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Emergence of Biological Function in the Framework of a Percolation Model

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The Reaction Center of Photosynthetic Purple Bacteria

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The Reaction Center of Photosynthetic Purple Bacteria

The bacterial reaction center isolated from Rhodopseudomonas viridis *is the essential site of the initial steps of photosynthetic conversion and storage of light energy. It is the first membrane protein from which a three-dimensional picture at atomic resolution has been obtained. The structure allows the visualization of the path of the ultrafast electron transfer events and suggests the role of the protein in fine-tuning the energy levels of the cofactors, in assisting the electron transfer, and in stabilizing the separated charges.*

BACKGROUND

Photosynthesis is the biological oxidoreduction process by which solar energy is converted into chemical energy. This is an essential process not only because it allows plants to grow but also because this stored chemical energy sustains all the other forms of life on earth. The emergence of photosynthesis more than three billion years ago occurred in the primitive reducing atmosphere, so reduced sulfur and organic compounds were used as sources of electrons. Subsequent evolution led to the now-dominant forms of photosynthesis in which water is the electron donor and molecular oxygen, the ultimate oxidation product, is released. The ensuing changes in the atmosphere's composition led the primitive nonoxygenic photosynthetic bacteria to retreat to ecological niches in which reducing conditions prevail, such as in sulfur springs or in the muddy layers at the bottom of lakes and ponds. There they are still abundant.

These bacteria have proved to be extremely useful in understanding how photosynthesis works, as they represent a simpler, less evolved form of photosynthesis than the one found in green plants. In particular, green plants contain two photosystems (Photosystem I and Photosystem II) that function in series, while photosynthetic bacteria contain only one. There exist two main classes of photosynthetic bacteria: the green bacteria, which bear analogies with Photosystem I, and the purple bacteria, which are closer to Photosystem II. In recent years considerable progress has been achieved in understanding the molecular structures and mechanisms involved in the primary processes of photosynthesis in the (purple bacteria rhodospirillales), and this article focuses on these developments.

Inside the cytoplasm of purple bacteria, an array of closed vesicles constitutes the photosynthetic apparatus. The membrane forming the interface of these vesicles contains, in addition to lipid molecules, specialized proteins that are directly responsible for the photosynthetic function. These transmembrane hydrophobic proteins, which bind the bacteriochlorophyll and carotenoid pigments (Fig. 1), and which are called the pigment-protein complexes, can be classified into two categories: reaction centers and antenna complexes. The reaction center is the site of the light-induced transmembrane charge separation. It is inserted in the membrane in such a way that one end of the reaction center is near the inner surface of the vesicle and the other end is near the outer surface. Each of the many reaction centers in a vesicle is surrounded by a large array of other pigment-protein complexes, the role of which is to absorb the energy of the incoming photon and to funnel this energy very efficiently (> 95%) to the reaction center. These pigment-protein complexes serve to increase the absorption cross section of the reaction center and are thus called antenna complexes. The ratio of antenna bacteriochlorophylls to reaction centers varies from species to species and according to the growth conditions. It ranges typically between 20 and 100.

The concepts and the evidence for antenna complexes and reaction centers in photosynthetic bacteria were derived in the 1950s from the early spectroscopic studies of Duysens. However, it was through the pioneering work of Reed and Clayton only 20 years ago that a reaction center was isolated for the first time (1). The purple bacteria from which it was derived, *Rhodobacter sphaeroides* (*Rb. sphaeroides*), contains bacteriochlorophyll *a* as the main pigment. Since its isolation, this reaction center has been extensively characterized in both biochemical and biophysical studies (2), and a large variety of spectroscopic techniques have been used to investigate both its structure and its function. From the genetic point of view, the closely related species *Rb. capsulatus* has been characterized in detail. The reaction

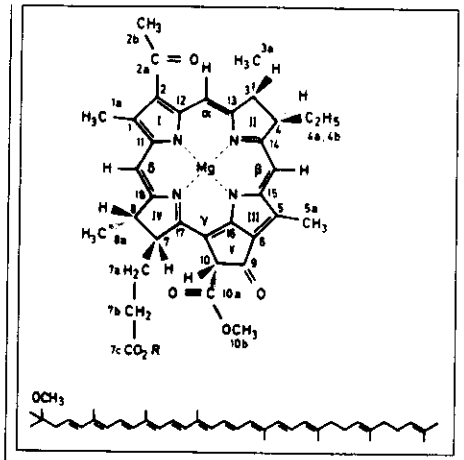

Figure 1 Structure of bacteriochlorophyll *a* (top) and of a carotenoid molecule (bottom) present in *Rb. sphaeroides*. The radical R in position 7c of bacteriochlorophyll *a* is a long and flexible phytol chain. In bacteriochlorophyll *b*, an ethylidene group (=CH-CH_3) is attached to ring II at position 4. In the bacteriopheophytins, the central Mg atom is lacking.

center from still another purple bacterium, *Rhodopseudomonas viridis* (*Rps. viridis*), which was isolated later (3), has also been quite extensively studied because its major pigment, bacteriochlorophyll *b*, has an absorption spectrum in the visible and near infrared that is significantly different from that of bacteriochlorophyll *a*. This gives a new set of spectroscopic data, allowing correlations between the properties of the two types of reaction centers to be derived. Furthermore, these two types of reaction centers differ in that only the one isolated from *Rps. viridis* functions with a tightly bound cytochrome.

CURRENT STATUS

The X-ray Structure of the Reaction Center

A major breakthrough in the field of photosynthesis research was the recent x-ray crystallographic determination at atomic resolution of the structure of the reaction center from *Rps. viridis* (4). The main difficulty was not so much the size of the protein (molecular weight of the order of 140 000) but the fact that, as an amphiphilic transmembrane protein, it requires detergents for isolation and purification. These detergent molecules, which somehow displace the lipid molecules normally responsible for the hydrophobic interactions inside the membrane and remain firmly bound to the reaction center, do not provide a well-defined surface coating of the protein. This usually prevents the formation of the very regular three-dimensional crystals required for high resolution studies. H. Michel pioneered the use of small amphiphilic molecules to replace the detergent in those positions in the crystals where they disturb the crystalline order. This technique allowed him to obtain crystals of this reaction center diffracting at better than 2.5 Å resolution. Normal photochemical activity of the crystalline material has been demonstrated. The x-ray structure, solved by J. Deisenhofer in R. Huber's laboratory,

Figure 2 Protein subunits (represented as ribbons) and prosthetic groups in the reaction center from *Rps. viridis*. The cytochrome subunit with the four hemes occupies roughly the upper 40% of the structure. Most of the H subunit extends below the Fe atom. The central core (made of the L and M subunits), which binds all the prosthetic groups but the four cytochrome hemes, is located in the middle. The pseudo C2 symmetry axis is in the plane of the figure and runs vertically from the Fe atom. Reproduced with permission from Deisenhofer et al. (4).

was first published at the end of 1984 and has now been refined to 2.3 Å. Subsequently, reaction centers from *Rb. sphaeroides* were crystallized in several laboratories; their structure, which is quite close to that of the reaction center from *Rps. viridis*, has been solved at about 3 Å by the molecular replacement method using the latter structure as a template, and is currently in the refinement process (5,6). It is hoped that the demonstration that well-ordered three-dimensional crystals of these integral membrane proteins can be obtained will lead to successful attempts with other membrane proteins. Precise knowledge of their molecular architecture is highly desirable, since membrane proteins have many important functions such as energy transduction, membrane trans-

port, stimulus reception, and cell-to-cell communications.

The electron density map calculated from x-ray analysis of the crystals of *Rps. viridis* reaction centers allows a good description of the overall organization of the cofactors (4 bacteriochlorophylls, 2 bacteriopheophytins, 1 carotenoid, 2 quinones with one site only partially occupied, 1 nonhemic iron, 4 hemes) and of the four polypeptide chains (named L, M, H, and Cyt) in the complex (Fig. 2). This complex has an elongated shape (~130 Å in length) with a central cylindrical core (approximately 50 Å long) presenting an elliptical cross section (~30 and 70Å for the lengths of the two axes). At each end of this cylindrical core, which corresponds to the L and M polypeptides, there is a flat face to which a globular subunit (Cyt on one side and most of the H polypeptide on the other) is attached. The central core is made up of two sets of five α-helical segments running preferentially parallel to the cylinder axis. Within these sets of α-helices, which are assigned to the L and M polypeptides, are located all the cofactors but the four hemes of Cyt. This central core, together with the single long α-helix of the H-subunit, consists almost exclusively of nonpolar amino acid residues, while the charged residues are located only in the two outer regions of the reaction center. The knowledge of the amino acid sequence for each polypeptide (obtained by analyzing the corresponding genes) was instrumental in determining the precise folding of the protein as well as the probable bonding interactions between the prosthetic groups and specific amino acid residues. When the reaction center from *Rb. sphaeroides* was analyzed by x-ray diffraction, a picture very similar to that obtained for the *Rps. viridis* reaction center emerged, indicating almost identical arrangements of the L, M, and H polypeptides and of the cofactors (5,6).

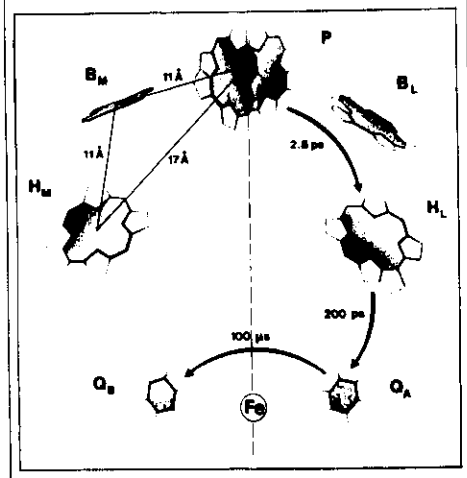

Figure 3 Schematic representation of the cofactors in the bacterial reaction center of *Rb. sphaeroides* (6). The C2 axis runs vertically from the Fe atom. For clarity, the carotenoid, the phytol chains of the chlorophylls, and the isoprenoid chains of the quinones have been removed.

One of the most striking features of the x-ray structural model of these bacterial reaction centers, shown schematically in Fig. 3, is the presence of a C2 symmetry axis running from the nonheme iron toward two closely associated bacteriochlorophylls. These two molecules have their pyrrole rings I stacked on top of each other (about 3 Å apart) and are related by the local two-fold rotation axis. They have been assigned to the special pair P, the existence of which had been inferred from spectroscopic investigations (7). To each bacteriochlorophyll of P is associated a monomeric bacteriochlorophyll (B_L, B_M) which forms sort of a bridge between P and the bacteriopheophytin (H_L, H_M). The Mg atoms of the four bacteriochlorophylls appear ligated to histidine residues. The nonheme iron, which is also bound by four histidines and by one glutamic acid, sits on the C_2 axis between the two quinones Q_A and Q_B. The symmetry axis relates not only the positions of the B and H molecules but also those of the main α-helical segments of the L and M polypeptides.

Although the symmetry axis is clearly recognized in the LM structure, a closer examination of the model reveals many important features that do not obey the C2 symmetry. Examples of such nonsymmetrically arranged elements are the phytol chains of the pigments, the carotenoid located close to B_M, and the lengths of the connections between the α-helices and the amino- and carboxy-terminal ends of the L and M polypeptides. While the model indicates that both polypeptides contribute equally to the binding of the pigments, the precise anchoring of the pigments to the polypeptidic backbone (which is assumed by multiple van der Waals interactions and hydrogen bonds) differs on the L and M branches (8). One of the clearest examples of this assymmetry is provided by the 9-keto carbonyl of H_L, which has a polar glutamic acid residue (L104) at an appropriate distance to form a hydrogen bond, while the residue in the homologous position on the M-branch (M131) is replaced by a nonpolar valine in *Rps. viridis* and by a threonine in *Rb. sphaeroides*. In addition, the distributions of the many aromatic amino acid residues—which, together with other nonpolar residues, constitute the medium in which the pigments are immersed—are different along the two branches.

Much structural information on the reaction center was already available prior to the x-ray crystallographic studies. For example, the locations of the 11 transmembrane α-helices had been inferred from hydropathy plots of the amino acid sequences of the L, M, and H polypeptides. Their average tilt in the membrane had been determined by infrared dichroism measurements (9). The distances between the pigments and their orientation had been correctly deduced from optical (10) and magnetic (11) spectroscopy. Also, the nature of the interaction between the polar C=O groups of the various cofactors and their protein binding sites had been investigated by techniques such as resonance Raman spectroscopy (12). The availability of the reaction center's x-ray structure and the recent refinements of this structure provides the framework that can be used to exploit these spectroscopic results more fully. The comparison of the structures and of the spectroscopic data represents an invaluable test case to renormalize the results of the spectroscopic investigations and thus to sharpen the interpretation of the data obtained on other pigment-protein complexes for which no high-resolution structure is yet available.

Although the conclusion that the C2 axis seen in the crystal is oriented in vivo along the normal to the plane of the photosynthetic membrane has been derived from our spectroscopic studies, it is of interest that the only major structural feature of the reaction center that had been altogether missed by the spectroscopists is the C2 symmetry axis itself. However, as already mentioned above (and as will become more evident in the discussion of the electron transfer pathway), it is now becoming clear that this C2 symmetry is apparent only in a picture at 3 to 4 Å resolution. This symmetry is broken at the more local level described by the recent x-ray studies at higher resolution and by the exquisitely sensitive spectroscopic techniques.

The Molecular Mechanisms of Photosynthesis

When the energy of a photon reaches the reaction center, following either excitation of an antenna or direct absorption, a transmembrane separation of electric charges occurs with great speed and efficiency. This stored electrical energy is then used up at a slower pace to drive the chemical steps leading to the synthesis of complex organic molecules. Redox reactions are important in many biological processes, including photosynthesis, and the electron transfer steps in the reaction center can be triggered with very short light pulses, thus allowing time-resolved studies. The path of the electron and hole within the cofactors and the time scale of these events have been investigated for many years. Together with the detailed structure of the reaction center, this now challenges us to rationalize the observed distances and kinetics and reconcile these observations with the current theories for electron transfer.

The first event in the electron transfer sequence starts at the primary donor P, where an excited state P^* is generated. The precise nature of P^*, notably the contribution to P^* of various charge transfer states, is still under investigation (13), and the unambiguous observation by time-resolved absorption spectroscopy of the initial products generated from P^* has been achieved only very recently (14–16). An electron is ejected from P^* and reaches the bacteriopheophytin H_L with a time constant of 2.8 ps (1 ps = 10^{-12} s) for both *Rb. sphaeroides* (15) and *Rps. viridis* (16) at room temperature (Fig. 3). Thus P^+ and H_L^- rise in concert with the decay of P^*. It is remarkable that no transient localization of the electron on B_L could be detected. The subsequent step, which had been characterized more than a decade ago (reviewed in 17), leads the electron from H_L to Q_A in about 200 ps and then to Q_B in about 100 µs. The hole on P^+ is neutralized by an electron coming from a cytochrome. This takes about 300 ns in *Rps. viridis*, where the cytochrome is bound to the reaction center, and about 2 µs in *Rb. sphaeroides*, where a water-soluble cytochrome binds electrostatically to the reaction center. The remarkable efficiency (~100%) of the overall charge separation process is mostly due to the large difference between the fast forward reactions and the much slower back reactions: ~20 ns for the decay of $P^+H_L^-$, ~60 ms for $P^+Q_A^-$, and seconds for $P^+Q_B^-$ in *Rb. sphaeroides*. In terms of energetic yield, about 50% of the energy of the photon used to excite P is stored in the separated charges.

The first striking feature that emerges from this brief description of the electron transfer pathway is the unidirectionality along the L-branch of cofactors (18). This unidirectionality must be the consequence of some intrinsic asymmetry in the arrangement of the cofactors themselves and of their local protein environment, including their binding site. It is probable that some of this asymmetry is already induced at the level of P^* itself, thus favoring unidirectionality in the nascent charge separation within the two bacteriochlorophylls constituting P. It has been noticed that the fluctuation of the position of the atoms along the M-branch was larger than for the L-branch. Furthermore, in terms of function, the cofactors involved in the earlier primary electron transfer steps obey the C2 symmetry better than those involved in the later stages.

A second interesting aspect concerns the role played by B_L, which bridges P and H_L. The fact that no electron localization on B_L is observed experimentally does not mean that this molecule is not involved in the electron transfer process. For example, a sequential two-step mechanism in which the second step ($P^+B_L^- \rightarrow P^+H_L^-$) is much faster than the first one ($P^* \rightarrow P^+B_L^-$) would lead to an undetectable transient population of B_L^-. However, recent spectroscopic measurements of the initial electron transfer at very low temperature (19) seem to exclude this possibility. At present two other mechanisms involving B_L are being considered: the one-step nonadiabatic/adiabatic process (20) and the superexchange in which B_L "catalyzes" the charge separation by enhancing the electronic overlap between P and H_L through the virtual state $P^+B_L^-$ (21).

In order to understand the electron transfer mechanism in the reaction center, one needs to know not only the atomic structure provided by the x-ray studies, but also the electronic structure of the cofactors within their binding site. Several groups are currently attempting such calculations (22,23). Furthermore, the x-ray picture is static and reflects neither the fluctuation of structure (which must play an essential role in fine-tuning the energy levels of the pigments and in assisting the electron transfer) nor the changes of structure that stabilize the separated charges. The combination of x-ray crystallography, theoretical calculations, and spectroscopy is needed to achieve a more complete description of the primary processes of photosynthesis.

FUTURE DIRECTIONS

Refined sets of coordinates are needed for more precise theoretical calculations (optical spectra, electron transfer, molecular dynamics, etc.).

Now that the structure of the reaction center is known in the neutral state, the many spectroscopic data that reflect the change of properties (electronic structure, environment, and so forth) of the cofactors accompanying charge separation must be rationalized in terms of structural changes. Furthermore, it might become possible, using synchrotron radiation, to determine the structure directly in one of the charge-separated states.

In order to identify the specific binding sites of the cofactors and the role of the symmetry-breaking elements in the unidirectionality of electron transfer, site-directed mutagenesis is being applied. By comparing the sequences of the L and M polypeptides from *Rps. viridis*, *Rb. sphaeroides*, and *Rb. capsulatus* and using the x-ray structures, one can identify the conserved amino acid residues, which could have important structural or mechanistic roles. Reaction centers from *Rb. capsulatus* carrying mutations directed at some of the key binding

sites for the cofactors have been isolated and are being spectroscopically characterized (24).

Regarding the general problem of understanding the folding of a protein from its primary sequence, the reaction center is the only membrane protein that has so far been solved by x-ray crystallography. This structure can thus be used for other membrane proteins for which only the primary sequence is known. It can also be used to study lipid-protein interactions and protein-protein interactions (such as docking of the cytochrome in *Rb. sphaeroides* (25), and interactions between the antenna and the reaction center).

Another active area concerns the analogy between the structures of the polypeptides L and M of the bacterial reaction center and those of D_1 and D_2 of the Photosystem II reaction center, which is the site of cleavage of water into molecular oxygen. A small Photosystem II reaction center that carries the primary photoactivity has recently been isolated (26) and is now under investigation in a number of laboratories.

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This is a partial list of individuals studying the main topics discussed in this article and of the major laboratories working in the field.

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* * *

The 1988 Nobel Prize in Chemistry has been awarded to H. Michel, J. Deisenhoser, and R. Huber for the determination of the three-dimensional structure of the reaction center from *Rhodopseudomonas viridis* by x-ray crystallography.

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4

Orientation of Photosynthetic Pigments *In Vivo*

JACQUES BRETON
ANDRÉ VERMEGLIO

ABBREVIATIONS

A	Absorption
B890,B800–B850	Light-harvesting complexes in bacterial photosynthesis
BChl	Bacteriochlorophyll
Bph	Bacteriopheophytin
Car	Carotenoid
CD	Circular dichroism
Chl	Chlorophyll
Chr	*Chromatium*
CPI-P700	Chlorophyll *a* complex of PSI
CPII	Light-harvesting chlorophyll *a*–*b* complex
Cyt	Cytochrome
ESR	Electron spin (paramagnetic) resonance
FP	Fluorescence polarization
I	Intermediary acceptor in bacterial photosynthesis
IR	Infrared

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LD	Linear dichroism
LHC	Light-harvesting complex
MCD	Magnetic circular dichroism
P	*Prosthecochloris*
P680	Primary donor of PSII
P700	Primary donor of PSI
PSI	Photosystem I
PSII	Photosystem II
Q_X, Q_Y	Electronic transition moments
RC	Reaction center
Rp.	*Rhodopseudomonas*
Rs.	*Rhodospirillum*
SP, P870, P960	Special pair, primary donor in bacterial photosynthesis
X, $A_{1,2}$	Intermediary acceptor of PSI detected by ESR

ABSTRACT

During the past decade, spectroscopy with linearly polarized light has been extensively used to probe the organization of pigments and of electron transfer components in the photosynthetic membrane. This organization can be recognized at different levels.

First, the relative orientation between pairs of transition moments of molecules (pigments and electron transfer components) within an isolated pigment–protein complex can be investigated. As compared to systems derived from green plants, our knowledge is much more advanced in the case of photosynthetic bacteria for which purified complexes containing a small number of chromophores in their native form have been isolated. Various intrinsic limitations (overlap of absorption bands, unknown nature of the interactions between the chromophores, and complex character of the absorption changes) lead, however, to a situation where this organization can be described only in qualitative terms.

Second, an orientation of the transition moments of the various pigments and electron transfer components with respect to the plane of the photosynthetic membrane has been described. Precise values of these angles can be obtained only when the degree of orientation of the membranes themselves is known. A similarity in the organization of the photosynthetic components in green plants and in bacteria is observed.

Finally, the possibility of investigating the mutual orientation of chromophores belonging to adjacent complexes in the photosynthetic membrane is discussed. We also describe how the concomitant analysis of these different levels of order together with distance parameters derived from other measurements can lead to a more precise model of the photosynthetic apparatus.

I. Introduction

Photosynthesis is the overall process by which the electromagnetic energy of light is converted into chemical free energy. The earliest steps of this complex conversion involve the absorption of a photon by the light-harvesting (antenna) pigments, the migration of the excitonic energy among other antenna pigments until it is trapped by the specialized reaction center (RC). A charge separation then occurs at the level of the RC and the transport of electrons and protons is initiated.

For all known photosynthetic organisms, these processes occur at the level of a membrane, which, like other biological membranes, is mainly built out of hydrophobic proteins partly or entirely embedded in a bilayer of lipids. In addition, it contains a variety of pigments and electron carriers that are mostly noncovalently attached to the proteins. In the course of evolution, the best adapted components have been selected to accomplish each elementary step: (bacterio)chlorophyll (Chl or BChl) and carotenoid (Car) molecules for the capture of light energy and its funneling towards the RC, cytochromes (Cyt), iron–sulfur (Fe-S) proteins, and quinones to carry charges and protons. The high efficiency of the conversion of solar energy by plants and certain bacteria is furthermore due to the precise organization of these components into the photosynthetic membranes. Actually, the photosynthetic membrane, with its system of closed vesicles limited by a thin bilayer of ordered lipids in which are anchored pebbles of Chl–protein complexes, bears some resemblance to both a liquid crystal and a solid-state device. In these integrated structures, fast reactions can take place without the rate-limiting step of the diffusion of the reactants usually encountered in solutions. The selective permeability of these membranes allows the stabilization of the separated charges, the compartmentation of the chemical species, and the storage of free energy under the form of electric potential or of protons gradient.

The notion of structure and organization appears at two different levels. In the pigment–protein complexes, the distances and relative orientations of the chromophores are intrinsic features that prevent wasteful quenching processes (light-harvesting (LH) complexes) and back reactions of the separated charges (RC complexes). In the highly anisotropic membrane itself, the localization of the various complexes, the distances between them, and their relative orientations are of prime importance for the transfer of energy, electrons, and protons.

A complete description and understanding of the process of photosynthesis necessitates not only the determination of the nature and the function of each individual component and the kinetics and thermodynamics of their reactions, but also the knowledge of their localization, positioning, and relative orientation. In general, a chemical approach in elucidating the architecture of biological membranes at the molecular level is rendered difficult because of the heterogeneity of the constituents and of the weakness of the chemical bonding (hydrophobic

interactions, hydrogen bonds, etc.) between them. Therefore, different physical techniques related to several forms of spectroscopy have been extensively used for the analysis of the structure of biological membranes. For example electron microscopy (Staehelin *et al.*, 1977), X-rays (Sadler *et al.*, 1973), and neutron scattering (D. Sadler and D. Worcester, personal communication) have been performed on photosynthetic membranes to obtain a gross view of the structure. The molecular organization is more difficult to analyze, although photosynthetic membranes offer a special case in the sense that a large number of components directly involved in the photosynthetic process, such as Chls, Cars, RC pigments, Cyts, and quinones, present characteristic electronic absorption spectra and/or ESR signals and can thus be used as intrinsic probes of the system. Absorption, fluorescence, flash spectroscopy, circular dichroism (CD), resonance Raman, as well as ESR techniques, have been widely used to determine the identity and concentration of the species present, their photochemical activity, and the interactions between them.

In Saclay, France, as well as in several other laboratories, different forms of spectroscopy involving plane-polarized light have been used on anisotropic samples to obtain information on the orientation of the chromophores in photosynthetic membranes and in systems derived from them. The purpose of this chapter is to critically discuss the structural information obtained by these techniques or by related approaches. Although a comprehensive review of the orientation of photosynthetic chromophores has not yet been published, several aspects of this subject have been partly covered in the following references: Hofrichter and Eaton (1976), Gregory (1977), Dutton *et al.*, (1979), Thornber and Barber (1979), Clayton (1980), and Hoff (1982).

In photosynthetic systems, different types of order can be outlined. The first type of order refers to the relative orientation between pairs of transitions belonging to distinct molecules in the same pigment–protein complex. The second type is related to the orientation of the transition moments of the various species (antenna pigments, RC chromophores, electron carriers, proteins) with respect to the membrane plane. Finally, a third degree of order is expected. It is called *local order* as it represents the mutual orientation of the chromophores in adjacent complexes (either of identical or different types). This last type of order represents a challenging area of research for which photosynthetic membranes, because of the unique property of extensive energy transfer among complexes, appear very promising. These three different types of order will be discussed in sequence in the following sections after the methodology underlying these determinations is described.

II. Methodology

A. General Approach

The determination of the orientation of some probes in a sample requires the use of a physical technique sensitive to the anisotropy of that probe and a sample that is oriented. The most straightforward technique is the X-ray crystallography. With photosynthetic material *in vivo* this has only been achieved so far for a BChl–protein complex (water soluble) isolated from *P. aestuarii* in which the BChl *a* molecules are very precisely positioned (2.8 Å resolution) with respect to the protein backbone (Matthews *et al.*, 1979). This technique however cannot be applied to the usual hydrophobic complexes, since they have not been crystallized. To circumvent this problem, the study of the orientation of different intrinsic probes has been performed on oriented particles or photosynthetic membranes by detecting their absorption, emission, or ESR anisotropic properties.

B. Techniques of Orientation

When the object to be investigated is optically resolvable, polarized spectroscopy (absorption or emission) can be performed on an immobile particle by using a microspectrophotometer. However, the lack of collimation in high resolution microscopes is an important source of potential errors (Hofrichter and Eaton, 1976). The objects must be oriented in a macroscopic array to overcome this problem or when they are too small to be seen in a microscope. This is achieved by using one of the following anisotropic properties of the object:

1. *Shape anisotropy.* A shear field can be applied to nonspherical (or deformable) particles. Flowing chloroplasts or bacteria in a narrow channel (Morita and Miyazaki, 1971; Breton *et al.*, 1973a) or in a special cylindrical cell (Sauer, 1965; Tjerneld *et al.*, 1977), and spreading intact chloroplasts, membranes, or particles with a small paint-brush (Breton and Roux, 1971; Penna *et al.*, 1975) will induce some orientation of the object. It is also possible to incorporate the particles in a plastic film (Rafferty and Clayton, 1978; Bolt and Sauer, 1979; Vermeglio *et al.*, 1980) and to stretch the film or to squeeze a polyacrylamide gel (Abdourakhmanov *et al.*, 1979). Another very straightforward and efficient way of orienting membranes is by air-drying a suspension on a flat surface (Morita and Miyazaki, 1971; Breton and Roux, 1971). Orientation of Chls (planar molecules) in liquid crystals has been reported (Journeaux and Viovy, 1978).

2. *Chemical anisotropy.* This has been used to orient isolated Chl either in collodion films (Breton *et al.*, 1972a), in lipids (Cherry *et al.*, 1972; Hoff, 1974), or in monolayers (Sperling and Ke, 1966; Breton *et al.*, 1972b).

3. *Magnetic anisotropy.* This elegant technique was first applied by Geacintov *et al.* (1972a) to chloroplasts and by Breton (1974) to photosynthetic bacteria. The membranes orient themselves so that their larger cross sections are perpendicular to the applied field direction. Details on the mechanisms and causes of the orientation of biological membranes in magnetic fields have been discussed (Hong *et al.*, 1971; Geacintov *et al.*, 1972b, 1974; Breton, 1974; Knox and Davidovich, 1978).

4. *Electric anisotropy.* This has been used on chloroplast fragments or chromatophores (Sauer and Calvin, 1962; Gagliano *et al.*, 1977) and also on isolated (bacterio)Chl–protein complexes (Whitten *et al.*, 1978; Gagliano *et al.*, 1979). Permanent and/or induced dipole moments are involved in this orientation process.

5. *Anisotropy of electronic absorption.* This interesting technique (photoselection) can be used on isolated molecules, complexes, or membranes. In this case the vectorial property of a polarized excitation beam is used to prepare an oriented sample. This technique will be described in more detail in a later section.

C. *Interaction of an Electromagnetic Wave with Electronic, Vibrational, or Spin Transitions in an Oriented Sample*

1. CASE OF PERFECT ORIENTATION

We will specifically examine the electronic absorption and then generalize for the other interactions. Electronic transitions usually correspond to changes in the distribution of the π-electron clouds in the molecules. For a fully allowed intense transition, this redistribution occurs in well-defined directions in the molecular framework along which the transition is said to be polarized. The geometric formula relating the anisotropic absorption of a molecule to the anisotropic absorption of a collection of these molecules fixed in crystals of different symmetry have been treated by Hofrichter and Eaton (1976). The usual case found for the orientation of the pigments *in vivo* is the case of uniaxial orientation. We will consider as an example an anisotropic particle containing one chromophore characterized by a single transition moment, which makes an angle θ with the long axis of the particle. We will further assume that all

the particles are mechanically aligned with their long axis parallel to the direction X of the mechanical force (Fig. 1). The absorption of the sample for light propagating perpendicular to X and polarized either parallel ($A_\parallel$) or perpendicular ($A_\perp$) to the direction X will be respectively:

$$A_\parallel = 3\,A\cos^2\theta; \qquad A_\perp = \frac{3}{2}A\sin^2\theta,$$

where A represents the absorption of the same ensemble of transition dipole moments randomly distributed in solution. The linear dichroism (LD), defined as $A_\parallel - A_\perp$, is then:

$$\mathrm{LD} = 3\,A\,\frac{3\cos^2\theta - 1}{2} \qquad (1)$$

If a single transition is involved, the LD spectrum will have the same shape as the absorption spectrum but its intensity and sign will be modulated by the function $S = (3\cos^2\theta - 1)/2$. The dependence of S as a function of θ is represented in Fig. 2. Note that for values of S close to either 1 or −0.5, the shape of the curve is such that a small imprecision in S will lead to a large uncertainty for θ; an opposite effect is observed when S is close to 0 ($\theta \sim 55°$). Other related expressions which can be found in the literature are the dichroic ratio $D = A_\parallel/A_\perp = \frac{1}{2}\cot^2\theta$ and the reduced dichroism LD/A. For other cases of orientation (flow or spreading, magnetic field, air-drying), formula analogous to Eq. (1) have been derived (Breton *et al.*, 1973a). All these calculations can be generalized to circularly degenerated transitions by considering the normal to the plane in which these transitions are located.

In the case of fluorescence, the electric vector of the emitted light has the same direction as the emission transition dipole moment of the molecule. It is thus possible to measure the orientation of an emitting dipole

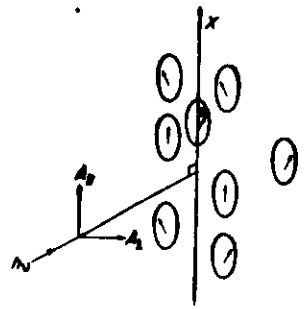

FIG. 1. Schematic representation of the measurement of LD ($A_\parallel - A_\perp$) on particles oriented along the axis X. Each particle contains one chromophore whose transition moment (represented by the arrow) makes an angle θ with the direction X.

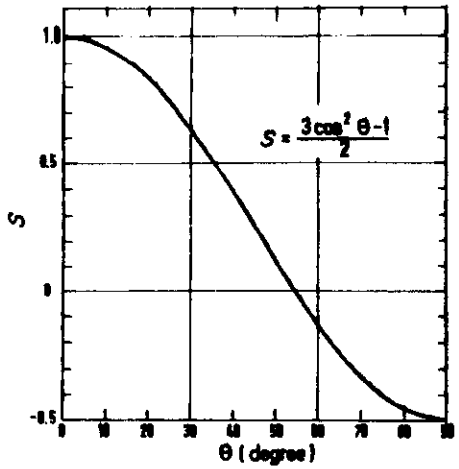

FIG. 2. Dependence of the quantity $S = (3\cos^2\theta - 1)/2 = (A_{\parallel} - A_{\perp})/3A$ on the angle θ between the direction of the transition moment and the orientation axis

by exciting with unpolarized light an oriented array of chromophores and monitoring the dichroism of the fluorescence (bifluorescence) with a polarizer. Under similar conditions the dichroism of some light-induced absorbance changes can be measured.

The interaction of light with an electronic transition dipole moment is only one form of a more general interaction of an electromagnetic wave with matter. When IR radiation interacts with molecules, the bonds between the atoms can undergo quantized transitions between several vibrational and rotational modes. If polarized IR light is used on oriented samples (IR dichroism), it is possible to monitor the orientation of some well-defined directions in the molecular framework.

Transitions between the different spin states of unpaired electrons can occur when microwaves are used to excite a sample. In this case, the magnetic vector of the radiation is involved in the interaction. For triplet states, it is possible to use optically detected magnetic resonance to monitor the transitions between the different spin sublevels. In ESR techniques, a permanent magnetic field is applied (usually parallel to the direction of propagation of the microwaves, although transverse modulation has also been used) in order to split the degenerated sublevels of the unpaired electron. When these anisotropic techniques are applied to an oriented sample, the orientation of paramagnetic probes rigidly bound to the system, and which possess g-tensor anisotropy can be determined.

2. DEGREE OF ORIENTATION

Most of the methods of orientation described earlier usually lead to an incomplete orientation of the sample. Even if a saturation is observed for the values of LD/A when increasing the orienting force, this behavior is not in itself a proof that a complete orientation is achieved, especially with heterogeneous systems. In such cases, it would be necessary to determine the final extent of orientation by another technique. X-rays and neutron scattering (Sadler *et al.*, 1973; D. Sadler and D. Worcester, private communication) are of a great potential use in this respect especially to determine the distribution of orientation of membranes (often called *mosaic spread*).

If the distribution of orientation of the objects is known, then one can calculate the exact θ angle by using Eq. (1). Otherwise uncorrected values of LD/A will lead to θ angles, which are always closer to 55° than the true values.

3. FLUCTUATIONS OF ORIENTATION

Owing to pigment–pigment as well as pigment–protein interactions, it is probable that the different chromophores in a well-defined pigment–protein complex are mutually oriented in a rather specific way (possibly including a complete disorder), and we will accordingly suppose that there is little variation in their relative orientation. In contrast, we do not know at the present time how unique is the orientation of the complexes themselves in the native membrane. This orientation can in principle fluctuate both in the time domain (especially if the membrane is fluid) and in space. Such fluctuations lead to the notion of a distribution of the orientation around an average position. It is worth mentioning that for a hydrophobic protein with polar region(s) protruding out of the bilayer, mostly lateral translation and/or rotation of the protein around the normal to the membrane plane can be thermodynamically expected (no "flip-flop" motion). However rocking motions could slightly change the orientation of the chromophores with respect to the membrane plane. For completely hydrophobic proteins, much larger fluctuations are possible.

When such fluctuations are present a measurement of the LD for a given chromophore will lead, after correction for the mosaic spread of the membranes, to a unique value of the order parameter $S = (3\cos^2\theta - 1)/2$. However this order parameter now includes the fluctuation of orientation, and the angle θ, which can be calculated, is only an apparent angle. This appears clearly in the limiting case where $S = 0$ (no LD); if

we deal with a single transition, two interpretations are possible: (1) the transitions are at random; (2) the transitions are perfectly oriented at 55° from the normal. For that reason the value of 55° is often referred to as the "magic" angle. It is only when the values of S are close to either 1 or −0.5 that the LD itself tells that the fluctuations are small.

In some cases the distribution of orientation of the probes can be estimated. ESR has been used for this purpose (Libertini *et al.*, 1969; Friesner *et al.*, 1979a) since paramagnetic species with anisotropic g tensor have resonance frequencies that are dependent upon the orientation of the principal axis with respect to the static magnetic field. With photosynthetic membranes, this information has also been obtained by optical techniques involving the Stark effect on the pigments (Paillotin and Breton, 1977).

4. PHOTOSELECTION

In this elegant technique outlined by Albrecht (1961), an excitation beam of linearly polarized light is used to create an anisotropy in a sample (isolated molecules, complexes, membranes). The sample is usually randomly oriented, but in some cases the objects are macroscopically oriented prior to the photoselection.

a. Photoselection on a Random Suspension of Chromophores. In this case the transition moments will be excited proportionally to the square of the cosine of their angle with respect to the direction of the electric vector of the polarized beam. Accordingly an anisotropy, corresponding to a known distribution of excited molecules, will be induced in the suspension. This anisotropy is probed by means of different physical properties: emission (the well-known polarization of fluorescence), absorption changes (photodichroism), or ESR spectroscopy (magnetophotoselection).

The photoselection techniques can give precise information on the relative angle between the transition implied in the absorption and the one involved in the detection, provided that several conditions are fulfilled. The rotational motion of the object must be much slower than the lifetime of the photoinduced species. (On the other hand, if the reverse condition is true, the rotational relaxation time can be followed.) Intermolecular energy transfer leading to a loss of the "memory" of the polarization of the excited species must be avoided. However this loss is of potential interest in studies of energy transfer and local order. Finally, excitation levels must be kept far below the saturation of the observed phenomenon.

A schematic representation of the usual right angle geometry used in photoselection experiments where fluorescence ($I_\parallel$, $I_\perp$) or absorbance changes ($\Delta A_\parallel$, $\Delta A_\perp$) are detected after excitation with vertically polarized light is given in Fig. 3a. If α is the angles between the absorbing dipole and the one involved in the emission (or the absorption change), the polarization value p is given by

$$p = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \quad \text{or} \quad \frac{\Delta A_\parallel - \Delta A_\perp}{\Delta A_\parallel + \Delta A_\perp} = \frac{3\cos^2\alpha - 1}{\cos^2\alpha + 3}$$

The analogous representation for magnetophotoselection is given in Fig. 3b. The ESR spectra have to be compared to calculated spectra in order to estimate the angle α_i between the excited optical transition **A** and each of the three principal magnetic axes of the detected paramagnetic species (D_i).

In photodichroism experiments, a single transition is usually detected and the absorbing dipole can be only positioned on a cone, making an angle α around the detected transition. This usually precludes any determination of the relative angle between two spectrally distinct transitions used for the excitation. However such information can be obtained by photodichroism experiments if two (or more) nonparallel transitions are used for the detection.

Owing to an ambiguity as to the sign of the projection of each optical transition onto the three principal magnetic axes, magnetophotoselection usually gives a set of four possible values for the angle between two

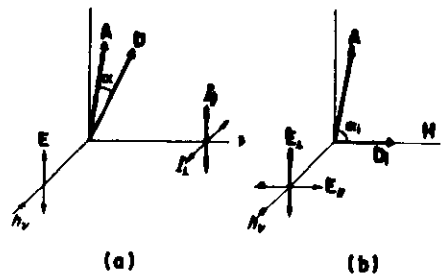

FIG. 3. Schematic representation of photoselection experiments: (a) Polarization of fluorescence and photodichroism: the electric vector of the polarized excitation light is represented by E. A and D represent, respectively, the transition moment of the sensitizing and detected chromophores; (b) Magnetophotoselection: the electric vectors of the polarized excitation light are represented by $E_\perp$ and $E_\parallel$ which are, respectively, perpendicular and parallel to the permanent magnetic field H. A represents the optical transition moment of the sensitizing chromophore and D_i the detected ESR transitions.

different transition moments used for the excitation (not including the complementary angles). Apart from this indetermination, the orientation of a given transition moment is fully determined with respect to the three magnetic axes. Consequently, it appears that the degrees of determination and of ambiguity are different in optical photoselection and in magnetophotoselection.

b. Photoselection of an Oriented Suspension of Objects. When the angle between the absorption and detected dipoles in the object is known, photoselection experiments can provide information on the distribution of the orientation of the pigments in the anisotropic system. This is due to the fact that the optical anisotropy is used twice, i.e., in the absorption and in the emission (or absorbance change) processes, and that there is a correlation between the two (Nishijima *et al.*, 1966). The distribution of orientation of the membranes of *Rp. viridis* in a magnetic field has been determined by a photodichroism experiment (Paillotin *et al.*, 1979).

D. *Spectroscopy of the Photosynthetic Components*

In order to relate the spectroscopic measurements described earlier to the orientation of the molecules *in vivo*, one must know the relationship between the studied transition (optical, vibrational, or magnetic) and the framework of the molecule. We will examine here some of the most interesting molecules in photosynthesis.

1. CYTOCHROMES

Because of the fourfold symmetry of the heme of these metalloporphyrins, the α, β, and γ transitions are predicted to be circularly degenerate in the heme plane (the *X* an *Y* axes being equivalent). However, imbalance along these two axes can arise from assymetric potential fields and electrostatic effects of the binding protein (Hofrichter and Eaton, 1976). Such an effect has been demonstrated by MCD, by low temperature absorption spectroscopy (Sutherland and Klein, 1972), and by LD studies on Cyt *c* oriented in a stretched polyvinyl alcohol film (Vermeglio *et al.*, 1980).

ESR studies of a crystal of oxidized Cyt *c* (Mailer and Taylor, 1972) has permitted to relate the three principal axes g_x, g_y, and g_z to the framework of the heme; g_x and g_y are in the plane (along the *N*–Fe–*N* directions), whereas g_z is normal to the heme.

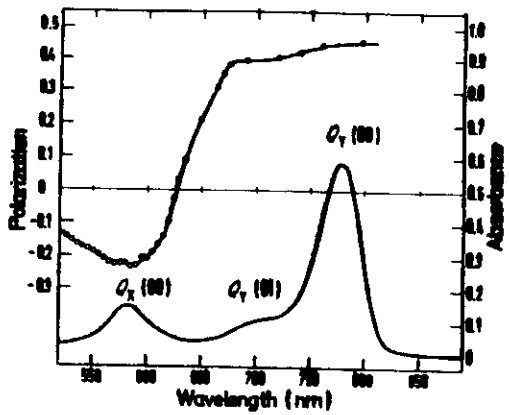

FIG. 4. Absorption (lower part) and fluorescence polarization (upper part) spectra of BChl *a* dissolved in cyclohexanol.

2. CHLOROPHYLLS

These molecules lack the high degree of symmetry of the porphyrins and their absorption bands are therefore associated with different directions in the molecular plane. The S_0 (ground) → S_1 (first excited state) lowest energy transition named Q_Y has been predicted to lie along the *Y* molecular axis, which joins the nitrogen atoms of pyrrol I and III (Gouterman, 1961). This is consistent with the high polarization of the fluorescence observed for this transition, in the case of BChl *a* (Fig. 4), Chl *a* (Fig. 5), and Chl *b* ($p = +.42$). The second $S_0 \rightarrow S_2$ electronic transition, named Q_X, is expected to occur in the visible spectral range. This transition is clearly seen in the case of BChl *a* (~ 580 nm) and of

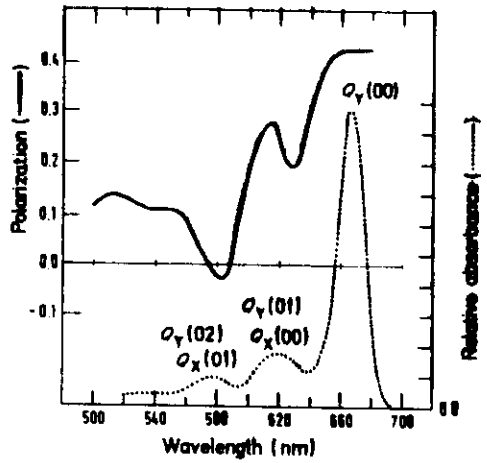

FIG. 5. Absorption (lower part) and fluorescence polarization (upper part) spectra of Chl *a* dissolved in castor oil. (Adapted with permission from Van Metter, 1977a.)

Bph *a* (~ 530 nm), and the *p* values of −.23 and −.21, respectively, indicate that these transitions are predominantely, although not entirely, directed along the *X* direction (perpendicular to *Y*). In the case of Chl *a* and *b*, the Q_X transition is very weak and not clearly resolved from vibrational components of the Q_Y transition. For Chl *a*, a possible decomposition is indicated in Fig. 5. This decomposition is also consistent with the LD results of Chl *a* in stretched plastic film (Breton *et al.*, 1972a). Furthermore, recent calculations (Petke *et al.*, 1979) seem to indicate that the direction of the $S_0 \rightarrow S_2$ transition of Chl *a* is closer to the *Y* direction than to the *X* direction. Accordingly, the interpretation of the data obtained by polarized light spectroscopy will be more conclusive for photosynthetic bacteria than for green plants. The Soret region of the Chls is composed of a rather complex set of numerous overlapping transitions with various polarizations; from different LD experiments (Breton *et al.*, 1972a; Breton, 1974; Journeaux and Viovy, 1978), the longest wavelength component appears as *X* polarized and then the polarization oscillates between *Y* and *X* toward the shorter wavelengths.

Thurnauer and Norris (1977) applied magnetophotoselection to the triplet state of various monomeric Chls. The magnetic transition with the largest splitting has been assigned to the *z* axis, normal to the molecule plane. They have also shown that for all the Chls the triplet *y* axis is parallel or close (< 35°) to the Q_Y transition moment.

3. CAROTENOIDS

These molecules present absorption bands in the spectral range 400–500 nm. When they are in all trans configuration, these linear structures containing a large number of π electrons present electronic transitions, which are polarized along the molecular axis (Salem, 1966).

4. PROTEINS

In the UV, the aromatic amino acid residues present transitions polarized in the plane of the cycle. In the IR, the α-helices display characteristic vibrational modes polarized parallel (amide A, 3300 cm^{-1} and amide 1, 1650 cm^{-1}) or perpendicular (amide 2, 1550 cm^{-1}) to the axis of the helix (Tsuboi, 1962).

III. Relative Orientation of Pigments within Isolated Photosynthetic Complexes

In vivo, the Chl molecules are generally associated with hydrophobic proteins, and the extraction and purification of these complexes (antenna and RC) require the use of detergents. These steps have been easier to achieve in photosynthetic bacteria than with higher plants or algae because of a greater (but unexplained) resistance to the denaturing effect of the detergents. Light-harvesting and RC complexes stable and apparently intact, as judged from several spectral characteristics or functional activities, have been purified from different photosynthetic bacteria species and chemically characterized. From a variety of green plants, two well-defined complexes have been obtained: a P700 Chl *a* complex (CPI) and a LH Chl *a,b* complex (CPII), whose true significance has been recently questioned (Thornber *et al.*, 1979). The analysis of various optical properties can give information on the structure of the different pigment–protein complexes, but it must be emphasized that the relevance of the proposed structures depends upon the retention of their *in vivo* characteristics.

A. Isolated Reaction Centers

1. PHOTOSYNTHETIC BACTERIA

All the RCs isolated from a variety of species contain four BChl molecules, two of them constituting the dimeric primary donor or special pair (SP), two Bph and one quinone (see Fig. 6 for their attributions in the absorption spectrum). (For details on RCs, see Okamura *et al.*, Chapter 5, this volume.) In some cases, one Car and/or several Cyts are also present. Both LD on oriented RC and photoselection studies have been performed to investigate the relative orientation of these various chromophores. There is good agreement between all the experimental results, and the controversy between the proposed models of RC structure arises mainly from the divergent attributions and interpretations of the RC spectral components and light-induced absorbance changes. In this section, we will summarize the LD results and emphasize those obtained by the more straightforward technique of photoselection, before presenting the controversial models of the structure of RC.

RCs from *Rp. sphaeroides* (R-26) have been oriented by a brush-spreading technique (Penna *et al.*, 1975), by stretching gelatin films (Rafferty and Clayton, 1978, 1979a), by squeezing polyacrylamide gels (Abdourakhmanov *et al.*, 1979), and by 8 kV, 3 msec pulsed electric field (A. Gagliano, A. Vermeglio, and J. Breton, unpublished results). Although the degree of orientation depends much on the technique (it is 100 times greater for stretched films than for the brush-spreading technique), the LD spectra present similar features. Figure 6 shows the polarized spectra of RC oriented in stretched films; large orientation ef-

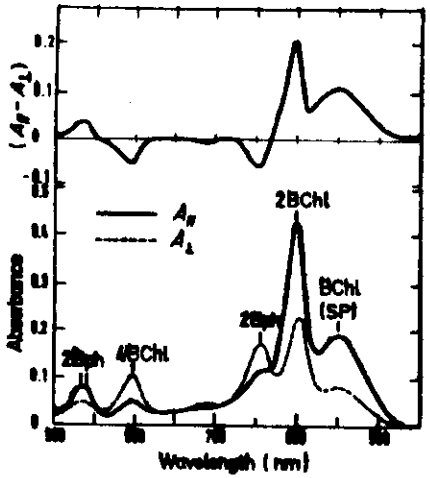

FIG. 6. Polarized absorption ($A_\parallel$ and $A_\perp$) (lower part) and LD ($A_\parallel - A_\perp$) (upper part) spectra of RC from *Rp. sphaeroides* R-26 in stretched gelatin film. For the $A_\parallel$ absorption spectrum measurement, the electric vector of the polarized measuring beam was parallel to both the film plane and the stretching direction. For the $A_\perp$ measurement, the electric vector was parallel to the film plane and perpendicular to the stretching direction. (Adapted with permission from Rafferty and Clayton, 1979a.) For a "genetic" schematic of a RC, see Fig. 2 in Kaplan and Arntzen, Chapter 3, this volume.

fects can be seen for all the transition moments. However, these different orientation techniques generally cannot reveal the mutual orientation between the chromophores, but rather orientation with respect to an unknown direction in the detergent–protein complex. Moreover, the degree of orientation is not known although some decomposition into ideally oriented and unoriented fractions can be made (Rafferty and Clayton, 1979a).

More conclusive results have been obtained by photoselection experiments: Polarization excitation spectra have been measured for both the fluorescence emitted from the 870 nm transition (Ebrey and Clayton, 1969) and the light-induced absorbance change related to this transition (Mar and Gingras, 1976; Shuvalov *et al.*, 1977; Vermeglio *et al.*, 1978) as shown in Fig. 7. A constant polarization value close to the theoretical limit was observed when exciting within the 870 nm band and detecting either the fluorescence ($p = +.5$) (Ebrey and Clayton, 1969) or the absorbance change occurring at 900 nm ($p = +.45, \pm .02$) (Vermeglio *et al.*, 1978; Rafferty and Clayton, 1979b). Previous reports of lower values of p ($p = +.22, +.25$) by Mar and Gingras (1976) and Shuvalov *et al.* (1977) have been shown by Vermeglio *et al.* (1978) to be due to an artifact arising from the saturation of a small fraction of RC with slow back-reactions, and the proper conditions for photoselection were thus strongly altered. After correction of the data of Shuvalov *et al.* (1977) for this artifact, there is good agreement between the polarization excitation spectrum reported by this group and the one by Vermeglio *et al.* (1978).

Several conclusions can be drawn from the spectra shown in Fig. 7. The constant p value ($p = +.45$) observed for the 870-nm band demonstrates that this transition of the SP is due to either a single transition or to two parallel transitions and therefore constitutes a well-defined direction in the RC complex. A determination of the angle between this direction and a single pure transition is possible only (for *Rp. sphaeroides*, 2:4:1) upon excitation in three spectral regions: (a) 450–500 nm where the Car absorbs; a p value of $-.24$ is obtained, leading to an angle of 75°. However, this value cannot be interpreted unambiguously as a (di)cis conformation has been demonstrated for the RC Car (Lutz *et al.*, 1978). (b) and (c) at ~ 530 and 546 nm where the Q_X of the two Bph molecules can be resolved at low temperature (Clayton and Yamamoto, 1976); these give p values of $+.05$ ($\alpha_{530-870} = 50°$) and -0.06 ($\alpha_{546-870} = 60°$), respectively (Vermeglio *et al.*, 1978). In other spectral regions, where several absorption bands overlap, the p values are only related to the average angle between the different transitions and the 870-nm direction. The average angle between the Q_Y transitions of the BChl molecules absorbing at 800-nm and the 870-nm transition is ~ 30°, whereas values of 65–70° can be calculated for the average angle of either the 4BChl Q_X transitions (around 600 nm) or the Q_Y transitions of the two Bph molecules with the 870-nm transition. The preceding angular relationships are direct deductions from simple photoselection experiments and fully compatible with the LD results on mechanically oriented RC (Clayton *et al.*, 1979). Therefore, we believe that all models of the organi-

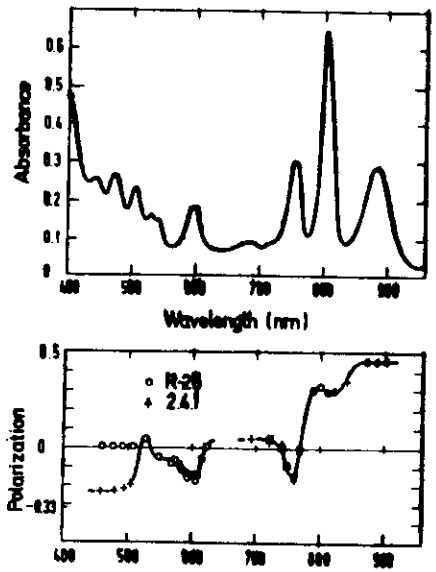

FIG. 7. (Upper part) Absorption spectrum of RC isolated from *Rp. sphaeroides* 2:4:1. (Lower part) Excitation polarization spectrum of the absorbance changes observed at 870 nm for RC from *Rp. sphaeroides* 2:4:1 (+) and R-26 (○). Both spectra were measured at 150° K. (From Vermeglio *et al.*, 1978.)

zation of the chromophores in RC should take into account these experimental results.

Additional information may be obtained upon excitation within a pure absorption band and observation of light-induced absorbance changes at different wavelengths. This is the case, for example, upon excitation within the two spectrally resolved Q_X transitions of the Bph molecules and observation of the shift of their Q_Y transition. From such an experiment, Vermeglio *et al.* (1978) were able to propose a model for the arrangement of the four transitions of the two Bph molecules relative to the 870-nm transition (Fig. 8). Excitation within the 870-nm band and detection of the absorbance changes of P^+ ($P870^+$) in the visible and near IR region has been reported by Shuvalov *et al.* (1977) and Vermeglio *et al.* (1978) and are shown in Fig. 9. After correction for the earlier artifact in the measurements of Shuvalov *et al.* (1977), the experimental results of both groups are in good agreement. However their interpretations of the spectra differed markedly. Shuvalov *et al.* (1977) proposed that the changes occurring around 800 nm upon photo-oxidation of the SP are linked to absorption band shifts of the two BChl molecules absorbing at this wavelength. These two BChl molecules are supposed to be in excitonic interaction because of the similar bandwidth of their respective contribution to the CD spectrum (Reed and Ke, 1973). In an alternative interpretation, Vermeglio *et al.* (1978) decomposed the absorbance changes occurring around 800 nm into an absorption band shift and the bleaching of a small band peaking at 805 nm, perpendicular to the 870-nm direction. This was taken to support the hypothesis of Vermeglio and Clayton (1976) that the 805 and 870-nm bands arise from excitonic coupling of the two BChl molecules of the SP. Both interpretations can explain the ΔCD (light-minus-dark) spectrum and the polarized absorbance changes depicted in Fig. 9.

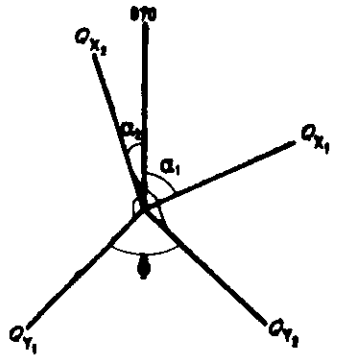

FIG 8. Schematic representation of the arrangement of the transition moments (870 nm, Bph 546 Q_{X1}, Q_{Y1} and Bph 530 Q_{X2}, Q_{Y2} within a RC. α_1 = 60°, α_2 = 30°, and Φ = 55° or 125°. [From Vermeglio *et al.* (1978).]

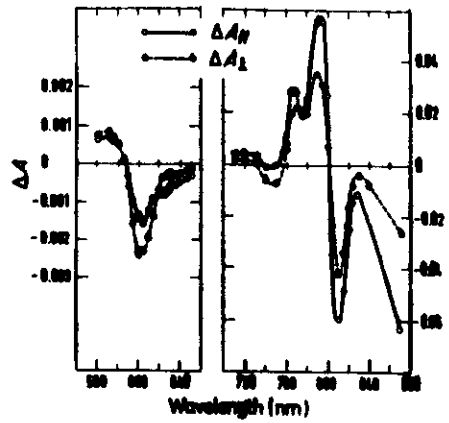

FIG. 9. Difference spectra induced by 900 nm vertically polarized light excitation for both parallel ($\Delta A_{\parallel}$) and perpendicular ($\Delta A_{\perp}$) polarization of the analyzing beam for RC isolated from *Rp. sphaeroides* R-26. [Visible part adapted with permission from Rafferty and Clayton (1979b); infrared part from Vermeglio *et al.* (1978).]

The interpretation of the polarized light-induced changes of the SP is less ambiguous in the Q_X region. Rafferty and Clayton (1979b) have detailed photoselection experiments in that region (Fig. 9) after their discovery of a weak band peaking at 630 nm in the polarized ΔA spectrum related to P^+ of RC in stretched gelatin films (Rafferty and Clayton, 1979a). They attributed the absorption bands peaking at 600 and 630 nm to the two excitonic components of the interacting Q_X transitions of the SP. Their photoselection experiments confirmed this hypothesis since they found an angle of 90° ± 15° between the 600- and 630-nm transitions as predicted by the exciton theory. Further support of that interpretation can be seen in the ΔCD spectrum of oxidized minus reduced RC, which presents a S-shaped signal with maximum and minimum at 600 and 630 nm, respectively (Reed and Ke, 1973).

Frank *et al.* (1979b) and Boxer and Roelofs (1979) performed magnetophotoselection experiments on isolated RC from the mutant *R-26* of *Rp. sphaeroides*. They both agree with the earlier conclusions of Thurnauer and Norris (1976) that the 870-nm transition lies predominantly along one of the principal magnetic axes of the SP triplet state. However some differences, probably reflecting the divergent methods of data interpretation, can be seen in the papers of Frank *et al.* (1979b) and of Boxer and Roelofs (1979). Nevertheless, the data of Frank *et al.* (1979b) are in agreement with the 60° angle found by Vermeglio *et al.* (1978) for the angle between the 550-nm transition (Q_X of Bph) and the 870-nm transition of the SP. The data of Boxer and Roelofs (1979) are in agreement with the observation of Rafferty and Clayton (1979b) of the composite nature of the 600–650 nm absorbing region.

The different interpretations of the light-induced absorption changes

occurring in the 800-nm region proposed by Shuvalov *et al.* (1977) and Vermeglio *et al.* (1978) lead to completely different pictures of the arrangement of the four BChl molecules in the RC. In Shuvalov's model, the two Q_Y transitions of the BChl molecules of the SP are parallel because their excitonic interaction gives rise to only one allowed transition moment (870 nm). The two transition moments absorbing at 800 nm of the other BChls are nearly parallel (790 nm) and nearly perpendicular (810 nm) to the 870-nm transition. In the alternate model of Vermeglio *et al.* (1978) and Rafferty and Clayton (1979a, b), the SP presents two distinct excitonic components (870 and 805 nm). From the relative values of the oscillator strength of the excitonic bands, the angle between the transitions of the two monomeric BChl in the SP can be estimated. This angle is ~ 25° between the Q_Y transitions, whereas it is ~ 40° for the Q_X transitions.

At present, it is difficult to make a definite choice between the two proposed models because of the consistency of both interpretations of the polarized ΔA spectra. In that context, photoselection studies on RC isolated from the BChl *b* containing species *Rp. viridis*, which presents a better spectral resolution in the 830-nm band (analogous to the 800-nm band of BChl *a* containing RC), could provide a more clear-cut interpretation of the absorbance changes related to state P^+. Such photoselection experiments have been reported by Shuvalov and Asadov (1979). The values of $(\Delta A_\parallel - \Delta A_\perp)/(\Delta A_\parallel + \Delta A_\perp)$ for the absorbance changes of P^+ upon excitation at 980 nm are plotted in Fig. 10 as a function of the analyzing wavelength. The low polarization value (p = +.25) obtained when exciting and observing within the 980-nm band was assumed by these authors to be due to depolarization by the artifact described earlier. We have repeated similar photoselection experiments under short illumination time conditions and indeed found a higher p value of +.45 (A. Vermeglio, G. Paillotin, and J. Breton, unpublished results) consistent with previous results on BChl *a* containing species (Vermeglio *et al.*, 1978; Rafferty and Clayton, 1979b). Our p values at other wavelengths are in good agreement with the ones reported in Fig. 10 after corrections.

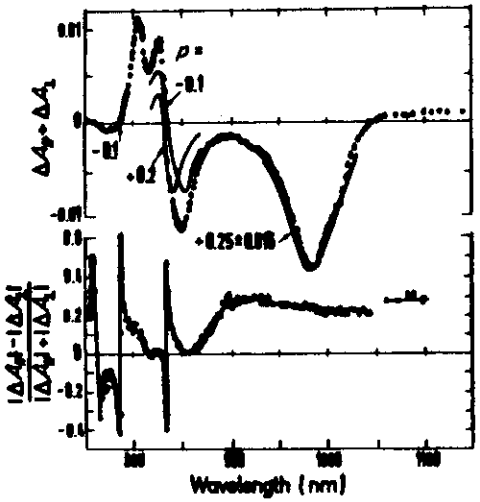

FIG. 10. Photoselection experiments performed on RC from *Rp. viridis* at 100°K. (Upper part) Spectrum of absorbance changes ($\Delta A_\parallel + \Delta A_\perp$) induced by exciting light at 980 nm, which was polarized either parallel ($\Delta A_\parallel$) or perpendicular ($\Delta A_\perp$) to the measuring light. The solid curves indicate the resolved components of the difference absorption spectrum with their attributed p values. (Lower part) Spectrum of p = $|\Delta A_\parallel| - |\Delta A_\perp| / |\Delta A_\parallel| + |\Delta A_\perp|$. (Adapted with permission from Shuvalov and Asadov, 1979.)

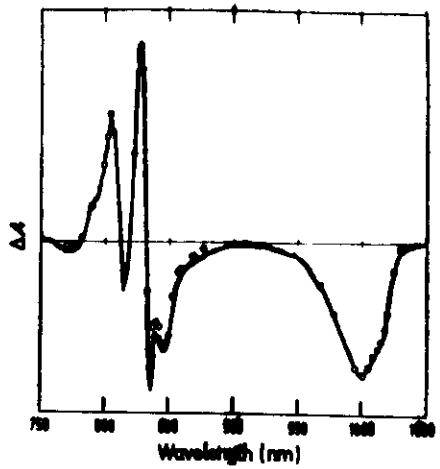

FIG. 11. Difference absorption spectrum measured at 4.2° K for *Rp. viridis* RC recorded with a Cary 17 D spectrophotometer in the dark and during continuous illumination. The dots correspond to laser-induced absorbance changes measured point-by-point.

The decomposition of the light-induced absorbance changes proposed by Shuvalov and Asadov (1979) is depicted in Fig. 10. The light-induced changes in the 820–880-nm region were attributed to two absorption band shifts in order to explain the sharp changes of the p values in the line of the decomposition proposed by the same authors (Shuvalov *et al.*, 1977) for *Rs. rubrum* species. One does, however, notice that the absorption band shifts are not symmetrical (see Fig. 10). From our photoselection study of *Rp. viridis* RC at 150°K, LD studies of oriented cells (see following section) and very low temperature (4°K) difference spectrum of isolated RC and chromatophores (Fig. 11), we conclude that the light-induced changes of P^+ are best fitted in the 820–880-nm region by the bleaching of a band centered at 850 nm with a negative polarization value and by two symmetrical absorption band shifts around 830 nm. The bleaching at 970 and 850 nm, clearly seen at 4°K for RC from *Rp. viridis* in the state P^+ (Fig. 11), is interpreted as the disappearance of the two excitonic components of the SP.

2. GREEN PLANTS

Up to now highly purified RC has not been isolated from green plants. Particles enriched in P700 are however available. They contain ~ 40 antenna Chl *a* and several Car molecules per P700. The spectroscopic work on these particles is far more complex than for RC isolated from photosynthetic bacteria for the obvious reason that the absorption of the antenna overwhelms the absorption of the RC.

Nevertheless some interesting information on the mutual arrangement of the pigments in the PSI particles of Bengis and Nelson (1975), which contain ~ 40 Chl *a* and ~ 13 Car per P700 has been gathered by Junge and his co-workers (Junge *et al.*, 1977; Junge and Schaffernicht, 1978, 1979) using a photoselection technique. By monitoring the polarization of the absorbance changes at 700 nm upon polarized light excitation in all the absorption bands (including the 700-nm band of the SP) they observed positive dichroic ratios that never exceed 4:3. This observation led them to two alternative possibilities for the structure of the SP: (1) If the photo-oxidized dimer is the only species absorbing at 700 nm, the Q_Y transition moments of the two Chls in the SP are mutually perpendicular; (2) If there are antennae absorbing at this wavelength in addition to the dimer, then the *Y* axis of the antennae is almost perpendicular to the former (Junge *et al.*, 1977). Furthermore, it has been observed that the average directions of both the Car transition moments and the Q_Y transitions of the antenna Chls absorbing above 690 nm lie in the same plane as the 700-nm transition of the SP.

B. *Isolated Antenna Complexes*

1. PHOTOSYNTHETIC BACTERIA

Thornber *et al.* (1978) proposed that the LH complexes of purple bacteria can be divided in two biochemically distinct categories: a B890 and a B800–B850 complex. (For further details, see Kaplan and Arntzen, Chapter 3, this volume.) The absorption maximum of the B890 complex is located between 850 and 890 nm depending upon the bacterial species. It contains two BChl *a* molecules in excitonic interaction, as shown by the characteristic CD signals in the Q_X and Q_Y absorption regions and one Car (which is missing in the case of the blue–green mutants). The B800–B850 complex contains two BChl *a* analogous to the ones of a B890 complex but absorbing around 850 nm (and in some cases at ~ 820 nm). A third one absorbs maximally at 800 nm and is apparently not in excitonic interaction with the other two. The molecular weight of the basic unit for both complexes is of the order of 20 kD but oligomeric forms have also been observed.

The LD of the B890 and B800–B850 complexes isolated from *Rp. sphaeroides* and oriented in stretched polyvinyl-alcohol films has been investigated by Bolt and Sauer (1979). The dichroic ratio of the B890 complex isolated from the *R-26* mutant varies across the Q_Y absorption region and presents two plateaus (D = 1.30 at 835 nm and D = 1.57 at 885 nm) on each side of the absorption maximum (853 nm). The midpoint of the dichroic ratio versus wavelength curve is located at ~ 860 nm. A similar behavior is observed for the B800–B850 complex (Fig. 12). In the Q_X region the dichroic ratio is constant (D = 0.43) for the B890 complex, whereas it varies for the B800–B850 complex (D = 0.38 at 610 nm and D = 0.84 at 575 nm). In addition, the dichroic ratio is 0.78 for the Car in the B800–B850 complex. Using a curve-fitting procedure on these polarized spectra, the two excitonic components in the Q_Y region of the B890 complex were located at ~ 852 and 867 nm with a ratio of their areas leading to an angle of 78° between the Q_Y transitions of the monomers. For the B800–B850 complex the Q_X region could also be analyzed in terms of a band (λ_{max} = 593 nm) representing the two exciton coupled transitions and a band (λ_{max} = 584 nm) corresponding to the isolated transition of the monomer-like BChl *a* absorbing at 800 nm (Bolt and Sauer, 1979). The angles between these transitions and the stretching axis were also calculated, but the rather narrow range of these angles (48°–68°) around the "magic" angle of 55° casts some doubts on their real significance. In the Q_X region very similar results have been obtained by using electric orientation of the complexes (A. Gagliano, A. Vermeglio, and J. Breton, unpublished results), although the dichroic ratios were smaller.

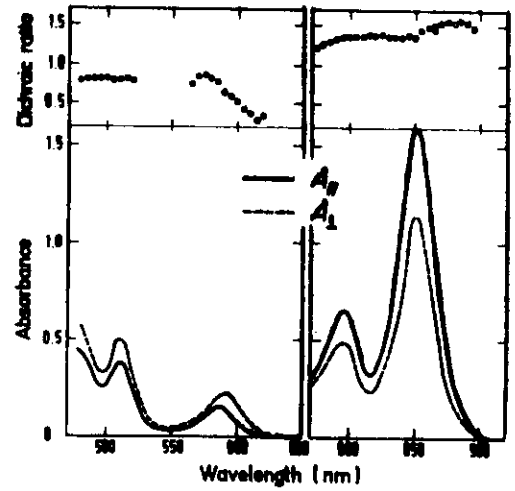

FIG. 12. Polarized absorption spectra measured parallel (—) and perpendicular (- - - -) to the stretch axis for light-harvesting complexes isolated from *Rp. sphaeroides* 2.4.1 embedded in polyvinyl alcohol. The stretching ratio was equal to 2.9. Open circles represent the dichroic ratios ($A_{\parallel}/A_{\perp}$) calculated from the polarized absorption spectra. (Adapted with permission from Bolt and Sauer, 1979.)

The polarization of the fluorescence of these complexes has been investigated by Breton *et al.* (1981). Upon excitation at several wavelengths within the longest wavelength band, a polarization of $+.13 \pm .01$ is observed throughout the fluorescence band. This result can be rationalized by assuming that the two (orthogonal) excitonic components have an equal probability to fluoresce, which causes them to behave like a circularly degenerated oscillator ($p = +.14$). Upon excitation at 800 nm the B800–B850 complex gives $p = +.13 \pm .01$ showing that the 800-nm transition is parallel to the plane of circular degeneracy of the long wavelength transitions. The fluorescence polarization (excitation) spectra of the two complexes are shown in the Q_X region (Fig. 13). With the B890 complex, a rather constant polarization ($p = -.17$), close to the limit of $-.23$ observed for isolated BChl *a*, shows that the dominant exciton component is almost perpendicular ($\theta \sim 70°$) to the plane of circular degeneracy, implying an almost parallel orientation of the Q_X transitions of the monomers. For the B800–B850 complex a similar behavior is found on the long wavelength side of the Q_X region, whereas the polarization rises significantly ($p = -.04$) on the short wavelength side of this band. A decomposition of this region into a band representing the two coupled molecules and a band (of one-half amplitude) corresponding to the third BChl *a* indicates that the Q_X direction of this latter molecule is tilted at less than 20° out of the plane of circular degeneracy. Upon excitation in the Car absorption bands of the B800–B850 complex isolated from *Rp. sphaeroides* (either 2:4:1 or G1C) p values of $-.09$ were obtained, indicating that the Car molecule is tilted at $\sim 45°$ out of the plane of circular degeneracy.

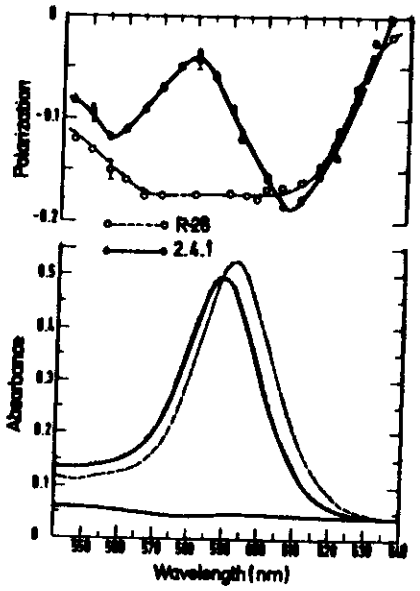

FIG. 13. Absorption (lower part) and fluorescence excitation (upper part) spectra in the Q_X region of light-harvesting complexes isolated from *Rp. sphaeroides* 2:4:1 (——) and R-26 (- - - -) suspended in 2 *M* sucrose, 0.1% LDAO.

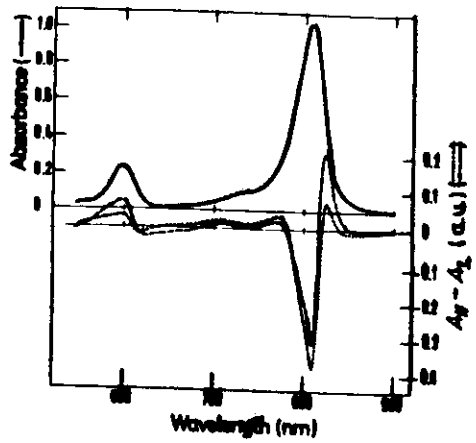

FIG. 14. Absorption (—) and LD of the soluble BChl *a*–protein complex isolated from *P. aestuarii* and oriented with low (- - -) and high (····) extent of hydration of the films prior to the stretching. The ∥ and ⊥ subscripts refer to the direction of the stretched axis. Spectra taken at 300°K.

The LD of crystals of the BChl *a*–protein complex from *P. aestuarii* has been investigated (Olson *et al.*, 1969; Olson, 1970). The 809-nm transition was found to be oriented at less than 55° of the crystal axis, whereas an opposite orientation was observed at 603 nm. Electric dichroism performed on solutions of this complex (Whitten *et al.*, 1978) has revealed that only one part of the long wavelength absorption band, namely the 813-nm component, exhibits a dichroism. Figure 14 shows two LD spectra obtained with stretched polyvinyl-alcohol films of this complex (J. Breton, unpublished results, 1979). Slightly different orientations were achieved depending upon the extent of hydration of the films prior to the stretching. Several spectral components could be discerned in both the Q_X and the Q_Y regions. Lowering the temperature to 100°K confirms this observation but does not resolve additional obvious components. The problem of relating the spectral components to each of the seven molecules of the monomer unit, as seen by X-ray crystallography, is fairly complex especially in view of the excitonic coupling between the BChl *a* molecules.

2. GREEN PLANTS

Van Metter (1977b) reported detailed optical measurements [absorption, CD, fluorescence polarization (FP)] on isolated CPII complexes. The CD spectrum is interpreted in terms of an excitonic interaction for

the three Chl *b* but not for the three Chl *a* molecules. The small value (p = +.02) observed for the FP upon excitation at 650 nm is indicative of a nearly spherical symmetry for the Chl *b* exciton states. This suggests a C_3 symmetry for the Chl *b* monomers with an angle of 125° between the symmetry axis and each transition of the exciton states, whereas the angle between the projection of these transitions on the plane of the trimer and the vector from the center of gravity to each monomer is between 0° and 60°. Several possible angles (always > 50°) between the transition moments of the Chl *a* can be calculated from the FP data, and a model is proposed in which the three Chl *a* are arranged at the periphery of a core of three *b* (Knox and Van Metter, 1979).

The CP1 complex exhibits a rather high FP (p = +.14 for 640-nm excitation) as shown by Vacek *et al.* (1977). Upon electric field orientation a LD signal (λ max 686 nm), which is narrower than the Q_Y absorption band (λ max 677 nm) has been observed (Gagliano *et al.*, 1979). From photoselection experiments (Junge *et al.*, 1977; Junge and Schaffernicht, 1978), it has been concluded that the ~ 13 Car molecules are within less than 30° from each other and that their average direction is approximately parallel to the one of the Q_Y transitions of the long wavelength forms of Chl.

IV. Orientation of Pigments with Respect to the Membrane Plane

A. Antenna Pigments

1. GREEN PLANTS

Studies of the dichroism of Chl *in vivo* have been conducted in the past by microspectrophotometry on large chloroplasts (Menke, 1943; Frey-Wyssling and Wuhrmann, 1947; Goedheer, 1955; Ruch, 1957; Butler *et al.*, 1964; Olson *et al.*, 1964). Whenever a dichroism was observed, it was interpreted as arising either from an "artifact" (form dichroism) or from a very small degree of orientation of some long wavelength form of Chl *a*. Similar conclusions were also drawn for mechanically oriented spinach chloroplasts (or chloroplast fragments) by Sauer and Calvin (1962), Sauer (1965), and Thomas *et al.* (1967). A critical review of these early works can be found in Breton (1977b).

The first reports of a significant orientation of Chl *in vivo* came in the early 1970s. Breton and Roux (1971) working with spinach chloroplasts oriented by air-drying or by spreading described their LD spectra in

terms of the orientation of the *X* and *Y* directions of several forms of Chl and of the Car molecules. Geacintov *et al.* (1972a), using magnetically oriented *Chlorella*, observed an orientation of the Q_Y transitions of Chl *a*. Since these early reports a large number of articles dealing with either the LD (Geacintov *et al.*, 1972b, 1974; Becker *et al.*, 1973; Breton *et al.*, 1973a; Faludi-Daniel and Breton, 1975; Demeter *et al.*, 1976; Vermeglio *et al.*, 1976; Gagliano *et al.*, 1977; Tjerneld *et al.*, 1977; Biggins and Svejkovsky, 1978) or the polarized fluorescence (Geacintov *et al.*, 1972a, b, 1974; Breton *et al.*, 1973b; Breton, 1975; Becker *et al.*, 1976; Garab and Breton, 1976) of oriented chloroplasts have appeared. As an example, Fig. 15 shows the polarized absorption spectra at low temperature for magnetically oriented spinach chloroplasts. Rather than analyzing each of these articles in detail we will summarize here what the present state of knowledge is on the orientation of the antenna pigments in the membrane of green plants.

1. The Chl *a* molecules absorbing between 680 and 730 nm have their Q_Y transition moments rather close to the membrane plane ($\theta > 65°$).
2. The Chl *a* molecules absorbing at shorter wavelengths (660–680 nm) have their Q_Y transition moments either making an angle θ close to 55° with the membrane normal or almost at random.
3. The *X*-polarized transitions of at least part of the Chl *a* molecules are oriented out of the membrane plane ($30° < \theta < 50°$).
4. The Chl *b* molecules are oriented in such a way that their *Y* directions are out of the membrane plane ($\theta < 55°$), whereas their *X* directions are closer to this plane ($\theta > 55°$).

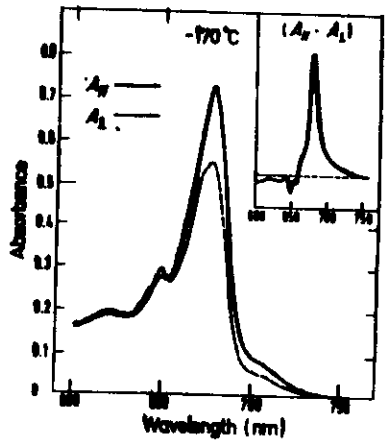

FIG. 15. Absorption spectra of a suspension of oriented chloroplasts measured at −170°C. The measuring beam was polarized either parallel or perpendicular to the chloroplast's membrane plane. The insert represents the LD spectrum ($A_\parallel - A_\perp$).

5. The transition moments of at least a fraction of the Car molecules are oriented close to the membrane plane ($\theta > 55°$).
6. The emission spectrum of *in vivo* Chl *a* is heterogeneous. The long wavelength emission originates from the pigments oriented the closest to the membrane plane. At least five different emitting species have been detected at low temperature by their different extent of orientation (Garab and Breton, 1976).

Apart from these basic observations one report indicates that divalent cations induce a reorientation of a form of Chl *a* absorbing around 690 nm (Biggins and Svejkovsky, 1978). However the possibility that the effect observed on the LD spectrum is due to an increase in light scattering is quite probable.

The dichroism of the transient absorbance changes linked to the Stark effect occurring on the antenna pigments when a short flash of light is used to induce a separation of charges across the photosynthetic membrane has been investigated on magnetically oriented spinach chloroplasts (Breton and Mathis, 1974; Breton and Paillotin, 1977). By considering both the absorption and the absorption changes spectra measured in polarized light and with the additional knowledge of the direction of the induced electric field (parallel to the membrane normal), Paillotin and Breton (1977) came to the conclusion that the observed effects were indicative of small fluctuations ($< \pm 5°$) in the orientation of the Chl pigments with respect to the normal. For example, this indicates that the Q_Y transitions of the Chl *a* molecules absorbing in the range 660–680 nm are oriented at about 55° from the normal rather than at random.

2. PHOTOSYNTHETIC BACTERIA

The antenna pigments of photosynthetic bacteria have been found to be specifically oriented in all the species which have been investigated by LD. Orientation has been achieved either with chromatophores by air-drying (Morita and Miyazaki, 1971, 1978; Breton, 1974; Vermeglio and Clayton, 1976), by electric fields (Gagliano *et al.*, 1977), or with intact cells by using flow (Morita and Miyazaki, 1971) or magnetic fields (Breton, 1974; Clement-Metral, 1975; Paillotin *et al.*, 1979). In all cases, the Q_Y transitions of the BChl *a* (or BChl *b* in the case of *Rp. viridis*) have been found to lie close to the membrane plane ($\theta > 65°–75°$), whereas their Q_X transitions are, on the average, almost perpendicular to this plane ($\theta < 30°$). This last observation is reinforced when one considers that the band at ~ 590 nm for isolated BChl *a* is not a pure *X* transition ($p = -.22$). Furthermore, an orientation out of the membrane plane ($\theta \sim$ 40°–45°) has been detected for the axis of the Car molecules. For those bacteria that contain B800–B850 antenna in addition to the B890 form, all the Q_Y transitions make approximately the same angle with the membrane plane. However, Vermeglio *et al.* (1979) observed that the LD/*A* in the Q_X absorbing region (~ 590 nm) was smaller for these bacteria than for the ones lacking the B800–B850 form and concluded that, in contrast to the other BChl *a* molecules, the *X* transitions of the BChl *a* molecules which absorb at 800 nm were oriented close the membrane plane ($\theta > 60°$).

B. Reaction Centers

1. GREEN PLANTS

Junge and Eckhof (1973, 1974) used a photoselection technique on randomly oriented chloroplasts to analyze the orientation of P700. By excitation with polarized light of wavelengths > 680 nm (for which the Q_Y transitions of the antenna Chl *a* are close to the membrane plane), an anisotropic set of excited membranes can be created. A dichroic ratio of 1.15 was observed at both 705 and 430 nm. The quantitative interpretation of these data necessitates additional information on the arrangement of the antenna Chl *a* around the P700. Assuming a circular degeneracy, which is substantiated by several experiments (Junge, 1975; Vacek *et al.*, 1977), it can be concluded that both the 705- and the 430-nm transition moments of P700 make an angle of less than 25° with the plane of the membrane. The conclusion that the whole P700 dimer is oriented approximately parallel to the membrane plane (Junge, 1975; Junge and Schaffernicht, 1978) would however require a further hypothesis concerning the *X* polarization of the 430-nm transition, which remains to be shown for a dimer of Chl *a*.

A more straightforward determination of the orientation of P700 was obtained by measuring the dichroic ratio of the absorbance changes on magnetically oriented chloroplasts (Breton *et al.*, 1975; Vermeglio *et al.*, 1976; Breton, 1977a). Large dichroic ratios ($D > 2.3$) were measured at 700, 685, and 820 nm, whereas a value lower than 1 ($D < 0.4$) was observed around 660 nm. Without any hypothesis on the arrangement of the antenna, these results indicate that the 700-nm transition is tilted at less than 20° from the membrane. A tilt angle of 50° with the membrane was found for the transition at 660 nm, which was ascribed to an excitonic component of either the Q_Y or Q_X transitions in the SP. This has been presented (Breton, 1977a) as an evidence that the two Chl rings in the SP cannot be both parallel to the membrane plane in contrast with

the model of Junge (1975). In order to resolve this discrepancy, an unambiguous interpretation of the absorbance changes linked to the photo-oxidation of P700 in terms of the bleaching of the dimer, of the apparition of a monomer-like absorption, of spectral shifts of the neighbor pigments, and of the excitonic components of the dimer is first required.

There is only one report on the orientation of PSII reaction center (P680) by Mathis *et al.* (1976). These authors measured the linear dichroic ratio at 825 nm in magnetically oriented chloroplasts trapped at low temperature (−170°C). The use of low temperature was necessary because of time resolution constraints of the P680 rereduction at room temperature. At such a low temperature, the absorbance change at 825 nm decays biphasically, the rapid phase (2.8 msec) being due to the rereduction of the photo-oxidized primary donor of PSII and the slow phase to its counterpart for PSI. The dichroic ratio was found to be similar for both phases, $D = 1.32$ and $1.40 \pm .05$ respectively. The authors therefore concluded that the Q_Y transition moment of the P680 has the same orientation, relatively, to the membrane plane, as the Q_Y transition moment of the P700, i.e., a tilt angle smaller than 20°.

2. PHOTOSYNTHETIC BACTERIA

Vermeglio and Clayton (1976) were the first to report on the orientation of the reaction center pigments with respect to the membrane plane. They measured the polarized absorption of air-dried chromatophores from *Rp. sphaeroides* (R-26) in which the antenna had been selectively oxidized by K_2IrCl_6. The 870-nm transition of the SP was found to be at less than 20° from the membrane plane, whereas the average direction of the Q_Y transitions of the two Bph molecules were tilted out of the membrane at an angle greater than 45°. The average angle between the Q_Y transition moments of the BChl molecules absorbing at 800 nm, and the membrane plane is smaller than 30°. Further information has been obtained from the LD of the light-induced absorbance changes related to the photo-oxidation of the SP (Fig. 16) (Vermeglio and Clayton, 1976; Rafferty and Clayton, 1979b), because these changes involve only some of the RC pigments. Vermeglio and Clayton (1976) observed that the LD had opposite signs for the increase at 790 nm (LD > 0) and the absorption decrease at 810 nm (LD < 0). This result is inconsistent with the generally assumed hypothesis of a single blue-shift of the 803 nm band in the state P^+. They proposed an alternative hypothesis in which the 860- and 810-nm absorption decreases were attributed to the disappearence of the two excitonic components of the

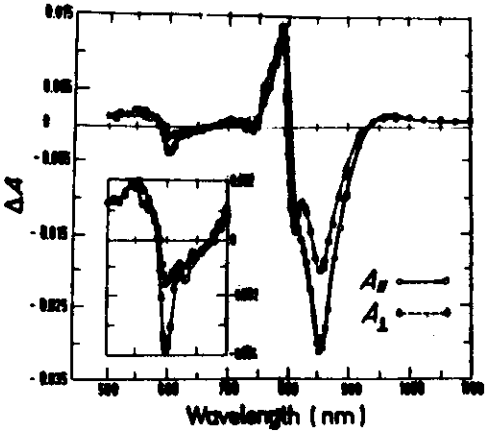

FIG. 16. Polarized light minus dark difference absorption spectra of chromatophores from *Rp. sphaeroides* oriented by drying on a glass slide. The plane of the slide was vertical and tilted at 30° from the direction of the measuring beam. The electric vector of the measuring beam was either parallel to the slide plane ($\Delta A_{\parallel}$) or at an angle of 60° with this plane ($\Delta A_{\perp}$). (Adapted with permission from Rafferty and Clayton, 1979b.)

SP, whereas the 790-nm increase reflected the appearance of a monomer-like BChl absorption band.

In a subsequent study, Rafferty and Clayton (1979b) detected a small absorption decrease at 630 nm besides the large one observed at 600 nm; both were attributed to the Q_X excitonic components of the SP. From their measurements (insert of Fig. 16), an angle of 18° with the membrane plane was calculated for the 630-nm transition, whereas the 600-nm transition was found to be tilted out of the membrane plane at more than 55° in agreement with the results of Vermeglio and Clayton (1976).

Similar polarization studies have been performed with *Rp. viridis* (Paillotin *et al.*, 1979). This species presents several advantages: Whole cells can be oriented in a magnetic field, both states P^+ and $P\,Bph^-$ can be photoinduced at room temperature, and the absorption bands around 830 nm of the RC pigments are better resolved than for BChl *a*-containing species and well-separated from the antenna absorption. The Q_Y transition moments of the two Bph molecules were found to be tilted out of the membrane plane, whereas the average direction for the Q_Y transitions of the two BChl molecules not involved in the SP is inclined at ~ 25° from the membrane plane. Figure 17 shows the polarized absorption changes linked to the state P^+. Qualitatively, these results are similar to the ones obtained on BChl *a*-containing species (see Fig. 16) with the 970-nm transition almost parallel to the membrane. In the 780–870-nm region, these absorption changes were interpreted (Paillotin *et al.*, 1979) as due to three different contributions: (a) bleaching at 845 nm of one excitonic component of the SP. This transition is tilted out of the membrane plane by more than 55°; (b) appearance at 805 nm of a monomer-like BChl transition making an angle smaller than 25° with the

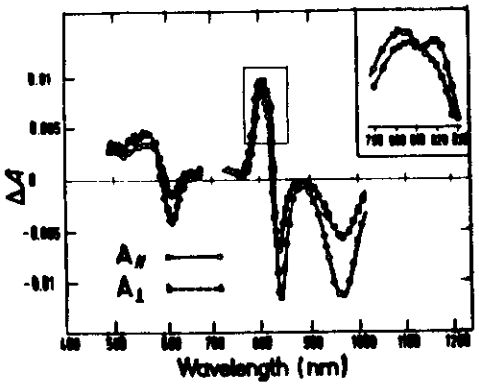

FIG. 17. Light-minus-dark difference spectra of oriented cells of *Rp. viridis* preferentially measured either parallel ($\Delta A_{\parallel}$) or perpendicular ($\Delta A_{\perp}$) to the chromatophore membrane plane. (From Paillotin *et al.*, 1979.)

plane of the membrane; and (c) shifts of the Q_Y transition moments of the two BChl molecules not involved in the SP; these shifts are in opposite directions. The Q_Y transition moment of one BChl is parallel to the membrane plane, whereas the transition of the other molecule makes an angle of 50° with this plane.

The polarization of the absorbance changes linked to the state P Bph$^-$ has also been measured on oriented cells of *Rp. viridis* (Paillotin *et al.*, 1979) and confirms the interpretation of Van Grondelle *et al.* (1976) of both a BChl band shift and a bleaching of one of the two Bph molecules. It has been possible to demonstrate that only the BChl with its Q_Y transition moment parallel to the membrane plane is shifted due to the presence of the negative charge in the RC. This interpretation is consistent with the photoselection study of Shuvalov and Asadov (1979) for state P Bph$^-$ which shows a constant polarization across the BChl band shift. The Bph molecule reduced in the state P Bph$^-$ has its plane nearly perpendicular to the membrane, its Q_Y and Q_X transitions being respectively perpendicular and parallel to this plane.

ESR studies of the triplet state of the SP in oriented membranes have also been reported. This approach, complementary to the LD studies, gives information about the three magnetic axes of the BChl dimer triplet state with respect to the membrane plane.

Frank *et al.* (1979a) have shown that ESR spectra of the BChl dimer triplet state of magnetically aligned whole cells of *Rp. viridis* and *Rp. palustris* display a marked dependence on the orientation between the analyzing static field and the membrane plane. For both BChl *a* (*Rp. palustris*) and BChl *b* (*Rp. viridis*) containing cells, the orientations of the triplet axes were found to be very similar. The *z* axis was found to lie (within 15°) preferentially in the membrane plane. The two other axes *x* and *y* make an angle of about 45° with this plane.

Hales and Das Gupta (1979a) have performed similar experiments on chromatophores of *Rs. rubrum* oriented by air-drying. Although they obtained qualitatively similar dependency of the ESR triplet signals upon the orientation of the static ESR field and the membrane plane than Frank *et al.* (1979a), they came to a somewhat different orientation of the *x*, *y*, *z* axes with the membrane plane: the *x* axis is parallel to that plane, whereas the *z* and *y* axes make, respectively, an angle of 10°–20° and 70°–80° with that plane. Frank *et al.*, (1979b) have shown by magnetophotoselection on purified RC from *Rp. sphaeroides* R-26 that the magnetic *x* axis is parallel to the long wavelength transition of the SP, in accord with results of Thurnauer and Norris (1976) and Boxer and Roelofs (1979). According to Vermeglio and Clayton (1976), Rafferty and Clayton (1979b), and Paillotin *et al.* (1979), this long wavelength transition lies preferentially in the membrane plane. Therefore the magnetic axis *x* must lie nearly parallel to the membrane plane, making it consistent only with the positioning of the SP proposed by Hales and Das Gupta (1979a).

C. *Other Constituents*

1. CYTOCHROMES

Several photosynthetic Cyts have been found to be oriented with respect to the membrane plane. The first reported studies have been done by ESR on air-dried chromtophores from *Rp. viridis* (Prince *et al.*, 1978) and *Chr. vinosum* (Tiede *et al.*, 1978). In the former species, the heme of Cyt *c*-558 has been found to be oriented perpendicular to the membrane plane. In the latter, a similar orientation has been observed for Cyt *c*-555, whereas the heme of the low potential Cyt *c*-553 was found to be parallel to the membrane plane.

Using light-induced polarized absorption changes on oriented samples, Vermeglio *et al.* (1980) have studied the orientation of the hemes of the high potential Cyt *c*-558 of *Rp. viridis* and Cyt *b*-559 of spinach chloroplasts. In contrast to ESR, which detects solely the oxidized species, these optical studies allow analysis of the difference spectrum of the reduced and the oxidized species. Although a slight complication arises from the partial overlapping of *X* and *Y* polarized transitions in the α and β bands, they compared the LD spectra obtained on magnetically oriented photosynthetic membranes to the LD of Cyt *c* (oxidized or reduced) in stretched films. It was concluded in both cases that the heme of the Cyts are oriented approximately perpendicular to the membrane plane.

2. QUINONES

There are good indications that the quinones are oriented with respect to the plane of photosynthetic membranes. Dismukes *et al.* (1979) found that the two ESR features associated with the reduction of the Fe–Q complex in whole cells of *Rp. viridis* display a marked dependence on the orientation of the sample. Hales and Das Gupta (1979a) described the orientation of the primary ubiquinone in oriented membranes reconstituted from an iron-depleted fraction of *Rs. rubrum*. From the computer simulation of their ESR spectra, they concluded that the plane of the primary ubiquinone acceptor molecule was parallel to the plane of the membrane.

Hales and Das Gupta (1979b) found that in chloroplast membranes the orientation dependence of the ESR signal II_{vf} ($D_{II,2}$) could be interpreted in terms of an orientation of the normal to the quinone plane at ~ 35° to the plane of the membrane.

3. OTHER MEMBRANE-BOUND RADICALS

The ESR of spinach chloroplasts oriented either by flow or by a magnetic field has been investigated (Dismukes and Sauer, 1978; Dismukes *et al.*, 1978; Friesner *et al.*, 1979b). The ESR signals of several membrane-bound radicals that possess *g*-tensor anisotropy display orientation effects. This is the case for *X* ($A_{1,2}$) an early acceptor of PSI whose identity is unknown, which is oriented with its g_x component predominantly perpendicular to the membrane plane and its g_y and g_z components close to the membrane plane.

The Fe–S protein B($A_{1,3}$) acting as an acceptor of PSI is oriented with its g_y component predominantly normal to the membrane. Another Fe–S protein functioning between PSI and PSII was also found to show orientation effects. McIntosh *et al.* (1979) reported that an acceptor more primary than *X* ($A_{1,2}$) and depicting large anisotropic effects could be detected by rapid ESR spectroscopy. In the same study, they also observed an orientation-dependent ESR signal from Mn^{2+}.

Slabas and Evans (1977) reported that ESR signals from an unknown paramagnetic species, probably reflecting the oxidation states of the oxygen-evolving system, in spinach chloroplast show strong orientation effects when they used an anisotropic sample. This was later confirmed by Dismukes and Sauer (1978). However, no further work is available on this system.

4. PROTEINS

A rather large fraction (~ 50%) of the intrinsic proteins of photosynthetic membranes is in α-helical conformation. By measuring the IR

spectra of chromatophores (from *Rp. sphaeroides* or *Rp. palustris*), which were either at random or oriented parallel to a plane disk, Breton (1979) measured the orientation of these α-helices. A comparison of these data to similar results obtained with purple membranes from *Halobacterium halobium*, in which the orientation of the α-helices was already known (Henderson, 1975), leads to the conclusion that the axes of the α-helices are on the average tilted at 20° ± 10° from the normal at the chromatophore membrane plane. A qualitatively similar observation has been seen for air-dried membranes of spinach chloroplasts by measuring directly the LD in the IR (Nabedryk and Breton 1981). This observation shows that a previous interpretation of the LD in the far UV (180–220 nm) as indicative of an orientation parallel to the membrane plane of the axis of the α-helices (Breton *et al.*, 1973a) was incorrect. Rather, the LD signals in this complex spectral range might reflect the various orientations of the several types of aromatic amino acid residues.

V. Local Order between Complexes in Photosynthetic Membranes

Photosynthetic membranes possess a unique characteristic for studying the problem of local order between adjacent complexes because of the transfer of excitation energy among them.

The measurement of the extent of depolarization of Chl fluorescence *in vivo* to estimate the number of transfer steps has been applied for a long time (Arnold and Meek, 1956; Goedheer, 1957, 1973; Teale, 1960; Lavorel, 1964; Ebrey and Clayton, 1969; Ebrey, 1971; Mar and Govindjee, 1972; Wong and Govindjee, 1981). However, two important facts were not clearly recognized at that time. First of all, a steady state measurement of fluorescence depolarization leads only to a convolution of the number of transfer steps by the averaged angular factor of the transition moments between which the transfer occurs. Second, the fluorescence polarization of a random suspension of membranes in which the pigments are oriented with respect to the membrane plane cannot be assimilated to an isotropic solution of pigments, since photoselection of membranes will occur and will appreciably distort the data. This effect was first described by Breton *et al.* (1973b), who have also shown that true depolarization by energy transfer between mutually oriented chromophores had to be studied on an oriented sample in which the fluorescence viewing direction was along the normal to the membrane plane. This photoselection effect can explain the results of Ebrey and Clayton (1969) on chromatophores isolated from photosynthetic bacteria where they observed upon excitation at 590 nm a polarization which was too

large as compared to the one measured when exciting at 850 nm. There is no need to invoke either anomalous energy transfer (Ebrey, 1971) or textural effects (Goedheer, 1973) to explain this discrepancy (Breton, 1977b).

It must be noted that the most readily interpretable data are those obtained upon excitation in a region where the intrinsic polarization of fluorescence for the isolated pigment is maximum and that the measurement of p for pigments in the membrane cannot be larger than the one determined for the isolated pigment–protein complexes.

In green plants there are some indications (Whitmarsh and Levine, 1974; Becker *et al.*, 1976, Garab and Breton, 1976) that the degree of local order between the Q_Y transitions moments of Chl *a* increases from the shorter toward the longer wavelength absorbing species (although a reduction in the number of transfer steps cannot be excluded). There is also evidence that the Car molecules and the Q_Y transition moments of Chl *a* tend to be mutually oriented at a small angle at a local level (Becker *et al.*, 1976). Only one magnetophotoselection experiment has been performed with photosynthetic bacteria which, although qualitatively, seems to indicate that local order is present between antenna complexes as well as between antenna and RC complexes (Thurnauer and Norris, 1976).

Finally, the use of singlet–singlet annihilation as a tool to reduce the extent of energy migration between the pigments complexes has been proposed in order to study the local order between subsets of neighboring pigments (Breton and Geacintov, 1979).

VI. Conclusions

During the last 10 years, the use of linearly polarized light has revealed a high degree of organization of the pigments and of the electron transfer components in photosynthetic material. However, the orientation of the chromophores either with respect to the plane of the photosynthetic membrane or within isolated pigment–protein complexes has been so far described in qualitative (or at the best semiquantitative) terms. Various intrinsic limitations like the overlap of several absorption bands, the lack of perfect polarization of some transitions, the divergent interpretations of the absorption changes are responsible for this imprecision. It is nevertheless interesting to note the similarity of the organization of photosynthetic membrane in bacteria and green plants. In both classes of organisms, most of the antenna Chls are found to be preferentially oriented perpendicular to the membrane plane; a similar

orientation is also deduced for the two Chl molecules of the primary donors. On the other hand, the Car molecules are closer to the membrane plane in green plants than in photosynthetic bacteria.

Beside the qualitative description of these orientations, we lack direct information on the distance between the chromophores and on the angles between the vectors joining the center of the different molecules. The absence of these two parameters precludes any complete description of the disposition of the chromophores. Different methods, however, permit an estimation of distances between molecules. For example, in the case of a dimer, the rotational strength of the CD bands depends upon a triple product involving the two transition moments of each monomer and a vector joining the center of the two molecules (Tinoco, 1963). The electronic interaction energy depends also on the geometrical relationship of the transition moment and their distances. These properties have been recently used to estimate a distance of ~ 10 Å between the antenna BChl *a* molecules in the B890 complex of R-26 (G. Paillotin, private communication). The distance between electron carriers can also be determined by measurements of the rate of electron transfer between these molecules (Hopfield, 1974; Jortner, 1976; Peters *et al.*, 1978) or by determination of the magnetic exchange coupling constant between two paramagnetic species (Tiede *et al.*, 1978; Okamura *et al.*, 1979a, b).

A hypothetical model of a photosynthetic membrane, which tentatively includes information on the orientations as well as on the distances of RC and antenna chromophores, is depicted in Fig. 18. Further ex-

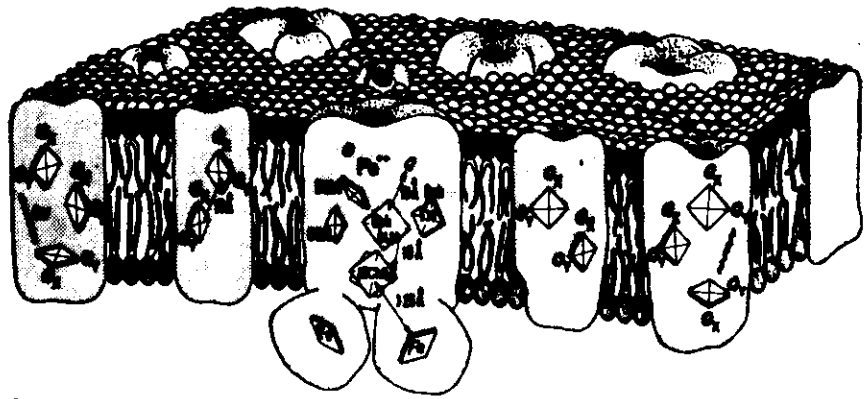

FIG. 18. Schematic representation of RC and LH chromophores in the membrane of a photosynthetic bacteria. Information obtained from different species have been pooled in drawing this model.

perimenits need to be designed in order to improve such models. This will necessitate the determination of the degree of orientation of the membranes or of the particles in the sample under investigation. The combination of ESR and absorption techniques, the availability of purified antenna and RC from green plants and a variety of refinements in the experimental conditions should increase our understanding of the orientation of the chromophores *in vivo*. However, further progress will probably require the use of some modeling. In this respect, the availability of various synthetic Chl dimers or complexes of known structure and bearing some resemblance with the models derived from the studies described in this chapter would be of considerable help. They would allow the development of proper theories for absorption, CD, and ESR, which could then be applied to the interpretation of the physical properties of natural Chl–protein complexes.

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