° \pm 5°$.

In the wavelength range from 655 to 670 nm, we have seen (under Order Parameter) that the order parameter is almost zero. Fig. 8 shows that the difference between Q_1D and QD_1 is negligible. From Eq. 16, this means that the chlorophylls contributing to the absorption in this range do not have randomly oriented transition moments. For example, chlorophyll a absorbing near 660 nm has an angle Φ close to the magic angle of $54.7°$. Similarly, for the chlorophyll absorbing near 670 nm the angle Φ is close to $58°$, the fluctuations of Φ in the latter case being less than $5°$.

The set of chlorophylls regarded as consisting of a pigment whose resonance frequency fluctuates only slightly shows some dispersion of the transition moment orientations: near 680 nm, Q_1D exceeds QD_1 and there is a shift of the peaks of the product of these two spectra (Linear Dichroism section).

The question is now whether the spatial fluctuations found are due only to the existence of several types of pigment, each with a transition moment precisely oriented with respect to ν, or to an actual disorder of these various moments. The first hypothesis is correct: from the values of $<s(\Phi)>$ (Fig. 4) and $Q_1(\omega)$ it is possible to calculate, e.g. by the method of moments (see Appendix), the spatial fluctuations of the transition moment orientations due simply to the existence of different types of chlorophyll a. If, as under Linear Dichroism, p_n is the proportion of the nth type and s_n its order parameter, the method of moments gives

$$\sum_n p_n s_n \approx 0.19, \tag{37a}$$

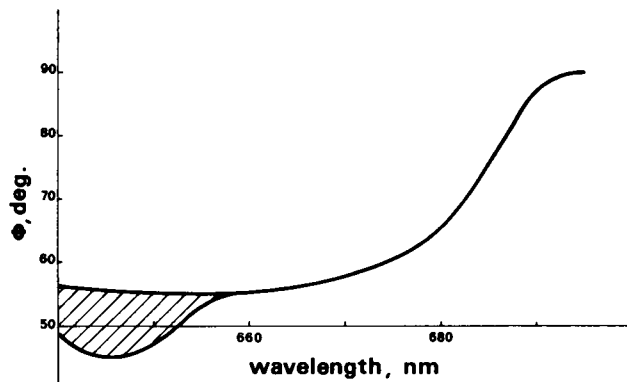$$\sum_n p_n s_n^2 \approx 5.8 \times 10^{-2}. \tag{37b}$$

FIGURE 9 Wavelength dependence of the angle Φ between the transition moment and the normal to the plane of the lamellae. The shaded area shows the fluctuations of Φ in the region belonging to chlorophyll b.

If we exclude the 692 nm band, negligible in any case as regards the products QD_1 and Q_1D, we find that, in the range where s_n is not negligible, $<c(\Phi,\psi)> \approx 1 - 2 <s(\Phi)> \approx <\cos^2\Phi>$. From the Linear Dichroism section, the ratio of the maxima of Q_1D and QD_1 is $R = [\max(Q_1D)/\max(QD_1)] = (\sum_{n,m} p_n p_m <s_n><c_m>)/(\sum_{n,m} p_n p_m <s_n c_n>)$. Where there are only frequency fluctuations, this ratio is

$$(\sum_{n,m} p_n p_m <s_n><c_m>)/(\sum_{n,m} p_n p_m <s_n><c_n>).$$

From Eqs. 37a and b, this ratio is 1.6, almost equal to the experimental value of R, 1.55. If there were an actual disorder of the transition moment orientations, R would have to be much greater than 1.6.

We can thus say that for the chlorophylls a considered as a whole, the angle Φ between the transition moments and the normal ν to the lamellae varies from 55° to 90°, but that for a given type of chlorophyll, i.e. a given resonance frequency, Φ has a definite value. This value is unambiguously derivable from $<s(\Phi)>$ and is shown in Fig. 9.

CONCLUSION

In analyzing the orientation of the chlorophyll transition moments in the photosynthetic membrane, we have been led to examine in detail the electrochromic effects shown by the photosynthetic apparatus. The investigation shows that these effects can in fact be attributed to the action of an electrostatic field (Stark effect). In particular, we have demonstrated that this electrochromism can be explained in terms of the overall optical properties of the photosynthetic membrane.

We have also confirmed that the transition moments reponsible for the absorption in the red are not all oriented in the same way relative to the normal ν to the plane of the membrane. The angle Φ between the direction of these moments and ν varies from 55° to 90° for chlorophylls a and from 43.5° to 53.5° for chlorophylls b. There

is, however, a great difference between these two types of pigment: for chlorophylls a, different values of Φ correspond to different values of the resonance frequencies, but for chlorophyll b they correspond to almost the same resonance frequency.

The following remarks may be made on these results. For chlorophyll a, two situations are possible. In one, all the optical properties are governed by the nature of the site it occupies. In this case, we must assume that the site is such as to determine both the orientation of the transition moment and the resonance frequency. In the other case, which we regard as correct on the basis of excitation energy transfer analysis (Paillotin, 1976), the optical properties of chlorophylls a are decided by the influence of molecule fixation sites and collective properties. In this case, the mode of fixing to the membrane would determine the orientation of the pigments, and the interactions of pigments with the same orientation would define the resonance frequencies.

Whichever hypothesis is accepted for chlorophyll a, the situation is different for chlorophyll b. Here we can suppose a less rigid fixation to the membrane or weaker electronic interactions between different chlorophylls b.

APPENDIX

Using the same notation as under Linear Dichroism, we can write the absorption $Q(\omega)$ and the linear dichroism $D(\omega)$ of a mixture of pigments as

$$Q(\omega) = (\omega/c)q(\omega), \quad q(\omega) = \sum_n p_n G_n(\omega - \omega_n), \tag{1}$$

$$D(\omega) = (\omega/c)d(\omega), \quad d(\omega) = \sum_n p_n s_n G_n(\omega - \omega_n). \tag{2}$$

$G_n(\omega - \omega_n)$ is a Gaussian curve centered at ω_n with half-width W_n; p_n is the probability of absorption by the nth pigment.

The Fourier transform $q(t)$ of $q(\omega)$ is

$$q(t) = \int e^{i\omega t} q(\omega) d\omega = \sum_n p_n \exp\left[(-W_n^2/4 \ell n\, 2)t + it\omega_n\right].$$

Expanding the two expressions for $q(t)$ in powers of t, we have:

$$\int \omega^k q(\omega) d\omega = \sum_n \sum_{2j \le k} (2j)!/j!(W_n^2/4\,\ell n\,2)^j \omega_n^{k-2j} C_k^{2j} p_n.$$

Thus, from the absorption spectra, neglecting the fluctuations of W_n ($W_n = W$), we can calculate the moments M_k by recurrence: $M_k = \sum_n p_n(\omega_n - \bar{\omega})^k$, where $\bar{\omega} = \sum_n p_n \omega_n$. Similarly, it is possible to evaluate the moments N_k from $d(\omega)$: $N_k = \sum_n p_n s_n(\omega_n - \bar{\omega})^k$.

Then, writing s_n as a polynomial in $\omega_n - \omega$, we can calculate its terms from the relations that must exist between the values of N_k and M_k.

Another method of evaluating s_n can be used when we regard s_n and p_n as continuous functions of ω_n. With an operator L defined by $L = (W_n^2/4\,\ell n\,2)(\partial^2/\partial\omega^2)$, Eqs. 1 and 2 be-

come $q = (\exp L)p(\omega)$, and $d = (\exp L)p(\omega)s(\omega)$, whence $s(\omega) = (\exp - L)d/(\exp - L)q$, and $s(\omega)$ is calculated from the successive even derivatives of d and q.

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