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STUDY OF PHOTOSYNTHETIC MATERIALS BY
CIRCULAR DICHROISM

Kenneth David Philipson
(Ph.D. thesis)

October 1972

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STUDY OF PHOTOSYNTHETIC MATERIALS BY CIRCULAR DICHROISM

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STUDY OF PHOTOSYNTHETIC MATERIALS BY CIRCULAR DICHROISM

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ABSTRACT

Circular dichroism (CD), a measure of the asymmetry of a system, is used to probe materials from the photosynthetic apparatus. The research can be divided into three parts.

(1) To provide a sound understanding for interpreting the CD spectra of complex photosynthetic membranes a theoretical study was first undertaken to calculate the CD properties of in vitro chlorophyll and related molecules. Agreement between calculated and observed rotational strengths for solution monomers of chlorophyll, bacteriochlorophyll, and related molecules has been improved over that obtained in a previous study (Houssier, C. and Sauer, K. (1970), J. Am. Chem. Soc. 92, 779) by an order of magnitude through the use of a model which distributes the $\pi-\pi^*$ transitions over the porphyrin ring. Using the Kirkwood-Tinoco coupled oscillator approach, the electronic transitions are described using point monopoles located at the porphyrin macrocycle nuclei.

(2) In order to explore the nature of the interactions among the chlorophyll molecules of the photosynthetic apparatus, a bacteriochlorophyll-protein complex which functions in vivo to transfer electronic excitation energy is studied. This bacteriochlorophyll-protein complex from the green bacterium Chloropseudomonas ethylica has previously been shown to consist of four subunits, each of which contains five bacteriochlorophyll a molecules. Interaction among the bacteriochlorophyll a molecules produces exciton splittings observable at 77°K in the long-wavelength (809 nm) absorption band of the bacteriochlorophyll-protein. An exciton contribution to the rotational strength results in five components in the CD spectrum in this region. The total exciton band splitting is 490 cm^{-1} . The separation of bacteriochlorophyll a molecules is estimated to 12-15 Å in one subunit of the protein.

(3) Reaction centers of photosynthetic organisms, complexes containing the primary photoreactants of photosynthesis, are known to exhibit light-induced, reversible absorption changes due to chlorophyll oxidation. We have found changes in the CD which accompany the absorption changes. These light-induced CD changes are examined in three different classes of photosynthetic organisms.

Preparations from a higher plant enriched 10-fold in the Photosystem I reaction center chlorophyll, P700, have been studied by observing the changes which occur in both the absorption and CD upon illumination. The results suggest that there are at least two chlorophyll a molecules within the reaction center and that a significant

exciton interaction exists among them. Upon photo-activation, one molecule within the array becomes oxidized, leading to the loss of exciton interaction.

The absorption and CD spectra of reaction centers from the purple photosynthetic bacteria, Rhodopseudomonas viridis, Rhodospirillum rubrum, and Chromatium are compared with those previously reported for Rhodopseudomonas spheroides. The reaction centers, with the exception of those from R. viridis, appear spectrally very similar. The spectral properties of the bacteriopheophytin present in the reaction centers give no indication that the bacteriopheophytin is strongly interacting with the reaction center bacteriochlorophyll and imply that the bacteriopheophytin has no function in the transfer of electrons.

A photochemically-active complex from the green bacterium Chlorobium thiosulfatophilum has been isolated and shown, on the basis of spectral measurements, to be structurally very similar to a complex previously obtained from Chloropseudomonas ethylica. The reaction center contains only a small portion of the BChl in this macromolecule with the BChl:P840 ratio being about 100:1. This P840-containing reaction center is characterized in terms of light-induced changes in the absorption and CD spectra.

On the basis of light-induced spectral changes the reaction centers from higher plants, purple bacteria, and green bacteria are compared with one another. It is concluded that significant structural differences exist among the reaction centers from these three classes of photosynthetic organisms.

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To Ms. Candy Mar.

ABBREVIATIONS

| | |
|------------|---|
| Chl | chlorophyll |
| BChl | bacteriochlorophyll |
| CD | circular dichroism |
| ORD | optical rotatory dispersion |
| A_L, A_R | absorbance of left and right circularly polarized light, respectively |
| BChl-RC | bacteriochlorophyll-reaction- center complex from green bacteria |
| BChl-P | bacteriochlorophyll-protein |
| BPh | bacteriopheophytin |
| SDS | sodium dodecyl sulfate |

DEDICATION

To my parents.

I. INTRODUCTION

The initial acts of photosynthesis - the absorption of light energy by chlorophyll (Chl) and the stabilization of this energy - take place within organized membranes of photosynthetic organisms. The intimate details of the architecture of these membranes are, with few exceptions, unknown. Although gross structures such as the grana and stroma lamellae of chloroplasts can be detected by electron microscopy and can be physically separated from one another (Sane et al., 1970), knowledge of these membranes on a finer scale remains to be elucidated. Specifically, an understanding is needed about the nature of the proteins and lipids which make up the membrane, the organization of these components with respect to one another, and the relationship of this organization to the electron transport reactions of photosynthesis.

Concurrent with this are problems concerning the state of Chl molecules within the membrane. Questions related to this area of much active research are: Does the Chl associate more closely with lipid or with protein? Do the Chl's aggregate with one another and, if so, what is the nature of this aggregation and the strength of Chl-Chl interactions within the aggregate? What is the mechanism of electronic excitation energy transfer amongst the Chl's? What is the mechanism which enables a specific Chl molecule to become oxidized upon excitation? Answering questions at this level of examination is the purpose of the bulk of this thesis.

A. The Photosynthetic Unit

It is known that Chl molecules are arranged in what is known as photosynthetic units. This concept originated with the experiments of Emerson and Arnold in 1932. These investigators measured the oxygen (a by-product of photosynthesis) evolved by the green alga Chlorella in response to brief ($\sim 10^{-5}$ sec) flashes of light of varying intensity. They found maximal yields of oxygen evolution at light intensities far too weak to excite all of the Chl. From these experiments it was inferred (and later confirmed) that photosynthesis operated in units with a large majority of the Chl's serving as "antenna" or light-harvesting pigment for a chemical reaction center. The photon energy of the antenna Chl would be collected at the reaction center and converted there into stable chemical energy. The discovery of small light-induced reversible absorbance changes in photosynthetic bacteria (Duysens, 1952) and higher plants (Kok, 1956) led to the concept that the reaction center contained a small number of BChl or Chl a molecules in an unspecified special environment. The small absorbance changes were interpreted as being due to the oxidation of a BChl or Chl molecule as an excited electron was given up to the primary acceptor of photosynthesis. These light-induced absorption changes, commonly referred to as P870 and P700 in purple bacteria and higher plants, respectively, could also be produced in the dark by using a chemical oxidant such as ferricyanide. Oxidation-reduction titration curves of these photo-active pigments show that they both have reversible mid-point potentials at about 0.43 V as they undergo one-electron oxidations (Kok, 1961; Kuntz, Loach and Calvin, 1964).

Chl's of the photosynthetic unit could now be conceptually separated into two functional types: antenna Chl's which functioned to absorb photons and transfer this energy, and reaction center Chl's where photochemistry was initiated. These experiments served as an impetus to research on the nature of in vivo Chl. The nature of the environments of antenna and reaction center Chl's remained to be explored.

To help solve this problem, photosynthetic membranes have, in recent years, been subjected to a wide variety of fractionation procedures. Methods of cell disruption (French press, sonication, detergent treatment) followed by separation techniques (column chromatography, ultracentrifugation) have been used in an attempt to break the membranes into their component parts. Such approaches have resulted in the isolation and characterization of several Chl-proteins. That is, homogeneous proteins are found with a specific number of non-covalently bound Chl molecules per macromolecule. Consistent with the concept of the photosynthetic unit, both light-harvesting Chl-proteins and reaction center Chl-proteins have been isolated. The best known of these are the light-harvesting BChl-protein, characterized by Olson (1966), from the green bacterium Chloropseudomonas ethylica and the BChl-containing reaction center from the purple bacterium Rhodopseudomonas spheroides studied by Clayton (1966a). Details of these (and other) protein complexes will be discussed below in conjunction with the CD experiments.

Electron microscopy studies, primarily those of Park and co-workers (Park and Pon, 1963; Park and Biggins, 1964) have found some evidence for a morphological expression of the photosynthetic unit. Small particles, about 200 Å in diameter, can be seen on the inner surfaces of

chloroplast membranes. These particles, called "quintasomes" by Park (1963), were calculated to contain about 230 Chl molecules (Park and Biggins, 1964); approximately the amount of Chl which the photosynthetic unit is thought to contain (Kok and Businger, 1957); Other workers (Howell and Moudrianakis, 1967; Karn and Moudrianakis, 1969) have disputed the existence of quintasomes, claiming that Park was actually observing ATPase particles on the outer membrane surface. Investigations have continued (Park and Pfeifhofer, 1969) in attempts to determine the relationship of quintasomes to photochemical activity.

B. Energy Transfer and Exciton Interaction

One immediate consequence of the photosynthetic unit concept is that photon energy absorbed by one Chl molecule must be transferred via some mechanism to the reaction center Chl's. Energy transfer by diffusion of atoms, radicals, or molecules has been ruled out as a feasible mechanism. This is clear from the fact that light energy can be efficiently transferred to a reaction center extremely rapidly at temperatures as low as 1°K (Clayton, 1962). Mechanisms of energy transfer based on the diffusion of electrons and holes following the photoionization of an antenna Chl molecule as proposed by earlier experimenters (Arnold and Maclay, 1959) also seemed unlikely. Experiments indicate that the efficiency of in vivo Chl photoionization is extremely low, probably less than 0.1% (Clayton, 1966b).

It seems probable that energy reaches the reaction center Chl's in the form of electronic excitation. That is, the Chl's of the photosynthetic unit are interacting through electric forces arising from the redistribution of charges in an electronic transition and, as a result

of this, the photon energy absorbed by one Chl is made accessible to other Chl molecules. This energy transfer must occur from either the singlet or triplet excited state of the molecule. Although efficient energy transfer by the triplet state is possible in molecular crystals (Robinson, 1963), the presence of triplet state energy transfer in photosynthetic tissues is improbable (but not impossible) on the basis of fluorescence experiments (Clayton, 1966b) and considerations of excited state lifetimes (Robinson, 1966).

Clayton reports that the fluorescence yield of in vivo antenna BChl increases as the reaction centers become saturated with incoming energy. Such variations in the decay of the singlet state would not be expected if the excitation is converted to the triplet state and then transferred to the reaction center. This leaves the excited singlet state as the dominant mode for the migration of photon energy in the photosynthetic unit.

Although this is agreed upon by most current researchers, the exact mechanism for the transfer of excited singlet state energy among the Chl's of the photosynthetic unit is a subject of some debate. This type of energy transfer is usually described by one of three quite different models depending largely on the strength of intermolecular interaction. The three types of interaction responsible for energy transfer are the strong exciton, the weak exciton, and inductive resonance, and the coupling between molecules in each case is described as strong, weak, and very weak, respectively. The criteria which separate these cases are:

| | |
|---|-----------|
| $2U \gg \Delta\epsilon$ | strong |
| $\Delta\epsilon \gg 2U \gg \Delta\epsilon'$ | weak |
| $\Delta\epsilon' \gg 2U$ | very weak |

where U is the interaction energy between molecules, $\Delta\epsilon$ is the Franck-Condon band width equal to the width of the entire absorption band of the isolated molecule, and $\Delta\epsilon'$ is the width of a single vibronic level. $\Delta\epsilon$ is usually taken to be about 1000 cm^{-1} and $\Delta\epsilon'$ is about 10 cm^{-1} .

It is the strength of interaction, U , between molecules which determines the rate at which energy is transferred. In the case of strong excitons, the coupling between molecules is so strong that it is impossible to excite an individual molecule. The excitation is delocalized over the array of interacting molecules which now has an excited state wave function characteristic of the aggregate of molecules. It is incorrect to speak of a rate of energy transfer since the excitation is never localized on a particular molecule. The strong exciton is sometimes descriptively referred to as a free exciton.

In the weak exciton situation the excitation resides on a molecule for a time longer than the period of a nuclear vibration (about 10^{-14} sec) but less than the time needed for vibrational relaxation (about 10^{-12} sec). Thus, the excitation transfer can take place from an upper vibrational level of the excited molecule. The weak exciton is sometimes called the localized exciton.

In the case of energy transfer by inductive resonance the excitation can be considered to be localized on one molecule for a time long enough to allow the molecule to relax into a lower vibrational level before energy transfer. This is the case often referred to as Förster transfer or vibrational-relaxation resonance transfer. [The nomenclature of Förster designates inductive resonance as the weak interaction case (1960) or as a very weak coupling case (1965).]

Inductive resonance energy transfer has an R^{-6} distance dependence as opposed to the R^{-3} dependence of both strong and weak exciton energy transfer.

The three cases are often difficult to distinguish from one another. In practice assignment is usually not made by use of the band width comparison criteria mentioned above. The strong exciton produces noticeable characteristic spectral effects such as the splitting of absorption bands into several components ("Davydov splitting"). The transition probabilities of each of these components will be determined by the geometrical arrangement of the interacting molecules. Strong coupling also results in a narrowing of the absorption components and in a characteristic CD spectrum (Tinoco, 1963). These CD effects will be described and put to use in later sections to help identify strong excitons in photosynthetic materials.

The spectral effects observed in absorption and CD disappear as the strength of the molecular interaction decreases and they become unobservable for both the weak exciton and inductive resonance cases. Theoretically these cases are distinguishable by the difference in the dependence of the rate of energy transfer on intermolecular distance (R^{-3} vs. R^{-6}). Obviously this criterion is difficult to apply to an in vivo photosynthetic material, where both the distance between Chl molecules and the rate of energy transfer are only very approximately known. Resonance transfer applies generally to energy transfer between dissimilar molecules or between like molecules in dilute solution. This probably does not apply to photosynthetic membranes where Chl concentrations run in excess of 0.1 M (Dratz et al., 1966).

Nevertheless, many authors, on little firm experimental basis, have assumed that resonance transfer dominates among in vivo Chl molecules (e.g., Hoch and Knox, 1968). This is not to say that resonance transfer has no role in photosynthesis. For example, it is probably the correct mechanism for describing energy transfer from accessory pigments, such as carotenoids to Chl and from Chl b to Chl a.

The recent evidence that Chl is not homogeneously distributed in the photosynthetic unit but rather is located inside specific proteins implies that perhaps more than one type of energy transfer operates in vivo (Philipson and Sauer, 1972). For example, strong excitons could dominate among the Chl's within one protein subunit and weak exciton and inductive resonance transfer might compete as the mechanism of energy transfer between Chl's in different proteins.

The information on electronic excitation transfer and exciton coupling in this section was taken from the following references: Davydov (1948), Simpson and Peterson (1957), Förster (1960), Kasha (1963), Hochstrasser and Kasha (1964), Förster (1965), Kasha et al. (1965), Clayton (1965), Pearlstein (1966) and Robinson (1966). The reader should be wary, however, that the notation used by these authors is not always uniform. The criteria used in these references should always be examined so that the reader will know which of the above cases is being discussed. In later sections when the term "exciton" is used in explaining absorption band splittings and other optical effects it will always refer to the strong exciton case described above.

C. The Aggregation of In Vivo Chlorophyll

It is the degree of aggregation and orientation of in vivo Chl which determines the mechanism of energy transfer. Little is known about the exact nature of this aggregation. For example, the distances between Chl molecules and the forces which hold the Chl's in specific relationships to one another are matters of conjecture. Fluorescence polarization (Olson et al., 1962) and linear dichroism (Sauer and Calvin, 1962) experiments on oriented chloroplasts or fragments reveal little long-range organization of the Chl molecules, although recent experiments on photosynthetic systems oriented by a magnetic field indicate greater Chl orientation than previously supposed (Geacintov et al., 1972).

The resolution of the long wavelength absorption bands of bacterial chromatophores (Vredenberg and Ames, 1966) and higher plant chloroplasts (French and Prager, 1969) into several components, especially at low temperatures, has been attributed to the existence of several BChl or Chl "forms". It has been a challenge, however, to determine the cause of these different spectral forms. That is, the multiple absorption components in these systems could be due either to exciton splittings among aggregated Chl molecules or to Chl molecules in different lipo-protein environments. To choose between these two cases some detailed information on the system under examination is needed. The number of different Chl-proteins contributing to the spectrum and the number of Chl's per protein would be helpful information in this regard. We shall see that CD spectroscopy, in certain cases, has been a potent tool in helping to distinguish the two cases.

Researchers, for many years, have made models of the internal membranes of the chloroplast. Typically, the Chl is considered to be organized in two dimensional arrays between layers of lipid and protein. The porphyrin ring of the Chl is in contact with the protein and the phytyl chain extends into the lipid layer. These models have become more detailed and sophisticated (Weier et al., 1966; Kreutz, 1970), but generally the evidence supporting any particular model is overextended.

The recent isolations of different Chl-proteins imply that most of the Chl is imbedded within the protein component of the membrane. Models of Chl molecules organized in homogeneous layers are no longer feasible. Future research must determine the interactions and relationships of the Chl-proteins so that more realistic models of the photosynthetic apparatus can be developed.

D. Outline of Research

Absorption and circular dichroism (CD) spectroscopy are the techniques used in this study. Optical spectroscopy, in all of its different forms, has been used for a long time in the study of photosynthesis, but only recently has optical activity (as measured by CD or optical rotatory dispersion (ORD)) been used to probe the photosynthetic apparatus. Initial reports of ORD in photosynthetic membrane fragments were made independently by Sauer (1965) and Ke (1965). Sauer suggested that Chl-Chl interactions were largely responsible for the observed spectra. Since then a number of CD studies on photosynthetic materials (Sauer, 1972) have been done. (CD and ORD spectra theoretically contain identical information; CD, however, is more easily interpreted.)

Photosynthetic materials are very amenable to study by optical techniques, especially CD. The highly colored Chl molecules are within essentially colorless lipid and protein, and the Chl serves as a probe to its surrounding environment. Optical activity, sensitive to chromophore-chlorophore or chromophore-protein interactions, has proven to be (as will be seen in this thesis) a potent tool for probing the photosynthetic apparatus.

The work to be presented can be divided into two main parts. In the first an attempt is made to understand more fully the optical activity of isolated Chl (and related molecules) in solution in the monomeric form. It was felt that an understanding of these properties was needed before CD spectra of more complicated systems could be interpreted with confidence. Calculations are made to see if the observed CD of these molecules can be accounted for in terms of existing theory (Philipson et al., 1971).

In the second part, the CD of materials closer to their native biological state is studied. The CD spectra of Chl- or BChl-protein complexes of two distinct functional types are presented. The absorption and CD properties of a BChl-protein from a green photosynthetic bacterium, which functions to transfer electronic excitation energy, is studied in detail at room and liquid nitrogen temperature (Philipson and Sauer, 1972). It is concluded on the basis of these experiments that the mode of energy transfer among the BChl molecules within this protein is via an exciton interaction, and that the BChl molecules within a subunit of this protein are located within 12-15 Å of one another.

The other type of Chl-protein examined is the reaction center. These complexes contain the primary photoreactants of photosynthesis

and are known to exhibit light-induced, reversible absorption changes when a Chl or BChl molecule within the reaction center loses an electron to a primary acceptor. Reversible CD changes can also be induced in these materials and useful information can be obtained from interpretation of these signals. Below, the reversible CD signals from the reaction centers of green bacteria, purple bacteria, and higher plants are discussed in detail (Philipson et al., 1972; Olson et al., 1972; Philipson and Sauer, 1973).

II. THEORETICAL CALCULATION OF THE CIRCULAR DICHROISM OF CHLOROPHYLL AND RELATED MOLECULES

A. Introduction to Circular Dichroism

Circular dichroism, the difference in absorption between left and right circularly polarized light ($A_L - A_R$), is a measure of the asymmetry of an electronic transition. To be optically active (*i.e.*, to exhibit CD) a molecule must be non-superimposable on its mirror image (*e.g.*, a carbon atom with four different substituents). Optical activity is determined by the geometrical arrangement of the parts of the molecule with respect to one another. This is what gives CD its extreme sensitivity to molecular geometry and makes it a powerful tool for studying structural relationships.

The fundamental equation of CD is (Rosenfeld, 1928):

$$R_A = \text{Im } \underline{\mu}_{0A} \cdot \underline{m}_{A0} \quad (\text{II-1})$$

where R_A is the rotational strength (a measure of the area under a CD curve) for a transition, Im means "the imaginary part of", $\underline{\mu}_{0A}$ represents the electron dipole transition moment for the transition $A \leftarrow 0$ where 0 is the ground state, and \underline{m}_{A0} is the magnetic dipole transition moment for the same electronic transition. Classically, what this equation means is that in order for a transition to be optically active, *i.e.*, $R_A \neq 0$, there must be a circular component (a magnetic dipole) in the motion of the electrons undergoing the transition in a plane perpendicular to a linear component (an electric dipole) of electronic motion. These

criteria describe a helical motion. In other words, a molecule must possess a helical asymmetry to be optically active, although, in many cases, the helical nature of an optically active molecule may not be obvious from its structure.

This is shown pictorially, for a classical case, in Figure 1. Consider the electric field, \underline{E} , of a plane polarized light wave impinging upon a helically shaped molecule in which the electrons are constrained to move along the spiral path of the molecule. Let the vector \underline{E} be parallel to the long axis of the molecule. An electric dipole moment, $\underline{\mu}$, will arise from the displacement of the electrons under the influence of the field \underline{E} . For both the right-handed and left-handed helical molecules, $\underline{\mu}$ will be parallel to the helix axis and pointing in the same direction. The circular motion of the electrons, however, will be in opposite directions for the mirror-image helices as the electrons are induced to move along the molecules. For the right-handed helical molecule (looking down the helix axis) the electrons will move in a clockwise manner as they are forced down the helix by the electric field vector, \underline{E} . In the case of a left-handed helical molecule, this motion will be counter-clockwise. The magnetic dipoles, \underline{m} , induced by \underline{E} will again be parallel to the helix axis but will point in opposite directions (as shown in Figure 1). R (a classical analogy to rotational strength), equal to the dot product of $\underline{\mu}$ and \underline{m} , is non-zero and is of opposite sign for right- and left-handed helical molecules. We see that a helix possesses the necessary asymmetry to produce optical activity and that for this case and for all mirror image molecules rotational strengths of equal magnitude and opposite signs will be observed.

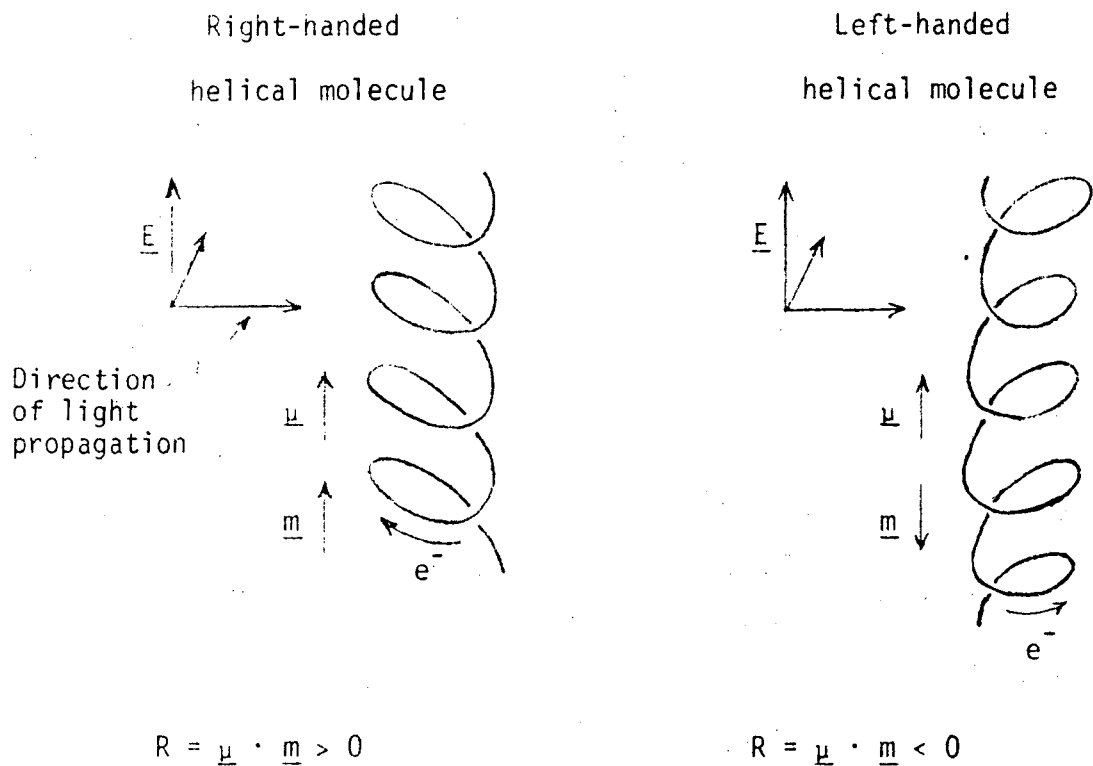


Figure 1. Classical representation of the optical activity of a helical molecule. \underline{E} is the electric field vector of a plane polarized light wave directed parallel to the helix axis. $\underline{\mu}$ and \underline{m} are electric and magnetic dipoles induced by \underline{E} . R is a classical analogy to rotational strength. See text for details.

Sources of optical activity in molecules can be divided into two limiting cases. The first is the case of the inherently asymmetric chromophore. The classic example of this is hexahelicene, a molecule which consists of six benzene rings fused into a left- or right-handed helix resembling a lock washer. Transitions are delocalized over the entire molecule, possess the needed helical quality, and demonstrate a huge optical activity (Moscowitz, 1961).

The second source of optical activity in molecules is the case in which an inherently symmetric chromophore is perturbed by an asymmetric environment. This asymmetry may be either inter- or intra-molecular. An example of optical activity induced by interaction with an asymmetric intramolecular perturbation is the 300 nm ketone carbonyl transition in steroids (Djerassi, 1960). The carbonyl transition by itself is symmetrical. It is only by interacting with the environment provided by the rest of the steroid molecule that the chromophore becomes optically active. The isolated Chl molecule falls into this category of optical activity (Houssier and Sauer, 1970; Philipson *et al.*, 1971). This will be discussed in detail below.

The CD induced in the transitions of the symmetric heme group by its protein surroundings in either myoglobin or hemoglobin (Hsu and Woody, 1971) is a good example of optical activity induced by inter-molecular interaction. A special case of this source of optical activity exists when the asymmetric interaction is between identical or very similar chromophores. This is the exciton interaction and can result in striking effects in the CD spectrum (Tinoco, 1963). It is these characteristic effects which will be utilized below to interpret

CD spectra of photosynthetic materials and to identify the existence of Chl-Chl interactions.

Reviews of various aspects of optical activity can be found in Kauzmann et al. (1940), Moscowitz (1962), Tinoco (1962), Schellmann (1966), and Deutsche et al. (1969).

B. Circular Dichroism of Chlorophyll and Related Molecules Calculated Using a Point Monopole Model for the Electronic Transitions

The application of circular dichroism measurements to materials of photosynthetic origin promises to provide important information about the internal organization and structure of the photoactive pigmented membranes (Sauer, 1972). In order to provide a sound basis for interpreting the CD spectra of these complex biological materials, it is important to develop a better understanding of the corresponding properties of the individual (isolated molecules).

The present study is an extension of a previous investigation of the origins of molecular optical activity in Chl and related molecules (Houssier and Sauer, 1970). These molecules consist of an extended, planar porphyrin chromophore, whose symmetry can be considered to be perturbed by substituents placed asymmetrically around the periphery. The absolute configuration at the asymmetric centers in many of these molecules is known (Fleming, 1967, Brockmann, Jr., 1968), which enables them to provide a rigorous test for the theoretical treatment. The agreement between experiment and calculations utilizing a model involving a point monopole approximation for the electric transition moment is a considerable improvement over that resulting from the use

of point dipole transition moments, but there is still some discrepancy in the quantitative nature of the calculations.

1. Theory

In the Kirkwood coupled oscillator model, the origin of optical activity is the interaction potential between electric transition dipoles located asymmetrically with respect to one another (Kirkwood, 1937). The equation needed for the calculation of the rotational strength (R_A) of a Chl-like molecule can be derived (Tinoco, 1962) from the fundamental equation of optical activity described above:

$$R_A = \text{Im } \underline{\mu}_{0A} \cdot \underline{m}_{A0} \quad (\text{II-1})$$

The electronic transition dipole moment

$$\underline{\mu}_{0A} = e \int \psi_0 \underline{r} \psi_A d\tau \quad (\text{II-2})$$

and is a measure of the electron redistribution which occurs during the transition $A \leftarrow 0$.

$$\underline{m}_{A0} = \frac{e}{2mc} \int \psi_A (\underline{r} \times \underline{p}) \psi_0 d\tau \quad (\text{II-3})$$

and, classically speaking, represents the angular momentum of the electrons during a transition. e is the charge on the electron, m is its mass, and c is the speed of light. The integrations are carried out over all space. \underline{r} is a position vector operator and \underline{p} is the linear momentum operator.

A molecule must possess nonperpendicular electric and magnetic dipole moments in order to be optically active [Eq. (II-1)]. Electrically allowed transitions of planar aromatic chromophores, such as the Q

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transitions of the simple porphyrins, cannot possess the required magnetic dipole from symmetry considerations. The necessary magnetic dipole, in the case of Chl and related biological pigments, is supplied as a perturbation resulting from interaction with asymmetrically-placed substituents.

The magnetic dipole moment is origin-dependent and has two contributions:

$$\underline{m} = \sum_j \underline{m}_j + \sum_j \underline{r}_j \times \underline{p}_j \quad (\text{II-4})$$

Each group, j , of a molecule makes two contributions to the angular momentum. There is the angular momentum of the group itself plus the product of the linear momentum of the group and its distance from an origin. We are treating the molecule as if it can be divided into independent groups. This treatment is valid if there is no significant electron exchange between groups. This is almost certainly true for Chl-like molecules where the electronic wave function for the porphyrin chromophore would not overlap with the wave functions of the asymmetrically-placed side groups. These are either aliphatic in nature or, in the case of esters, they are separated by insulating aliphatic linkages.

Using first-order perturbation theory and the substitution (Bohm, 1951),

$$\underline{p}_{OA} = -(2\pi im/e)v_a \underline{\mu}_{OA} \quad (\text{II-5})$$

we can now calculate the magnetic transition dipole moment for the whole molecule (Tinoco, 1962):

$$\underline{m}_{AO} = -\left(\frac{\pi i}{c}\right) \sum_{j \neq i} \sum_{b \neq a} \frac{[V_{ioa;job} (\underline{R}_j - \underline{R}_i) \times \underline{\mu}_{job} v_b]}{h[2v_b^2 - v_a^2]} \quad (\text{II-6a})$$

$$-\left(\frac{\pi i}{c}\right) \sum_{j \neq i} \sum_{b \neq a} \frac{(V_{ioa;job} \mu_{jbo})}{h[2\nu_a / (\nu_b^2 - \nu_a^2)]} \quad (\text{II-6b})$$

The summations are over all groups, j , except i (the porphyrin ring) and all transitions, b , except a (e.g., one of the Q transitions of the porphyrin). This indicates that the transition of interest, μ_{ioa} , is interacting with all of the transitions of each of the asymmetrically-placed substituents. $V_{ioa;job}$ is the coulomb potential energy due to the interaction of transition charge densities in group i with those in group j . This will be discussed in greater detail below. μ_{jbo} and m_{jbo} are the electric and magnetic transition dipoles for the transitions of group, j , in the static field of the surrounding molecule (Tinoco, 1962). h is Planck's constant and ν_a and ν_b are the frequencies of transitions $a \leftarrow o$ and $b \leftarrow o$ respectively. \underline{R}_j and \underline{R}_i are position vectors of the j^{th} and i^{th} group, respectively.

The two terms (II-6a and II-6b) are due to the interaction of electronic and magnetic transition dipoles, respectively in the asymmetrically-placed side groups with the electronic transition dipole of transition $a \leftarrow o$ on group i (a Q transition of the porphyrin macrocycle). We have assumed that the transitions of interest (Q transitions of Chl) are non-degenerate and magnetically forbidden, and the origin of the molecule has been placed at the center of group i . This results in the dropping of some terms from the more general equations of Tinoco (1962).

The predominant contribution to the electronic transition moment of the Chl, μ_{OA} , will be from the transition localized in the porphyrin ring, μ_{ioa} . We will take these quantities to be equal

$$\mu_{OA} = \mu_{ioa} \quad (\text{II-7})$$

Now, multiplying Eq. II-6a and Eq. II-6b, above, with Eq. II-7, we obtain the pertinent equation:

$$R_A = - \left[\frac{2\pi}{c} \sum_{j \neq i} \sum_{b \neq a} \frac{V_{ioa;job} \nu_a \nu_b (R_j - R_i) \cdot (\mu_{job} \times \mu_{ioa})}{h(\nu_b^2 - \nu_a^2)} \right] \quad (\text{II-8a})$$

$$+ 2 \sum_{j \neq i} \sum_{b \neq a} \frac{\text{Im } V_{ioa;job} \nu_a \mu_{ioa} \cdot m_{jbo}}{h(\nu_b^2 - \nu_a^2)} \quad (\text{II-8b})$$

The second term in this formula is extremely difficult to calculate and is usually ignored. It is expected on theoretical grounds (Moffitt, 1956) to be much smaller than the first term and one explicit calculation (Jones, 1961) has it accounting for about 1/10 of the observed rotational strength in cyclopentanone derivatives. Hence, only the more familiar first term, equivalent to the Kirkwood contribution, will be considered in this discussion.

For each of the pigments, the long wavelength Q_y and Q_x transitions are examined (Gouterman, 1961; 1959). These electrically allowed transitions are $\pi-\pi^*$ in nature and are delocalized over the porphyrin chromophore. For each of the molecules considered here the Q_y band is lower in energy and has a greater absorption intensity (Table 1). The x and y axes are defined in Figure 2. Typical absorption and circular dichroism spectra are shown in Figure 3. The band assignments are from experimental studies of fluorescence polarization and linear dichroism (Goedheer, 1966; Gouterman and Stryer, 1962), and from the theoretical studies of Gouterman (1961; 1959). Each of the bands is slightly complicated by higher vibrational components. These components, however, are not strongly polarized (Gouterman, 1959), due to mixing with higher

Table 1. Experimental Values of Absorption and Rotational Strengths (in Ether)

| | | <u>Chl a</u> | <u>pyroChl a</u> | <u>BChl</u> | <u>pyroBChl</u> |
|---|-------|--------------|------------------|-------------|-----------------|
| λ_{\max} , nm | Q_y | 661 | 661 | 770 | 770 |
| | Q_x | 575 | 578 | 570 | 583 |
| ω_{\max} , cm^{-1} | Q_y | 15,130 | 15,130 | 12,987 | 12,987 |
| | Q_x | 17,390 | 17,300 | 17,540 | 17,150 |
| Band width, $\Delta\omega$, cm^{-1} | Q_y | 390 | 420 | 550 | 490 |
| | Q_x | 760 | 780 | 910 | 920 |
| $10^{-3} \epsilon_{\max}$, $(\text{mol/l.})^{-1} \text{cm}^{-1}$ | Q_y | 86.3 | 80 | 96 | 96 |
| | Q_x | 6.8 | 8 | 22 | 22 |
| Oscillator strength, $f = 4.33 \times 10^{-9} \int \epsilon(\omega) d\omega$ | Q_y | 0.155 | 0.155 | 0.309 | 0.30 |
| | Q_x | 0.024 | 0.029 | 0.110 | 0.110 |
| Rotational strength, $10^{40} R_A$, cgs | Q_y | -8.7 | -14.3 | 5.0 | |
| | Q_x | ~ 0 | ~ 0 | -4.4 | |

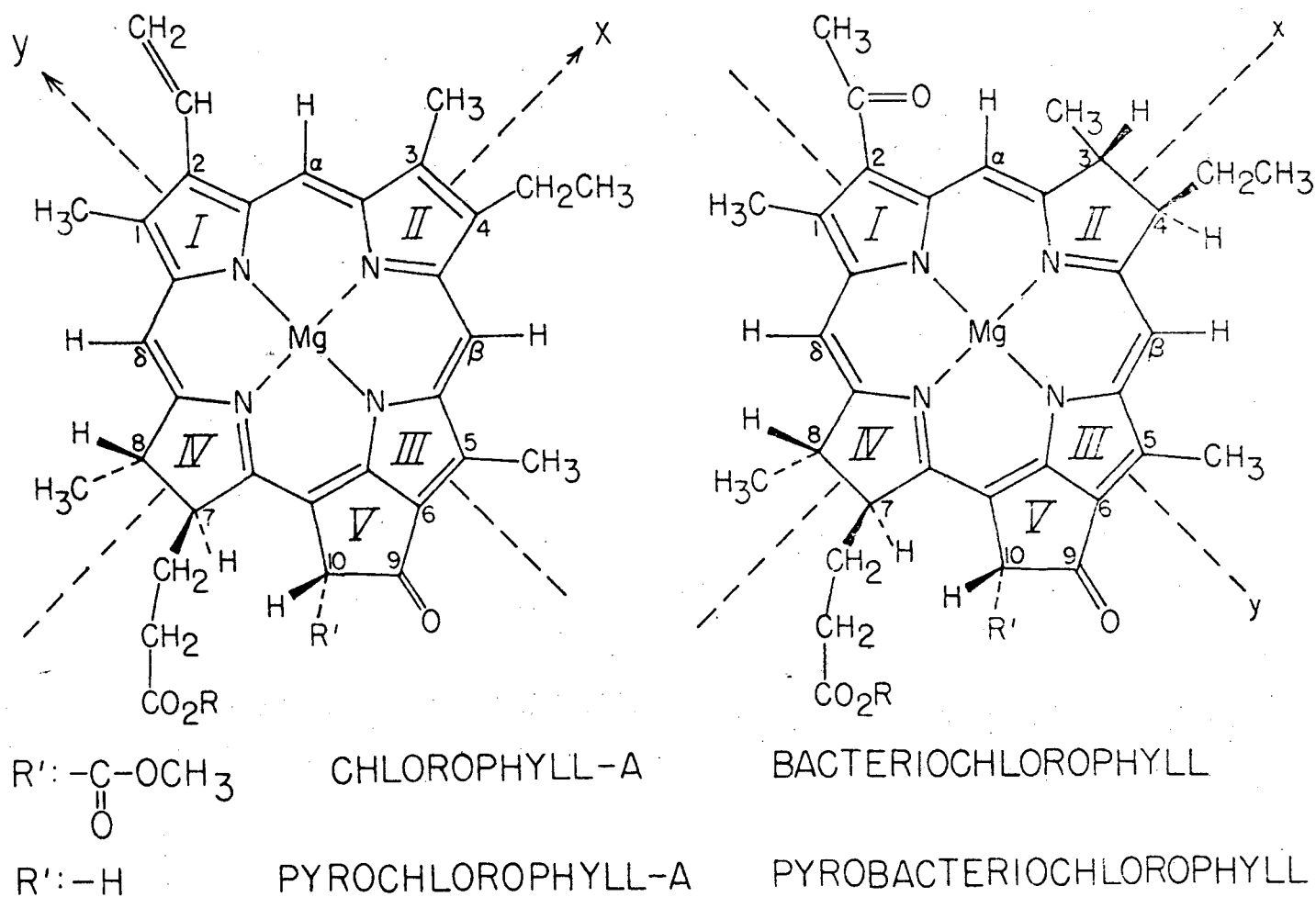
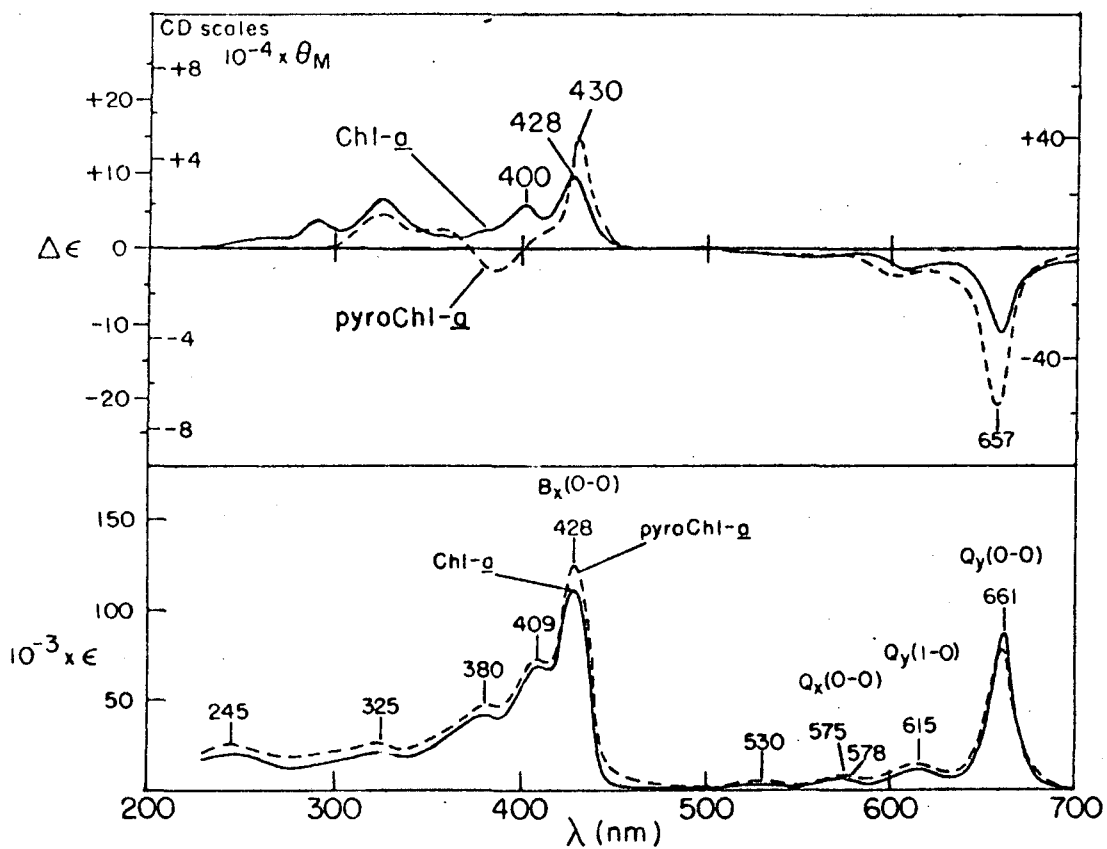


Figure 2. Molecular structures of Chl a, pyroChl a, BChl, and pyroBChl, showing the absolute configuration of the asymmetrically placed ring substituents, R = phytol.

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Optical activity of Chl-a and pyroChl-a in ether



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Figure 3. Circular dichroism and absorption spectra of Chl a and pyroChl A in ether (Houssier and Sauer, 1970).

electronic states. Because of this, the higher vibrational components tend not to contribute to the circular dichroism and are ignored in the calculations. The shorter wavelength Soret bands (occurring in the range 350-450 nm) overlap one another strongly, and no calculations were attempted on them.

The specific molecules for which calculations were done are chlorophyll a (Chl a), pyrochlorophyll a (pyroChl a), bacteriochlorophyll a (BChl), and pyrobacteriochlorophyll a (pyroBChl). Calculated rotational strength is compared with experimental circular dichroism (CD) results for the first three of these for both the Q_y and Q_x bands. The relevant experimental absorption and CD data for these molecules are shown in Table 1. The molecular structures of these molecules differ in two important respects: (1) the number of asymmetric substituents, and (2) the number of rings which have reduced outer bonds. As shown in Figure 2, BChl has five porphyrin ring carbon atoms substituted asymmetrically at positions C-3, C-4, C-7, C-8, and C-10. In pyroBChl, the carboxymethyl group is replaced with a hydrogen atom and so the C-10 position is no longer asymmetrically substituted. Likewise Chl a and pyroChl a are asymmetrically substituted at positions C-7, C-8, C-10, and at C-7, C-8, respectively. The absorption spectra of these molecules, in contrast to the CD, are relatively insensitive to the nature of the asymmetric substituents and are more a function of the extent of unsaturation of the pyrrole rings. Therefore, it is expected, and found experimentally, that the absorption spectrum of Chl a closely resembles that of pyroChl a. The same relationship holds between BChl and pyroBChl.

In a previous model used to calculate the interaction potential, $V_{ioa;job}$ was evaluated by treating the porphyrin transitions as point

dipoles, substituting a dipole-dipole interaction expression in Eq. II-8a and then using the Kirkwood polarizability approximation (Houssier and Sauer, 1970). The actual $\pi-\pi^*$ transitions involved, however, are delocalized over the entire porphyrin ring, which has a radius of the order of 5 Å, while the distance between the asymmetric centers and the nearest carbon of the aromatic part of the porphyrin ring can be as small as 2 Å. Thus, a more realistic attempt must take into account the effect of this delocalization of the transition on the interaction potential. For the results presented here, this was done by considering the transition dipole to be the sum of transition electric monopole moments located at each of the conjugated atoms of the ring. The potential can then be determined by treating separately the interaction of each of the monopoles of the porphyrin ring with the effective transition dipoles of the asymmetric centers according to the equation

$$V_{ioa;job} = - \sum_t \frac{q_{it0a} \underline{R}_{it0a;j} \cdot \underline{\mu}_{job}}{|\underline{R}_{it0a;j}|^3} \quad (\text{II-9})$$

where q_{it0a} is the electric transition monopole located at the t^{th} atom for transition $a \leftarrow 0$ (the Q_y or Q_x transition) for group i (the porphyrin ring) and $\underline{R}_{it0a;j}$ is a position vector from the j^{th} asymmetrically-placed substituent to monopole t .

The transition monopoles were obtained from the self-consistent field molecular orbital calculations of Weiss (unpublished data), who used the configuration interaction method of Pariser, Parr, and Pople (SCMO-PPP-CI) (Parr, 1963). Good qualitative agreement with the visible and near-UV absorption spectra was obtained. The monopole charge at the t^{th} atom of a π system is proportional to $C_{to} C_{ta}$ for a transition from

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the ground state to a singly excited state, a, where C_{to} and C_{ta} are, respectively, the molecular orbital coefficients at the t^{th} center for the ground- and excited-state molecular orbitals. The monopoles (Table 2) for BChl and pyroBChl were derived from a calculation for 2,6-dicarbonyl OPP-tetrahydroporphin and those for Chl a and pyroChl a from 2-vinyl-6-carbonylchlorin. The calculated monopoles were scaled so that the summation

$$\sum_t R_{it0a} q_{it0a}$$

equaled the experimental value for μ_{i0a} . In order to improve the approximation to the transition charge densities of π -type orbitals, each monopole strength was separated into two parts. New monopoles, with magnitudes equal to 1/2 those of the calculated monopoles, were placed 1 Å above and below the conjugated atom (Woody, 1968). The number of monopoles used, therefore, was actually twice the number of conjugated atoms. Molecular orbital calculations on protochlorophyll were not very successful. For this more symmetric molecule polarized transitions for the long wavelength absorption bands, in contrast to experiment (Houssier and Sauer, 1970), were not predicted and therefore a rotational strength calculation was not attempted.

If we now substitute Eq. II-9 into Eq. II-8a,

$$R_A = -\frac{2\pi}{c} \sum_{j \neq i} \sum_{b \neq a} \sum_t \frac{v_a v_b}{h(v_b^2 - v_a^2)} \frac{q_{it0a}}{|R_{it0a;j}|^3} \times$$

$$(\underline{R}_{it0a;j} \cdot \underline{\mu}_{job}) [\underline{\mu}_{job} \cdot (\underline{R}_{i0a;j} \times \underline{\mu}_{i0a})] \quad (\text{II-10})$$

we find that any calculation would be extremely difficult, because knowledge of the transition dipoles of each of the asymmetric groups, j, for

Table 2. Atomic Coordinates and Transition Monopoles

| Atom ^a | Coordinates, Å, for methyl pheophorbide ^b | Transition monopoles ^c | | | |
|------------------------------|---|-----------------------------------|----------------|----------------|----------------|
| | | BChl | | Chl a | |
| | | Q _x | Q _y | Q _x | Q _y |
| N ₁ | (0.20, -2.01, 0.02) | -0.035 | 0.007 | -0.049 | 0.001 |
| N ₂ | (2.10, 0.00, -0.04) | 0.001 | -0.115 | 0.005 | -0.055 |
| N ₃ | (-0.13, 2.07, -0.04) | 0.035 | -0.007 | 0.047 | 0.004 |
| N ₄ | (-2.16, -0.06, 0.06) | -0.001 | 0.115 | 0.004 | 0.109 |
| C _α | (2.33, 2.46, -0.03) | 0.035 | -0.129 | 0.107 | -0.112 |
| C _β | (2.63, -2.40, 0.03) | -0.033 | -0.124 | -0.086 | -0.136 |
| C _γ | (-2.30, -2.45, -0.02) | -0.035 | 0.129 | -0.054 | 0.108 |
| C _δ | (-2.57, 2.35, 0.03) | 0.033 | 0.123 | 0.044 | 0.109 |
| C ₁ | (-0.85, 4.25, 0.02) | -0.051 | 0.037 | -0.039 | 0.029 |
| C ₂ | (0.52, 4.26, -0.01) | -0.046 | -0.035 | -0.028 | -0.041 |
| C ₃ | (4.26, 0.83, 0.07) | 0.000 | 0.000 | 0.040 | 0.027 |
| C ₄ | (4.36, -0.54, 0.07) | 0.000 | 0.000 | -0.046 | 0.017 |
| C ₅ | (0.80, -4.20, -0.02) | 0.051 | -0.037 | 0.045 | -0.037 |
| C ₆ | (-0.61, -4.11, -0.03) | 0.046 | 0.035 | 0.036 | 0.031 |
| C ₇ | (-4.83, -0.92, 0.30) | 0.000 | 0.000 | 0.000 | 0.000 |
| C ₈ | (-4.46, 0.61, 0.01) | 0.000 | 0.000 | 0.000 | 0.000 |
| C ₉ | (-1.83, -4.88, -0.09) | 0.000 | 0.001 | -0.001 | 0.002 |
| C ₁₀ | (-3.02, -3.81, -0.13) | 0.000 | 0.000 | 0.000 | 0.000 |
| C ₁₁ | (-0.93, -2.73, -0.02) | 0.081 | -0.078 | 0.067 | -0.079 |
| C ₁₂ | (1.30, -2.89, 0.02) | 0.091 | 0.070 | 0.075 | 0.059 |
| C ₁₃ | (2.99, -1.06, 0.01) | 0.112 | 0.055 | 0.069 | 0.062 |
| C ₁₄ | (2.85, 1.13, 0.01) | -0.103 | 0.063 | -0.084 | 0.050 |
| C ₁₅ | (1.00, 2.87, -0.02) | -0.081 | 0.078 | -0.085 | 0.050 |
| C ₁₆ | (-1.26, 2.84, 0.00) | -0.091 | -0.070 | -0.075 | -0.085 |
| C ₁₇ | (-3.01, 1.00, 0.04) | -0.112 | -0.055 | -0.111 | -0.058 |
| C ₁₈ | (-2.92, -1.19, 0.08) | 0.103 | -0.063 | 0.106 | -0.056 |
| C ₁₉ | (1.43, 5.43, 0.00) | 0.000 | -0.001 | 0.000 | 0.000 |
| C ₂₀ ^d | (1.12, 6.61, -0.22) | -0.015 | -0.008 | 0.000 | 0.000 |
| O ₁ | (-2.01, -6.09, -0.11) | 0.015 | 0.008 | 0.019 | 0.010 |

^aSee Figure 2 for numbering system. ^bSee Fischer (1969). ^cFrom Weiss (unpublished data).

^dC₂₀ is replaced with an oxygen in BChl.

all transitions, $b \neq 0$, is needed. A majority of these transitions occur in the far-ultraviolet region and have never been classified. To overcome this, we use Kirkwood's polarizability approximation

$$(2/h\nu_0) \sum_b \underline{\mu}_{j0b} \underline{\mu}_{j0b} = (\alpha_{33} - \alpha_{11})_j \underline{e}_j \underline{e}_j \quad (\text{II-11})$$

where ν_0 is an average frequency of the transitions, α_{33} and α_{11} are the polarizabilities parallel and perpendicular to the axis of symmetry for each group (assuming cylindrical symmetry), and \underline{e}_j is a unit vector pointing along the axis of cylindrical symmetry. Each covalent bond in an asymmetric center is considered in the calculations to be a group, j . A polarizability anisotropy $(\alpha_{33} - \alpha_{11})_j$ is associated with each bond, and its magnitude is based on values found in the literature. By considering each bond separately, and therefore distributing each asymmetric substituent in space, better results can be expected than if each group is approximated by a single polarizability value. Bond polarizabilities are difficult to determine and considerable variation in values is found in the literature. The values used for C-H (Vickery and Denbigh, 1949; Yoshino and Bernstein, 1958; Amos and Hall, 1966) C-C (Bunn and Daubeny, 1954; LeFevre and LeFevre, 1955), C=O (LeFevre and LeFevre, 1955; Denbigh, 1940), and C-O (LeFevre and LeFevre, 1955; Denbigh, 1940) bonds are shown in Table 3 and are judged to be the best available. By putting Eq. II-11 into II-10 and by using the good approximation

$$\frac{\nu_0^2}{\nu_0^2 - \nu_a^2} \approx 1$$

We obtain our final equation

$$R_A = -\frac{\pi}{c} \sum_{j \neq i} \sum_t \frac{v_a^{q_{i\text{toa}}}}{|R_{i\text{toa};j}|^3} (\alpha_{33} - \alpha_{11})_j \times$$

$$(\underline{R}_{i\text{toa};j} \cdot \underline{e}_j) [\underline{e}_j \cdot (\underline{R}_j - \underline{R}_{i\text{oa}}) \times \underline{\mu}_{i\text{oa}}] \quad (\text{II-12})$$

Table 3. Bond-Bond Polarizabilities

| Bond | Polarizability anisotropy $10^{24} (\alpha_{33} - \alpha_{11}), \text{cm}^3$ |
|------|---|
| C-C | 0.71 |
| C=O | 1.24 |
| C-O | 0.96 |
| C-H | -0.312 |

Coordinates for the molecules (Table 2) are taken from a recent crystal structure determination of methylpheophorbide a (Fischer, 1969) (Chl a with the central Mg atom removed and with a methyl group replacing the phytol chain). Thus, the geometry of the side groups in solution is taken to be the same as that in the crystal. Although this may seem to be a crude approximation, molecular models show that each side group has significant steric hindrances. Thus, the assumption used is that, even in solution, the side groups are unable to rotate freely. Because Eq. II-12 predicts a dependence of rotational strength on the inverse square of the distance of separation, the part of the side group which makes the greatest contribution to the optical activity is expected to be that which is closest to the conjugated ring, and it is precisely this part of the group which will feel the bulk of the steric forces.

Calculations in which all side groups were allowed to rotate freely (Table 4) were also attempted. In all cases the agreement with experiment was not so good as that in which the "crystal conformation in solution" was assumed. In certain cases the results did not even agree with the sign of the experimental rotational strength.

The phytol chain is ignored in the calculations. This probably introduces no serious error since this substituent is a larger distance from the porphyrin ring than any other group, thereby decreasing its interaction potential. Evidence from NMR measurements (Sauer et al., 1966; Closs et al., 1963) indicates that the phytol does not strongly interact with the other substituents. Measurements in this laboratory show that the CD of methylchlorophyllide a is identical with that of Chl a, implying that the phytol may safely be ignored in a theoretical treatment. This result would seem to resolve a conflict between the results of Houssier and Sauer (1970) on pheophytin a and those of Briat et al. (1967), on methylpheophorbide a (pheophytin a with a methyl replacing the phytol). The difference in the magnitude of the CD reported for these molecules can most likely be attributed to a calibration problem of one of the spectrometers or to a partial epimerization of the substituents at C-10, as has already been suggested (Houssier and Sauer, 1970).

2. Results

Results of the calculations are given in Table 4. All experimental measurements (Houssier and Sauer, 1970; Coyne, unpublished data) were made on solutions in ether and, to account for the dielectric effect of the solvent (Moscowitz, 1962), all calculated rotational strengths were

Table 4. Comparison of Calculated and Experimental Rotational Strengths

| Molecule | Transition | Point dipole model, ^a $R_A \times 10^{40}$ cgs | Point monopole model | | | | | Calculated sum, $R_A \times 10^{40}$ cgs | Experimental, $R_A \times 10^{40}$ cgs |
|---|------------|---|--|-------|------|------|------|--|--|
| | | | Calculated $R_A \times 10^{40}$ cgs contribution for each asymmetry center | | | | | | |
| | | | C-3 | C-4 | C-7 | C-8 | C-10 | | |
| BChl | Q_y | +1.8 | 1.9 | 1.9 | -3.3 | -2.3 | 5.2 | 3.4 | 5.0 |
| | Q_x | -1.0 | -0.005 | -0.31 | 0.14 | 0.20 | -3.1 | -3.1 | -4.4 |
| pyroBChl | Q_y | 0 | 1.9 | 1.9 | -3.3 | -2.3 | | -1.8 | |
| | Q_x | 0 | -0.005 | -0.31 | 0.14 | 0.20 | | 0.03 | |
| Chl a | Q_y | 0.5 | | | -2.9 | -2.1 | 3.6 | -1.4 | -8.7 |
| | Q_x | 0.02 | | | 0.07 | 0.1 | -1.6 | -1.4 | ~0 |
| pyroChl a | Q_y | -0.4 | | | -2.9 | -2.1 | | -5.0 | -14.3 |
| | Q_x | 0.3 | | | 0.07 | 0.1 | | 0.2 | ~0 |
| BChl (monopoles in plane) | Q_y | | 3.2 | 3.9 | -5.0 | -4.1 | 5.2 | 2.8 | 5.0 |
| Chl a (with BChl monopoles) | Q_y | | | | -1.6 | -1.0 | 2.4 | -0.2 | -8.7 |
| BChl (with freely rotating side groups) | Q_y | | 0.1 | 0.8 | -0.4 | -0.8 | 0.4 | 0.1 | 5.0 |

^aCalculated from data in Houssier and Sauer (1970).

multiplied by $(n^2 + 2)/3$, where n is the refractive index of the solvent. In all cases there is qualitative agreement between the theoretically derived rotational strengths and the experimental values. In the previous study (Houssier and Sauer, 1970), in which the Q transitions were approximated by a point dipole, the experimental measurements gave larger rotational strengths than the calculations by factors from 8 to 40. In one case (Chl a; Q_y transition) the wrong sign was predicted. In the point monopole calculations (Table 4) the range of discrepancies is reduced to 1.5-6 and the signs are all correctly predicted. Most of the improvement reported here can be attributed to the use of monopoles and the use of a fixed geometry for the substituents. It is clear that in calculations such as these, a highly delocalized transition cannot be accurately represented as a point dipole.

Results are also presented for a calculation in which all monopoles were placed in the plane of the chromophore (for all other calculations, monopoles were divided by two and placed 1 \AA above and below the plane; see above). It is seen that this can change the contribution of an individual asymmetric center by as much as a factor of 2. The implication from this is that if the true wave function (with the transition charge densities distributed continuously through space) were used to calculate the interaction potential, the result would be an improvement over that obtained by using monopoles (even if the monopoles were an extremely accurate set). That is, there is still an inherently large approximation in the use of point monopoles.

A calculation is presented for Chl a in which BChl monopoles were used. This changes the result by a large amount and appreciably reduces the agreement with experiment. Since Chl a and BChl are closely related

molecules with monopole patterns which are rather similar, this test shows that relatively small changes in monopoles can cause large changes in the calculated rotational strengths. Fairly accurate monopoles are, therefore, essential for this type of calculation.

Attempts were made to prepare and to obtain a CD spectrum for pyroBChl. For this molecule the contribution to the rotational strength induced by the groups at C-3 and C-4 is expected approximately to cancel the contributions of the asymmetric centers at C-7 and C-8 (these groups are related by an approximate center of inversion) and a small CD signal is expected. However, the rotational strength of the Q_y transition of pyroBChl was observed to be generally greater than that for BChl and with a large variability from sample to sample. Although the infrared spectrum confirmed the absence of the C-10 carboxymethyl group following pyrolysis and the visible and near-infrared absorption was virtually indistinguishable from that of BChl, the CD results lead us to doubt whether we had the correct molecule. As a consequence, the experimental rotational strengths for pyroBChl are not included in Table 4.

3. Conclusions

The nature of the optical activity of the chlorophyll molecules studies can now be considered to be understood qualitatively. The general features of the CD spectra can be accounted for in terms of the interactions described by the Kirkwood-Tinoco approach. It is possible that this type of calculation may prove useful in assigning absolute configurations to molecules where this information is not known.

The origin of the circular dichroism of chlorophylls in photosynthetic membranes differs from that described here. Exciton interactions

between chlorophylls cause rotational strengths of a much larger magnitude than those exhibited by monomers. Nevertheless, the theory for such interactions has much in common with that presented here. The results presented above thus make calculations on more complicated systems feasible.

III. EXCITON INTERACTION IN A BACTERIOCHLOROPHYLL-PROTEIN
FROM CHLOROPSEUDOMONAS ETHYLICA

Photosynthetic organisms typically contain one or more types of chlorophyll-related molecules incorporated into a protein or a lipoprotein matrix. The nature of the binding of the chlorophyll and the relative orientations and distances between chlorophylls inside the protein are largely unknown. Questions closely related to these relationships are the strength of the interaction between chlorophylls and the mode of electronic excitation energy transfer in vivo. Since the detailed structure of no chlorophyll-protein is known, models for the pigment-lipoproteins have been constructed primarily on the basis of theoretical considerations (Weier et al., 1966; Kreutz, 1970).

The chlorophyll-protein which has been studied in most detail and which has been best characterized is the bacteriochlorophyll-protein (BChl-P) complex from the green photosynthetic bacterium, Chloropseudomonas ethylica (Cps. ethylica) (Olson, 1966; Thornber and Olson, 1968; Olson, 1971). This BChl-P is crystallizable and has four subunits, each containing five bacteriochlorophyll a (BChl a) molecules. The complex is blue-green in color and has a molecular weight of 152,000. In vivo the BChl a in the protein accepts excitation energy from chlorobium chlorophyll (which forms the bulk of the antenna pigment in green bacteria) and transfers this energy to the reaction center bacteriochlorophyll, P840.

The BChl-P from Cps. ethylica is water soluble and, unlike most chlorophyll-containing proteins, can be isolated without the use of solubilizing detergents, which might alter its structure. Electron microscopy of BChl-P crystals (Labaw and Olson, 1970) suggests that the shape of the protein is a noncompact sphere of 81.5 Å. The resistance of the BChl-P to pheophytinization at low pH implies that the BChl a molecules are imbedded within the protein (Ghosh et al., 1968) rather than located on its surface. The BChl a molecules are apparently bound by peptide chain folding and not by covalent bond linkages (Thornber and Olson, 1968).

In this section we present the results of optical studies on the BChl-P. Absorption and CD spectra at room temperature and at liquid nitrogen temperature have been measured for the long-wavelength Q_y and Q_x transitions of the BChl a chromophores. The fact that BChl-P has been relatively well characterized enables us to interpret the optical spectra with some confidence. The resolving power of CD, especially at liquid nitrogen temperature, gives direct evidence for the presence of five components in the Q_y (809 nm) transition of the BChl-P. The five transitions are interpreted as the result primarily of exciton interactions among the five BChl a molecules located within each of the subunits of the protein. From the magnitude of the splitting between component peaks of the absorption band we estimate the distances between BChl a molecules to be 12-15 Å. These results imply that, at least within a subunit of the BChl-P, energy transfer is by exciton coupling rather than by a Förster resonance transfer mechanism (Kasha, 1963).

1. Materials and Methods

The BChl-P from Cps. ethylica was prepared (Olson, 1971) in the laboratory of J. M. Olson and was stored as a solid precipitate in an ammonium sulfate solution. Before use, the sample was resuspended in 0.01 M Tris buffer (pH 7.5) with a salt concentration of 0.2 M NaCl. The absorbance ratio A_{809}/A_{371} has been used as a criterion for the integrity of the complex (Olson, 1971), and for all spectra shown here this ratio was 2.15 or larger. Concentrations were determined by using $154 \text{ mM}^{-1} \text{ cm}^{-1}$ as the extinction coefficient of the 809 nm peak (Olson, 1966). The BChl-P demonstrates a marked hyperchromism for the transition at 809 nm. The measured oscillator strength is 0.40 (Olson, 1966) per BChl a molecule, whereas this number is 0.309 for BChl a in solution (Philipson et al., 1971). This is presumably due to interactions of the BChl a molecules with the surrounding environment (Rhodes, 1961).

In order to obtain satisfactory spectra at 77°K, a solvent must be used which forms a clear glass and does not denature the protein or otherwise introduce artifacts into the CD spectrum. A solvent which meets these criteria is a mixture of 75% potassium glycerophosphate in water (K & K Laboratories)-aqueous solution containing the sample of interest-glycerol (Baker analyzed reagent) (2:1:0.5) (Vredenberg, 1965). For the 77°K spectra reported here, samples were immersed rapidly in liquid nitrogen.

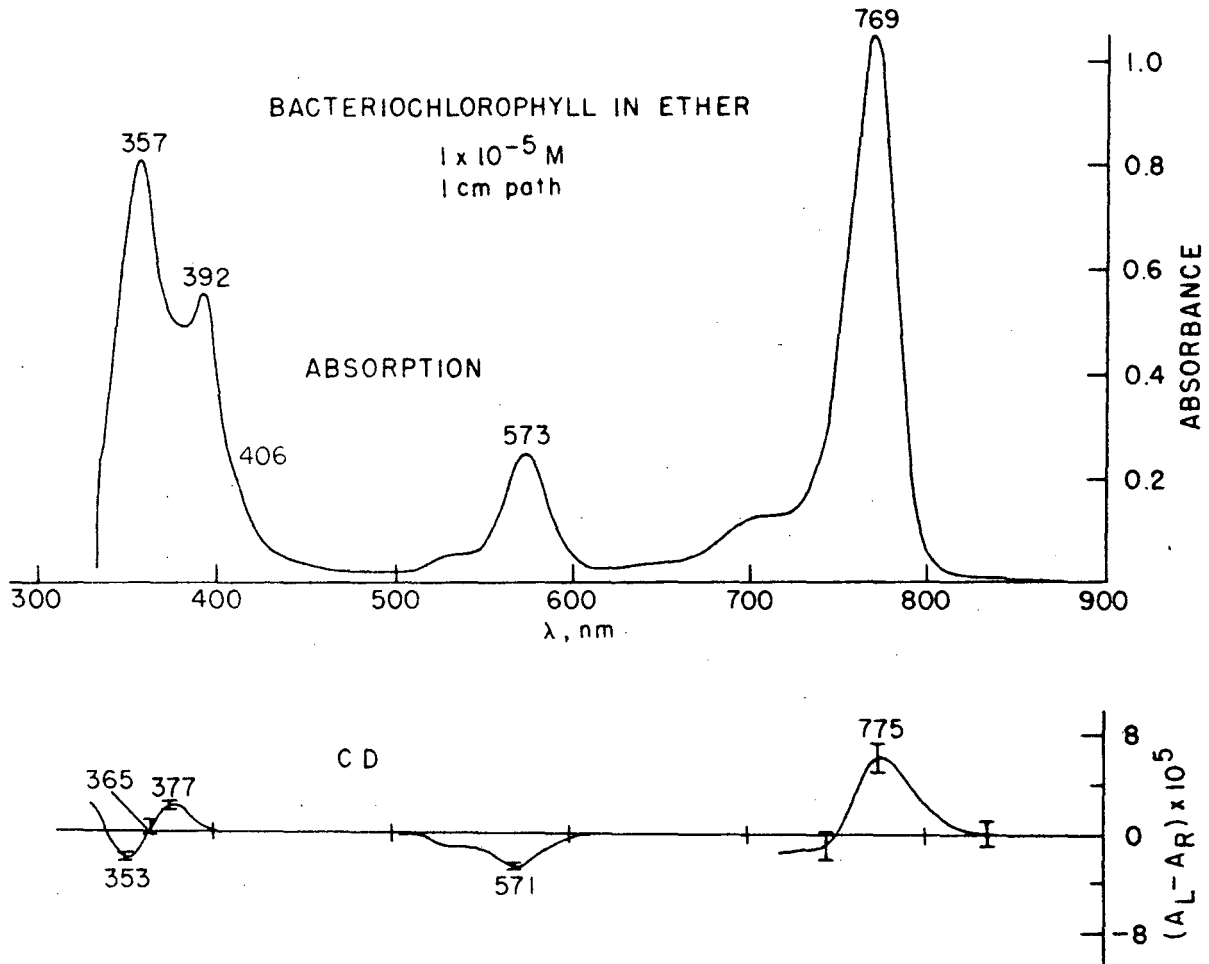
Unfortunately, the BChl-P is of limited solubility in this solvent and CD spectra of the weak Q_x band of the BChl-P could not be obtained at 77°K. A 50% mixture of glycerol and water (a common solvent for low-temperature absorption measurements on biological materials) was

unsuitable for CD measurements, as it produced a large baseline shift and excessive noise as the temperature approached 77°K. The CD spectrum of the Q_x band of the BChl-P at -120°C was taken in glycerol-water. At this temperature the baseline shift did not occur.

All absorption spectra were recorded on a Cary 14R spectrophotometer. The CD spectra in Figures 4, 5, and 6 were recorded on an instrument built in this laboratory (Dratz, 1966). The CD spectrum in Figure 7 was recorded on a Durrum-Jasco J-20 CD spectrometer with far-red sensitivity extended to 1000 nm. The magnitude of the CD signals was calibrated with an aqueous (1 mg/ml) solution of (d)-10-camphorsulfonic acid. For all spectra the spectral bandwidth was 3 nm or less.

2. Results

The CD and absorption spectra of BChl a in its monomeric form in ether are shown in Figure 4. The long-wavelength Q_y transition at 769 nm and the Q_x transition at 573 nm are electrically allowed and are polarized nearly perpendicularly to one another. The band assignments are from fluorescence polarization measurements (Goedheer, 1966) and from theoretical considerations (Gouterman, 1961). The short wavelength shoulder on each band is due to higher vibrational components. The separation of these electronic transitions from other absorption bands enables the effects of perturbations to be studied without the complications of band overlap. The CD band in the Q_y region of the BChl a spectrum is a weak positive peak barely above the noise level of the measurement. The Q_x CD is a small negative peak. The small rotational strengths observed are the result of perturbations supplied by interactions of the asymmetrically placed side groups with the inherently symmetric porphyrin chromophore (Houssier and Sauer, 1970; Philipson et al., 1971).

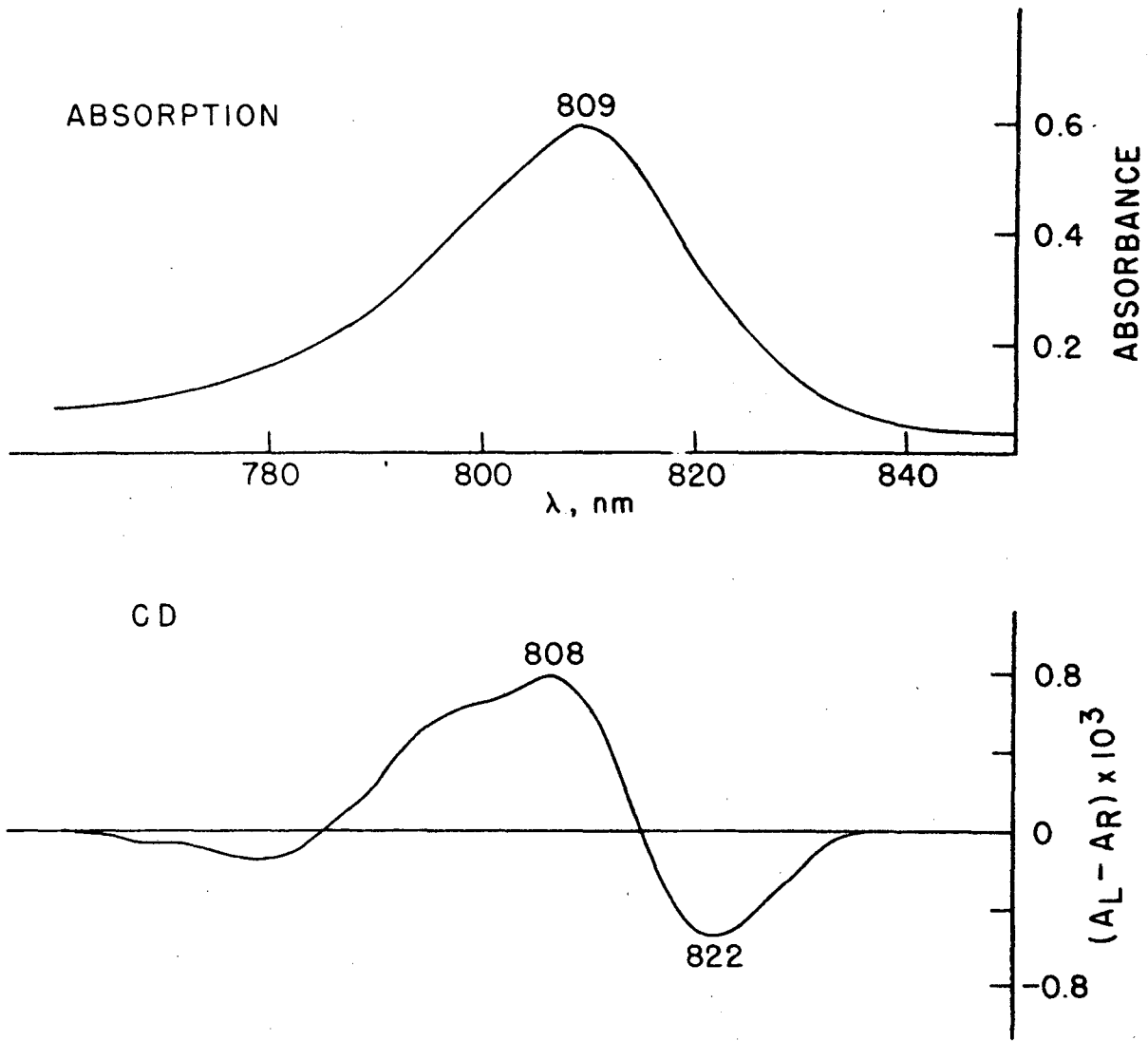


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Figure 4. Absorption and CD spectra of BChl a in ether at room temperature; concentration 1×10^{-5} M and path length 1 cm (Coyne, unpublished data, 1968).

In Figures 5 and 6 we show the absorption and CD of the Q_y transition of BChl-P at room and liquid nitrogen temperatures, respectively. A detailed interpretation of these spectra is reserved for a later section but several features are of note. The absorption peak in Figure 5 is shifted to longer wavelength (809 nm) compared to that of BChl a in solution (Figure 4), but the shape of the absorption band at room temperature is little altered by the change in environment. The CD, however, has changed drastically; its magnitude is greater in the BChl-P by more than an order of magnitude, and three components are clearly visible (at 822, 808, and 778 nm). A similar spectrum has previously been reported (Kim and Ke, 1970). At 77°K (Figure 6) increased resolution uncovers more structure. Absorption reveals four resolved components (at 789, 804.5, 813.5, and 824.5 nm) (Olson, 1971); the Q_y transition of monomeric BChl a in solution shows no hint of having more than one peak at 77°K (Olson, 1966). At 77°K the CD spectrum exhibits four components (786, 799.5, 811.5, and 823 nm) and a shoulder (817 nm) implying the existence of a fifth component. The magnitude of the CD for BChl-P at 77°K is more than double that at room temperature. This is mostly due to band narrowing which increases the amplitudes and decreases the overlap of neighboring positive and negative peaks.

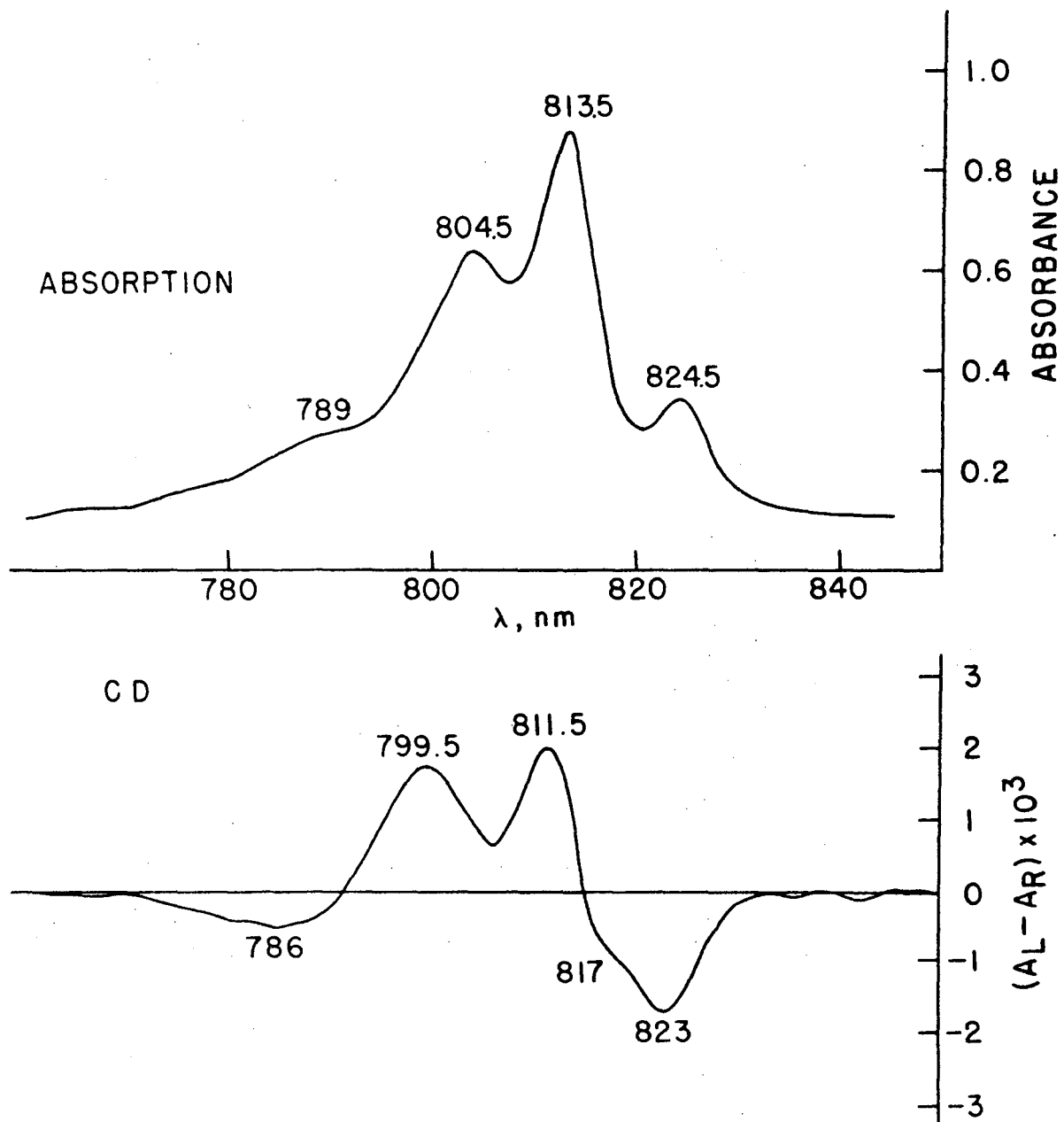
Similar effects are seen in the weaker Q_x band (603 nm) of the BChl-P at room temperature (Figure 7). The single small negative CD peak of BChl a in ether (Figure 4) is replaced by a complex band of three components (593, 605, and 616 nm). The effects of temperature lowering are not so drastic in this case as with the Q_y band. At temperatures down to -120° the CD shows no evidence of additional peaks.



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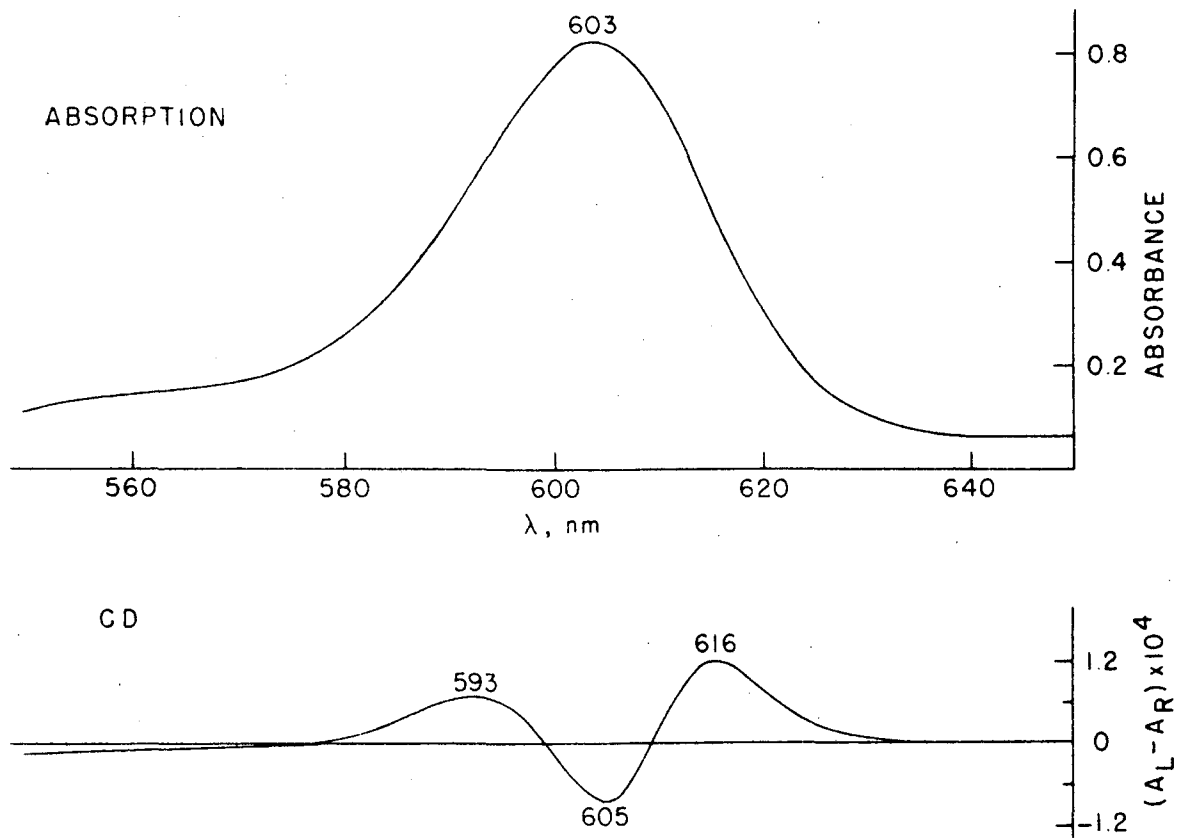
Figure 5. Absorption and CD spectra of Q_y band of BChl-P at room temperature in potassium glycerophosphate, Tris buffer (pH 7.5, 0.2 M NaCl), glycerol; BChl a concentration 1.3×10^{-5} M and path length 3 mm.

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Figure 6. Absorption and CD spectra of Q_y band of BChl-P at 77°K in potassium glycerophosphate, Tris buffer (pH 7.5, 0.2 M NaCl), glycerol; BChl a concentration 1.3×10^{-5} M (uncorrected for solvent shrinkage) and path length 3 mm.



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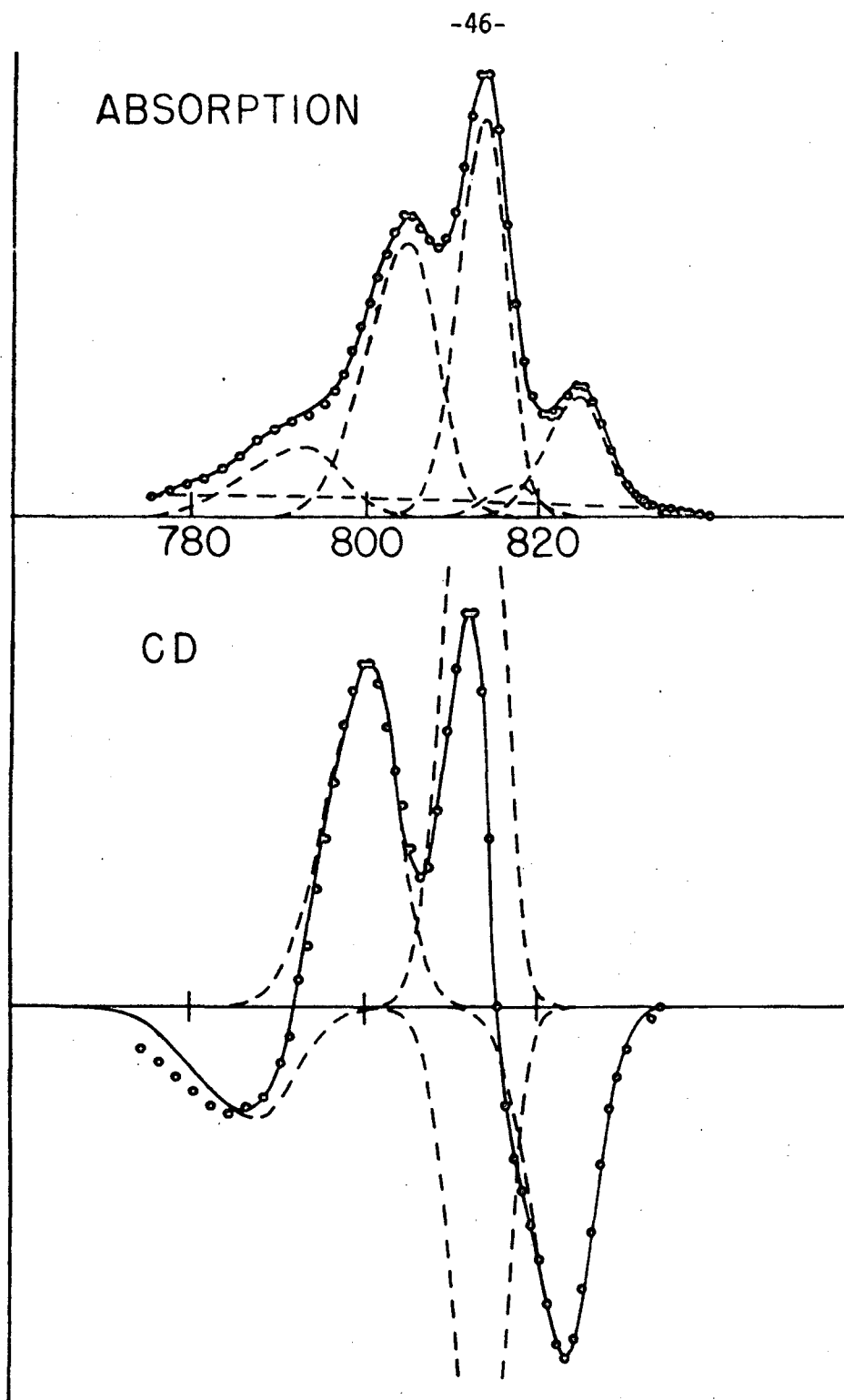
Figure 7. Absorption and CD spectra of Q_x band of BChl-P at room temperature in glycerol, Tris buffer (pH 7.5, 0.2 M NaCl); BChl a concentration 5.2×10^{-5} M and path length 5.7 mm.

The absorption at liquid nitrogen temperature shows the main peak with weak shoulders on both its long- and short-wavelength sides (Olson, 1971).

Comparison of the spectral properties of the BChl-P with the BChl a monomers makes it clear that specific interactions with the protein matrix are perturbing the BChl a chromophores.

The CD of the BChl-P shows small variations from one sample to another with respect to exact position and magnitude of its component peaks. We attribute this to small conformational differences of the protein (see Discussion).

The absorption and CD spectra of the Q_y band of the BChl-P at 77°K (Fig. 6) were computer resolved (see Appendix) into component peaks (Fig. 8 and Table 5). Since the absorption peak of monomeric solution BChl a is asymmetric (the half-width of the short-wavelength side is significantly larger than the long-wavelength side), gaussian curves of asymmetric shape were used for the fitting. The computer program did simultaneous fitting of the absorption and CD. Initially we required that each absorption component have a corresponding CD component at the same wavelength and of the same half-width. With these restrictions we were unable to obtain a precise fit of the experimental data. The requirement that the centers of the absorption and CD components occur at the same wavelength was then relaxed, and it was required only that corresponding absorption and CD components have the same half-width and skew (a measure of the asymmetry of the gaussian curve used for the fit). In this manner the computer fit shown in Figure 8 was obtained. The long sloping line in the upper half on Figure 8, which is the tail of a gaussian curve centered at 770 nm, is intended to represent the contributions from the absorption of higher vibrational components and from a small amount of scattering. It is needed to improve the fit in the



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Figure 8. The absorption and CD of the Q_y band of the BChl-P at 77°K resolved into asymmetric gaussians. See text and Table 5. Experimental data, \bullet ; gaussian component, ---; sum of gaussians, —.

Table 5. Parameters of the Computer-Resolved Components of the Absorption and CD Spectra of the Q_y Band of the BChl-P at 77°K (see Figure 8) and Data on the Q_y Band of BChl a in Acetone at 299 and 180°K.

| λ (nm) | | Dipole strength ^a (debye ²) | Rotational strength ^a (debye magneton) | Full-width at half-maximum (nm) | Skew ^b |
|---|-----|---|--|------------------------------------|-------------------|
| Abs | CD | | | | |
| <u>BChl-P at 77°K</u> | | | | | |
| 792 | 787 | 9 | -0.6 | 13.2 | 0.65 |
| 804 | 800 | 26 | 1.3 | 9.8 | 0.65 |
| 813 | 812 | 25 | 2.5 | 6.6 | 0.75 |
| 817 | 814 | 2 | -1.6 | 6.6 | 0.65 |
| 824 | 823 | 9 | -1.2 | 8.3 | 0.82 |
| <u>BChl a in Acetone at 299°K^c</u> | | | | | |
| 770 | 772 | 49 | 0.06 | 44.0 | 0.69 |
| <u>BChl a in Acetone at 180°C^c</u> | | | | | |
| 771 | | 50 | | 36.5 | 0.74 |

^aPer molecule of BChl a. ^bHalf-width of long-wavelength side of gaussian = (skew) x (half-width of short-wavelength side).

^cJ. R. Lindsay and K. Sauer, 1966, unpublished data.

short wavelength region of the absorption spectrum. The CD spectrum also exhibits its poorest fit in the short wavelength region. As seen in Table 5, the gaussian curves used to fit the shortest wavelength absorption and CD components are of a much larger half-width (13.2 nm) than needed to fit any of the other components. This is probably due to the effect of the higher vibrational components. For all five of the major components the CD component was placed by the computer a few nanometers (from 1 to 5 nm) to shorter wavelength than the corresponding absorption component. This is outside the range of likely discrepancies in the wavelength calibration of the spectrometers used. It has been found in this laboratory that the position of absorption and CD bands for monomeric chlorophylls, where there is only one component to the electronic transition band, do not always correspond; the direction of the discrepancy varies from case to case (see the spectra in Houssier and Sauer, 1970, for examples).

3. Discussion

The multiple components in the absorption and CD spectra of the BChl-P are most readily interpreted in terms of an exciton model. We define an exciton state as the collective excitation of an assembly of molecules, by contrast with the localized excitation of each individual member of the assembly. This is the molecular exciton model of Kasha (Kasha et al., 1965), first formally developed by Davydov (1948). The like molecules are resonantly coupled to one another so strongly that it is not possible to excite one molecule individually. The source of this coupling is in electrostatic interactions between transition charge distributions usually approximated as transition dipole-transition dipole

interactions. These interactions result in the splitting of energy levels, where the number of new levels equals the number of molecules interacting. Each of these energy levels will have a characteristic transition probability; i.e., the dipole strength will be redistributed among these new energy levels as determined by the geometrical relationships among the molecules (Kasha, 1963; Kasha et al., 1965).

The equations for calculating these exciton spectral properties (assuming that the spectroscopic properties of the isolated molecule are known) are relatively straightforward (Tinoco, 1963). The wave function for an exciton state is written as a linear combination states in which the excitation is localized on one of the molecules of the aggregate:

$$\psi_K = \sum_{j=1}^N C_{jK} \psi_j \quad (K = 1, \dots, N) \quad (\text{III-1})$$

where N equals the number of identical molecules in the assembly, ψ_K is the wave function for each of the K exciton states, ψ_j is the wave function for the assembly with the excitation localized on the j^{th} molecule with all the other molecules in their ground state. The coefficients C_{jK} and the frequency of transition, ν_K , of each of the exciton states are obtained as the eigenvalues and eigenvectors of the perturbation matrix, V_{ij} , whose elements are the transition interaction energies among all of the like molecules.

Once the perturbation matrix has been solved and the energy levels obtained, the optical properties of the molecular aggregate can be characterized by the dipole strength, D_K , and rotational strength, R_K at each frequency ν_K :

$$D_K = \underline{\mu}_K \cdot \underline{\mu}_K = \left(\sum_{j=1}^N C_{jK} \underline{\mu}_j \right) \cdot \left(\sum_{j=1}^N C_{jK}^* \underline{\mu}_j \right) \quad (\text{III-2})$$

$$R_K = \text{Im } \underline{\mu}_K \cdot \underline{m}_K = \text{Im} \left(\sum_{j=1}^N C_{jK} \underline{\mu}_j \right) \cdot \left\{ \sum_{j=1}^N C_{jK}^* (\pi \nu_0 i/c) (\underline{R}_j \times \underline{\mu}_j) \right\} \quad (\text{III-3})$$

where $\underline{\mu}_j$ is the transition electric dipole moment of molecule j , Im means the imaginary part of, \underline{m}_K represents the transition magnetic moment for the K^{th} transition level of the aggregate, ν_0 is the molecular absorption frequency before exciton interaction, and \underline{R}_j is a vector to molecule j from an arbitrary origin.

As seen from the vector dot and cross products in Eq. III-3, the CD band resulting from exciton interaction will be highly dependent on the specific geometric arrangement of the interacting molecules. This equation represents a new source of rotational strength which has been introduced to the system; the CD is now more a function of the asymmetrical arrangement of the interacting molecules of the aggregate than of the inherent asymmetry of the individual molecules. The CD spectrum over the electronic transition band is now characterized by having multiple components of both positive and negative sign. The exciton contribution to the rotational strength is conservative (Tinoco, 1962), meaning that the rotational strength must sum to zero over the exciton band (i.e., $\sum_K R_K = 0$). Thus, it is characteristic of exciton interactions that the different components within the one electronic band will be of opposing sign.

Other contributions, besides exciton interaction, to the rotational strength of the BChl a transitions within the protein include the asymmetry of the individual molecules (roughly of the same magnitude and sign as seen in Figure 4) and asymmetric interactions of each of the BChl a molecules with transitions in the protein matrix and with non-degenerate transitions in the other BChl a molecules. The magnitudes and signs of these last contributions are difficult to estimate (Hsu and Woody, 1971a,b) without knowing the detailed molecular structure of the BChl-P, but evidence is given below which implies that they are not the major source of the BChl-P rotational strength.

The absorption bands which result from exciton interaction also depend on geometrical considerations. This dependence is through the perturbation matrix, V_{ij} , which determines the coefficients, C_{jK} , in Eq. III-2. The effect of the exciton interaction is to remove the degeneracy which exists among the like molecules and to create new energy levels. The number of component peaks observed will be equal to or less than (in the case where adjacent components are not sufficiently resolved from one another) the number of molecules interacting (or, more rigorously, the number of molecules per unit cell in the lattice (Hochstrasser and Kasha, 1964)). Each allowed component will have its own characteristic transition probability proportional to D_K in Eq. III-2. Thus, another characteristic of exciton interaction is the redistribution of dipole strength amongst the exciton energy levels dependent on the specific geometry under consideration.

As mentioned above, the interaction potential is most simply calculated by a point dipole approach. That is, the porphyrin transitions are approximated as point dipoles located at the ring center and the

interaction with other point dipoles or other molecules is then calculated. The $\pi-\pi^*$ transitions actually involved, however, are delocalized over the entire porphyrin ring with radius of the order of 5 Å. Thus, more realistic calculations must take into account the effect of this delocalization on the interaction potential. In the calculations to be presented below this was done by considering the transition dipole to be the sum of transition electric monopole moments located at each of the conjugated atoms of the ring. Each of the elements of the perturbation matrix can then be determined by:

$$V_{ij} = \sum_{r,s} \frac{q_{ir}q_{js}}{|R_{ir}-R_{js}|} \quad (\text{III-4})$$

where q_{ir} is the electric transition monopole located at conjugated atom r on molecule i , and $|R_{ir}-R_{js}|$ is the distance between monopoles q_{ir} and q_{js} . The monopoles for the Q_y and Q_x transitions of BChl were calculated by Weiss and are described by Philipson *et al.* (1971).

The molecular exciton model provides a consistent explanation of the band splittings observed in the BChl-P spectra. Five resolved components are visible in the CD of Figure 6. The CD for both the Q_y and Q_x bands is greatly enhanced as compared with the BChl a solution monomers (Figure 4) and the characteristic positive and negative components of a conservative exciton contribution to rotational strength are present.

In the past, multiple components in absorption spectra of photosynthetic materials have been assigned to chlorophylls in different environments (Vredenberg and Amesz, 1966; Clayton, 1966). We feel that our data are better explained by exciton interaction than by BChl a molecules being in different local protein environments. If the primary

perturbation on the BChl a molecules which causes the band splitting at 77°K (Figure 5) is the specific protein environment of each BChl a, then it would be expected that each of the component absorption peaks would be of approximately the same area. Studies of various chlorophylls in solution show that the dipole strengths (absorption intensities) are virtually invariant in different solvents, although the band maxima may differ in position by 10-20 nm (Seely and Jensen, 1965; Sauer *et al.*, 1966). Although multiple protein environments are undoubtedly present for the differently situated BChl a molecules in the BChl-P, the exciton model provides the only satisfactory explanation for the widely different intensities (band areas) found for each of the absorption components shown in Figure 6 and listed in Table 5.

Using Eqs. III-1 through III-4, calculations were undertaken to see if the distances between BChl a molecules could be estimated. For these model calculations the centers of the five BChl a molecules were arbitrarily fixed at the coordinates of a regular array (either a trigonal bipyramid or a linear array). The distance between molecules was allowed to vary and for each different distance, five orientations of each BChl a molecule were supplied in a random manner by the computer program. Using these geometries and the monopoles for the Q_y transition (Philipson *et al.*, 1971), the calculated exciton bandwidth (the energy difference between the highest and lowest components) was then compared with that found experimentally (490 cm^{-1}) for the BChl-P. Agreement between the calculated and experimental band splitting was found only when the molecules were between 12 and 15 Å apart, regardless of their orientation. On this basis we estimate the distance between BChl a centers to be between 12 and 15 Å. We consider this

estimate to be reliable unless the BChl a molecules are in an unusual geometrical arrangement (e.g., all transition oscillators oriented approximately parallel to one another) such as to cause inordinately large or small band splittings. The CD and absorption are inconsistent with highly symmetrical molecular arrangements, however.

The diameter of this noncompact, spherical protein, as mentioned earlier, is 81.5 \AA and its molecular volume is $193,000 \text{ \AA}^3$ (Olson and Thornber, 1971). The calculated diameter of one subunit (assuming a spherical shape) of the protein is, therefore, 45 \AA . These dimensions for the subunit will suffice to hold five molecules of BChl a with centers $12\text{-}15 \text{ \AA}$ from one another. Thus, the distances estimated from the exciton calculations above are consistent with what is known about the protein structure. Because of the dependence of the interaction potential on the inverse cube of the distance, exciton interactions between BChl a molecules in adjacent subunits are unlikely to result in additional observable optical effects. This assumes that BChl a molecules are not located very near the intersubunit interfaces.

The magnitude of the observed splitting indicates that we are dealing with strong coupling according to the criteria of Simpson and Peterson (1957); this case is designated weak coupling by Förster (1965). Qualitatively, this requires that the exciton bandwidth be greater than the bandwidth for an individual molecule and implies that excitation is delocalized over the array. Additional mechanisms for energy transfer (e.g., localized exciton migration or Förster's inductive resonance) must exist in vivo, where some of the intermolecular distances are greater and the interactions are not so strong as within the BChl-P subunits. In most photosynthetic organelles there is probably a

heterogeneous arrangement of the photosynthetic pigments, with strong exciton delocalization predominant among chlorophylls in close contact and Förster resonance transfer occurring among the more widely separated subunits.

In order to fit the shoulder on the long-wavelength negative component in the CD (Figure 6), it was necessary to use two large gaussian curves of opposite sign almost on top of one another. These are the components in Figure 8 (located at 812 and 814 nm) which largely cancel one another. This feature is interesting in that it would seem to explain the variability (see Results) which is sometimes found in the CD spectra of different samples of the BChl-P. That is, if there are large components in the spectrum which largely cancel each other, a small shift in protein conformation which slightly alters transition energies would result in relatively large changes in this sensitive region of the CD spectrum. The computer curve resolution implies that two of the CD components may be only 2 nm apart. This small separation leads to an observable inflection in the 77°K CD spectrum (the 817 nm feature in Figure 6) and is an impressive demonstration of the superior ability of CD to resolve components which are undetectable in absorption measurements.

The narrowness of the gaussian curves (Table 5) used in the resolution of the spectrum is unusual. The half-widths of these components (as small as 6.6 nm) are less than would be expected on the basis of studies of the spectrum of monomeric BChl a as a function of temperature. The half-widths of the Q_y band of BChl a in ether at 299 and 180°K are 44.0 and 36.5 nm, respectively (Lindsay Smith and Sauer, unpublished data). The very narrow components in the BChl-P may be the result of

the rigid protein matrix or an exciton effect (Kasha, 1963). In cases of strong coupling, where the excitation is spread over several molecules, the internuclear distances will be little affected by excitation into vibrationally excited components of the upper electronic state. That is, it is incorrect to think of the excitation residing in one molecule long enough for the molecule to relax into an excited state internuclear configuration. By the Franck-Condon principle, this will mean that the 0-0 vibrationless electronic transition will tend to predominate and a characteristic band narrowing will be observed. Thus, the surprisingly small bandwidths found for the BChl-P may be another manifestation of the strong exciton interactions present in this system.

The possibility of electronic excitation energy transfer by an exciton mechanism and its implications within the photosynthetic unit have been discussed in a theoretical paper by Robinson (1966). Recent work on various chlorophyll-proteins provides evidence that exciton interactions play an important role in vivo. In particular, the absorption and CD of reaction center preparations from the purple bacteria Rhodospseudomonas spheroides (Sauer et al., 1968) and Rhodospirillum rubrum (Philipson and Sauer, 1973) have been interpreted in terms of exciton interactions among three BChl molecules in the reduced form and between two molecules in the oxidized form. Spectral changes accompanying the protochlorophyllide to chlorophyllide transformation in holochromes indicate exciton effects (Schultz and Sauer, 1972).

CD has proved to be a useful tool in detecting exciton interaction, owing both to its sensitivity to intermolecular interaction and to its ability to resolve closely spaced components. Since CD closely reflects the geometric arrangement of molecules, it should also prove useful in

detecting similarities between chlorophyll-proteins from different organisms. Using this method, reaction centers from R. spheroides and R. rubrum appear to be similar and a BChl-P from Chlorobium thiosulfatophilum has been found to be closely related to the BChl-P from Cps. ethylica (Olson et al., 1972) discussed here. Thus, a CD study can reveal more detailed similarities than would be possible through biochemical techniques.

IV. EXCITON INTERACTION IN THE REACTION CENTERS OF
PHOTOSYNTHETIC ORGANISMS

The Chl molecules found within the membranes of photosynthetic organisms are of two distinct functional types. The large majority of pigment molecules are antenna, or bulk, Chl's, and a smaller fraction is contained in what is known as the reaction center. The function of the antenna Chl's is to absorb incident light photons and to transfer this energy, in the form of electronic excitation, to the reaction center Chl's where the primary photoproducts of photosynthesis are found. These reaction centers are complexes containing protein, Chl, and other primary photoreactants of photosynthesis. The reaction center exhibits light-induced, reversible absorption changes when a Chl molecule within the reaction center loses an electron to the primary acceptor.

Photosynthetic organisms can be divided into three categories according to the nature of the absorption changes which accompany light-induced oxidation. (1) Algae and higher plants comprise the first category with an absorption decrease at about 700 nm (referred to as P700) due to Chl oxidation. (2) In purple bacteria the reversible, long wavelength absorption changes have a characteristic complex shape with three components. There is a large trough in the light-minus-dark difference absorption spectrum of reaction centers from these organisms at approximately 870 nm (referred to as P870). (A

notable exception to this is Rhodopseudomonas viridis, a purple bacterium, in which this long wavelength trough occurs at about 960 nm. The overall shape of the absorption difference spectrum from this organism, however, is characteristic of the purple bacteria.)

(3) Green bacteria make up the third category of photosynthetic organisms, although the absorption changes in these organisms have been studied less than those of higher plants and purple bacteria. BChl oxidation in the reaction centers of green bacteria results in a decrease in absorption centered near 840 nm (usually called P840).

We have found that the absorption changes which accompany the oxidation of reaction center pigments also result in an altered CD spectrum. These reversible CD changes provide a useful probe of reaction center structure. In the following sections, we discuss, in turn, the observed reversible CD changes of higher plants, purple bacteria, and green bacteria.

A. Higher Plants

A light-induced reversible absorbance change at 700 nm in higher photosynthetic organisms, initially reported by Kok in 1956, has been associated with the oxidation of a specific Chl a molecule (P700) within the reaction center of Photosystem I (Kok, 1956). The concentration of P700 within the chloroplast, however, is low (ca. 1 P700 per 400-600 Chl's) and, despite much effort, little is known of the molecular architecture of this reaction center.

The use of organic solvents and solubilizing detergents to extract the bulk Chl's has allowed the preparation of fractions enriched

in P700, greatly facilitating the examination of this photoactive reaction center pigment. In contrast, however, to bacterial systems from which highly purified reaction centers have been obtained (Clayton, 1963), no completely purified preparation of green plant reaction centers has yet been achieved. Recent preparations contain Chl to P700 ratios of 15-30/1 (Sane and Park, 1970; Ogawa and Vernon, 1969; Yamamoto and Vernon, 1969). This has presented a serious obstacle to the characterization of P700 within the reaction center, for the presence of large amounts of bulk Chl often presents difficulties in the interpretation of optical data.

Studies of purified bacterial reaction centers have shown that circular dichroism spectroscopy can provide new insights into the organization of pigments within the reaction center (Sauer et al., 1968). These studies on the BChl's of purple bacteria suggested a model in which the reaction center consists of at least three strongly interacting BChl molecules, one of which is oxidized by light.

In this section the results of some similar studies with CD and absorption spectroscopy on a highly enriched preparation of green plant reaction centers are considered. The spectral changes observed upon illumination of these P700 particles strongly support a model for the Photosystem I reaction center which involves exciton interaction between at least two molecules of Chl a.

1. Materials and Methods

Preparations of enriched P700 particles (HP700), fractionated from spinach by treatment with organic solvents, Triton X-100, and sucrose density centrifugation were described by Yamamoto and Vernon

(1969). We found, however, that the use of 0.025% Triton X-100 in tricine buffer for the initial detergent treatment of the hexane/acetone extracted chloroplasts was sufficient to release residual accessory pigments and bulk Chl. The 0.25% Triton concentration reported by Yamamoto and Vernon frequently resulted in low yields of the HP700 fraction, presumably due to the harsher action of the higher detergent concentration. Purified preparations were stored in darkness at -20°C , under which conditions they were stable for two weeks. Samples obtained had Chl/P700 ratios of 37-60/1.

Absorption spectra were measured on a Cary 14R recording spectrophotometer fitted with a 150 Watt tungsten lamp for side illumination of the sample cuvette. For the light-minus-dark spectra, the photomultiplier was shielded with a Corning 2-58 filter, and the sample illuminated with actinic light filtered through a Baird Atomic 436 nm interference filter and a Corning 5-60 filter. The resultant light had an intensity of $5 \times 10^4 \text{ ergs cm}^{-2} \text{ sec}^{-1}$. Samples were chemically reduced with an excess of sodium ascorbate added in the solid form.

The Chl to P700 ratio was determined by directly comparing the absorbance at 672 nm with the magnitude of the light-induced absorbance change at 697 nm.

CD spectra were recorded on a Durrum-Jasco J-20 CD spectrometer, also adapted for side illumination. The same filter combinations and light intensity cited for the Cary spectrophotometer were used here. The CD spectra are given in terms of $A_L - A_R$, where A_L and A_R equal the absorbance for left and right circularly polarized light, respectively.

Because it was found that continual illumination of a P700-containing sample for the duration of a CD spectral scan resulted in

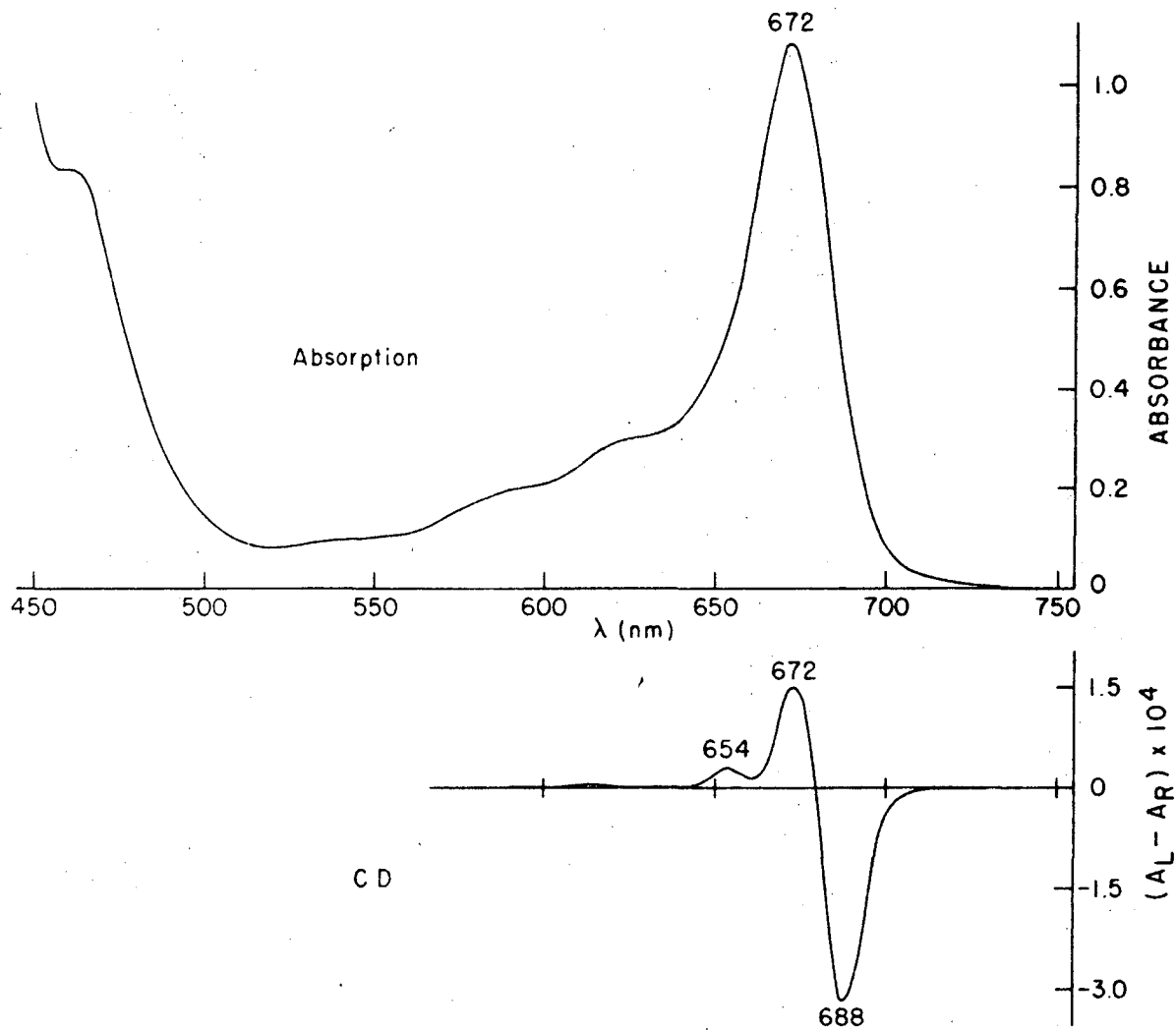
some irreversible bleaching of the sample, the light-minus-dark difference CD spectra were recorded in the following manner. Each ascorbate-reduced sample was allowed to equilibrate in the dark for several minutes, after which the dark CD signal at a particular wavelength was recorded. Actinic illumination was then begun, and the change induced in the CD signal of the sample monitored. The illumination period was approximately two minutes. Following illumination, each sample was allowed to recover in the dark, while the return of the CD signal to the dark level was recorded. This procedure was repeated at intervals of a few nanometers in order to determine the light-minus-dark difference CD spectrum.

Because of the small signal size [$\Delta(A_L - A_R) \cong 2.5 \times 10^{-5}$], special care was taken to exclude the possibility of signal artifacts created by the illumination procedure. Samples known to possess no photoactivity were subjected repeatedly to the dark-light-dark sequence described above, with no indication of CD changes induced by the procedure.

2. Results

Absorption and CD of reduced HP700. The absorption spectrum of an HP700 preparation which has been reduced with ascorbate is given in Figure 9. The peak of the absorption is at 672 nm, as compared with the 680 nm normally seen in intact chloroplasts. This shift to shorter wavelengths in the HP700 fraction is consistent with that reported by Yamamoto and Vernon (1969), although they found the peak at 676 nm. There is also a rather large Chl b absorption shoulder at 645 nm, indicating a higher concentration of this pigment in our

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Figure 9. Absorption and circular dichroism spectra of reduced HP700 particles; phosphate buffer (pH 7.4, 0.01 M KCl); 1 cm pathlength and 25°C; ChI to P700 ratio is 60 to 1.

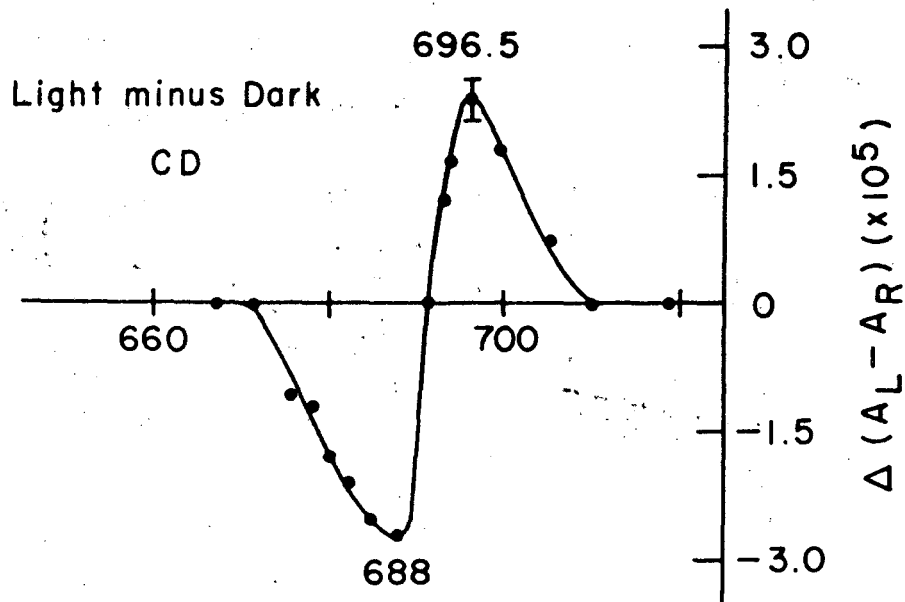
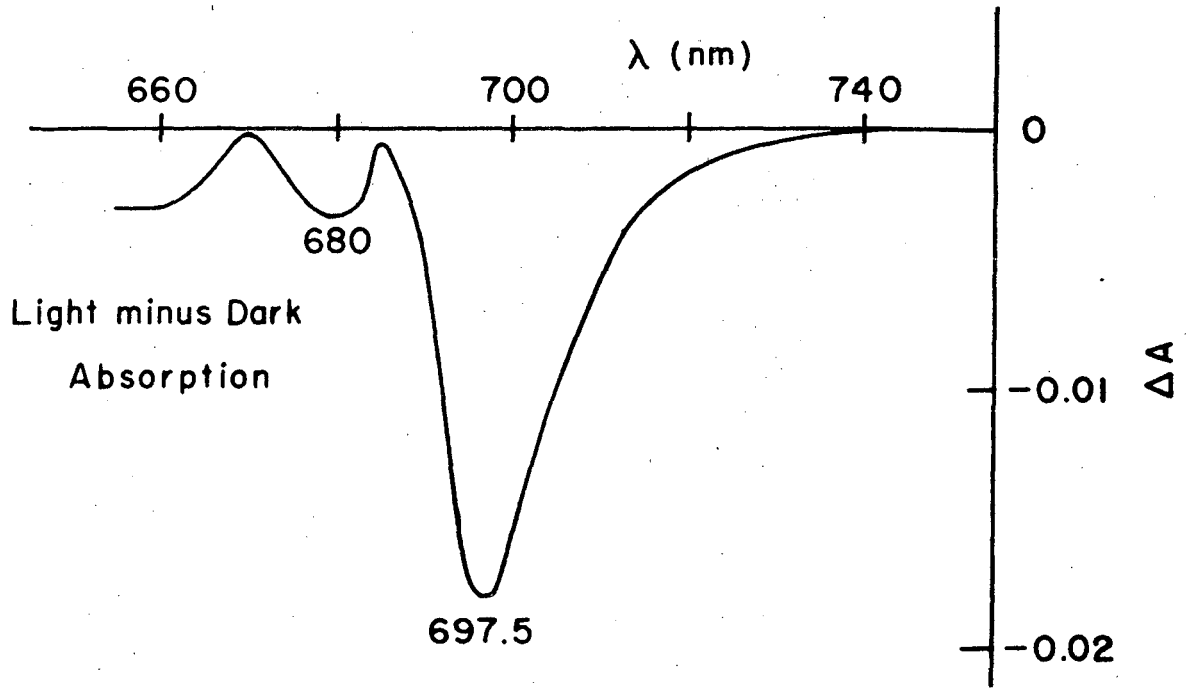
preparation than has been previously reported (Yamamoto and Vernon, 1969). The absence of any absorption around 500 nm, however, does indicate the removal of carotenoids from this fraction.

It should also be noted that the detergent treatment results in an HP700 preparation which exhibits very little turbidity.

The CD spectrum of an ascorbate-reduced HP700 preparation is shown in Figure 9, and three bands are apparent in the long wavelength region. These components are at 688 nm(-), 672 nm(+), and 654 nm(+), where (+) and (-) indicate the signs of the CD signal. Although the CD spectrum does vary from one preparation of HP700 to another (as does the ratio of P700 to total Chl), these basic features are retained.

Light-minus-dark absorption and CD. The light-minus-dark absorption and CD spectra are both given in Figure 10. The absorption difference spectrum reveals two bands, a major decrease at 697.5 nm, and a smaller change at 680 nm. Both components are light-induced absorbance changes which are reversible in the dark. Although there is some variability in the size of the 680 nm feature, as is also noted by other investigators (Vernon et al., 1969), the reversibility of the absorbance change strongly suggests that the peak does not represent non-specific bleaching of bulk Chl. It is significant that the asymmetry in the long wavelength component of the spectrum is not normally observed for this Chl absorption band.

The shape of the absorption difference spectrum given here is consistent with that reported for P700 by several other laboratories working on different types of preparations. The shape of the difference spectrum (one major band near 700 nm, another minor band near 680 nm) appears to be the same in preparations ranging in purity from untreated



XBL725-4648

Figure 10. Absorption and CD difference spectra of HP700 particles.

$A_{672} = 1.08$; phosphate buffer (pH 7.4, 0.01 M KCl); 1 cm pathlength and 25°C; Chl to P700 ratio is 60 to 1; see text for details.

chloroplast fragments (Kok and Hoch, 1961) to the enriched HP700 particles (Ogawa and Vernon, 1969; Yamamoto and Vernon, 1969), although there is a shift of the major peak to shorter wavelengths (703 nm in chloroplast fragments, 697.5 nm in HP700) in fractionated preparations.

The light-minus-dark CD spectrum of the HP700 particles (Figure 10) exhibits two components at 696.5 nm(+), and 688 nm(-) of approximately equal areas but of opposite sign. Like the absorbance changes, these changes in the CD upon illumination are also reversible in the ensuing dark period. We observed similar changes in hexane/acetone-extracted chloroplasts which had not yet been subjected to detergent. Thus, this effect is not an artifact of the Triton X-100 treatment. Attempts to observe the effect in unextracted chloroplast fragments, with low P700 concentration, were limited by instrumental sensitivity. The long wavelength positive CD change could be seen, but excessive noise, due to rising sample absorbance, made the accompanying negative change unobservable.

3. Discussion

Several laboratories have been successful in preparing highly enriched fractions of P700 which range in purity from 1 P700/80 Chl molecules (Kok, 1961) to 1 P700/15 Chl molecules (Sane and Park, 1970). Optical studies on the P700 of both chloroplast fragments (Kok and Hoch, 1961) and the enriched preparations are consistent in revealing the light-minus-dark difference spectrum with the shape shown in Figure 10. On the basis of the two bands (680 nm and 697.5 nm) evident in this difference spectrum, several workers (Murata and Takamiya, 1969; Döring et al., 1968; Vernon et al., 1969) have suggested a model for

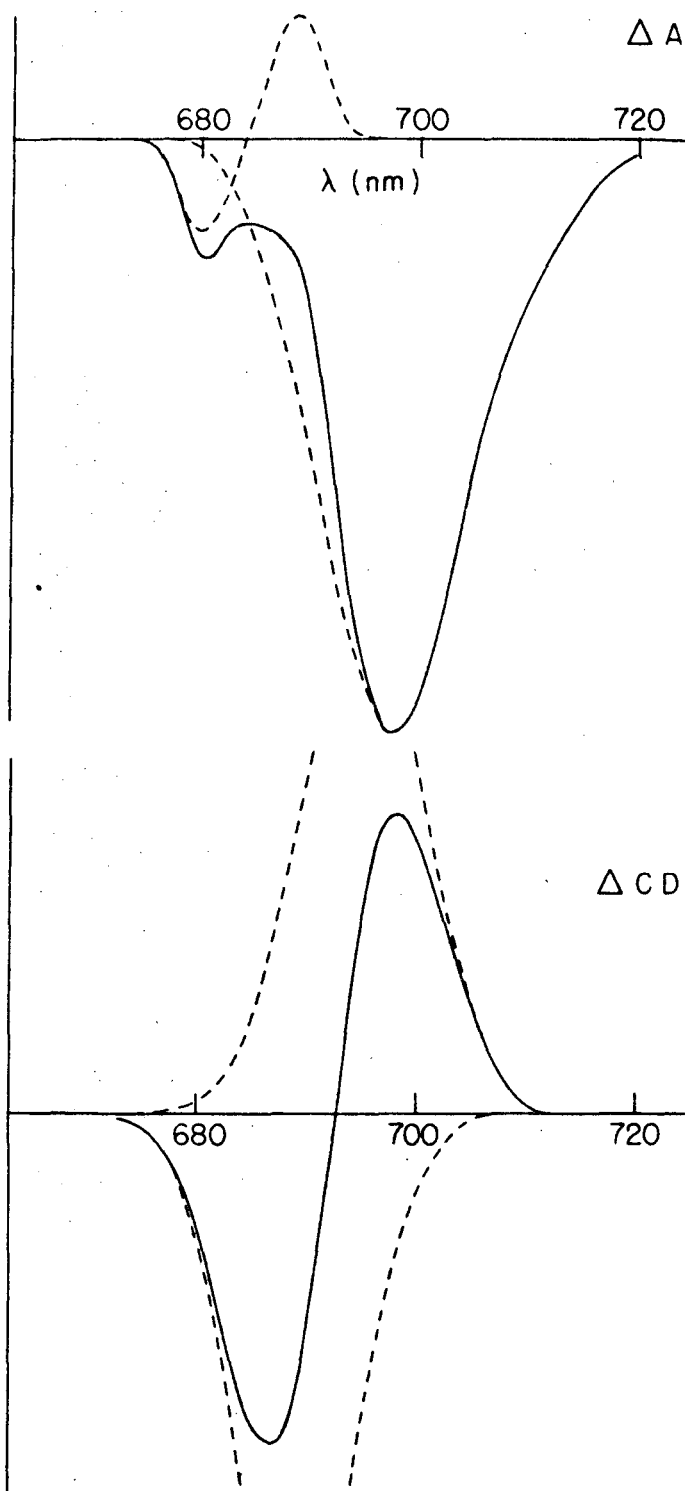
the Photosystem I reaction center which involves at least two molecules. Characterization of the oxidation-reduction properties of the absorbance change at 680 nm (Murata and Takamiya, 1969) revealed extensive similarities to those found at 700 nm. These two absorbance changes exhibited very similar kinetic behavior (Döring *et al.*, 1968). From these observations it has been suggested that the absorbance change at 680 nm is another expression of that seen at 700 nm.

Using the technique of difference circular dichroism spectroscopy, we have obtained results which imply that at least two Chl a molecules are involved in the Photosystem I reaction center; these molecules are apparently coupled by an exciton interaction (Kasha *et al.*, 1965). Under the conditions of exciton coupling, the electronic interaction among the chromophores is so strong that photon absorption results in the excitation of the array of reaction center Chl molecules. Thus, it is not possible to excite an individual Chl molecule within the array. A splitting of energy levels occurs as a result of this interaction, with the number of new energy levels (*i.e.*, absorption bands) equalling the number of molecules involved. The signs and rotational strengths of the CD band components resulting from exciton interaction are determined by the specific geometric arrangement of the interacting molecules with respect to one another, rather than by the inherent asymmetry of the individual molecules. The exciton contribution to the rotational strength is conservative (Tinoco, Jr., 1963), meaning that its contribution to rotational strength should sum to zero over the exciton band. Thus, it is characteristic of exciton interactions for the different components within the one electronic band to have both positive and negative signs.

The presence of two components of approximately equal area but opposite sign in the light-minus-dark CD spectrum (Figure 10) strongly implies that an exciton interaction exists among the pigment molecules of the reaction center in the reduced state. Upon illumination, the oxidation of a specific Chl a within the reaction center results in the loss of resonant coupling; the exciton states no longer exist. Thus, the conservative CD, due to exciton interaction in the reduced reaction center, must disappear upon light oxidation. The loss of this exciton contribution will be reflected in a conservative light-minus-dark CD spectrum.

The magnitude of the CD change [$\Delta(A_L - A_R) \approx 2.5 \times 10^{-5}$] which is seen in the HP700 particles is appreciable. Because the spectrum shown in Figure 10 was obtained from a preparation containing only 1 P700 per 60 Chl's, the fraction of chromophores in the sample which actually contribute to the signal is quite small. In addition, the magnitude of the difference CD signal is further reduced by the overlap of components of opposite sign. As can be seen from the curve resolution shown in Figure 11, this results in cancellation of a major part of the CD components. A CD change of this magnitude is consistent with a loss of an exciton contribution upon light oxidation.

We wish to emphasize that the foregoing discussion is based upon an analysis of a CD difference spectrum, in which advantage is taken of the reversible light-induced properties inherent in a photosynthetic reaction center. The CD of the reduced HP700 particles (Figure 9) provides little information about the reaction center itself; it is primarily an expression of the large number of bulk Chl's present in the preparation. It is also important to note that the long-wavelength Q_y

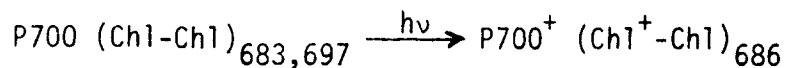


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Figure 11. Resolution into components (-----) of curves (——) which are representative of the experimental absorption and CD difference spectra for HP700 particles. The components in the ΔA spectrum are at 683(-), 686(+), and 698(-) nm; the latter has a half-width of 16.6 nm. For the ΔCD spectrum the components are centered at 689(-) and 695(+) nm. Both components have half-widths of 13.3 nm. The experimental results could not be resolved well into Gaussian components, partly due to scatter in the data points; thus, the sums of these components (——) are not quantitative fits of the actual data. See text for details.

transition of Chl a is electronically allowed and non-degenerate. This excludes the possibility of multiple CD components arising from one molecule; there is at least one molecule present for each component observed.

The light-induced absorbance changes exhibited by the HP700 particles are consistent with the existence of an exciton interaction which is lost upon oxidation of one Chl molecule. Two negative components (680 nm and 697.5 nm) are seen in the absorption difference spectrum (Figure 10), the larger of which exhibits a marked asymmetry with the steep portion on the short-wavelength side. These spectral properties can be accounted for by the light-induced disappearance of the two observable exciton components and the associated appearance of a new absorption band due to a remaining, non-interacting Chl. The bleaching of the exciton components is responsible for both negative changes. It is suggested by the skewed shape of the change at 697.5 nm that the absorption band of the non-interacting Chl overlaps, and is therefore cancelled by, the larger negative components. A diagrammatic representation is given in Figure 11 (upper curves). The inflexed curve centered at 684.5 nm represents the band shift to longer wavelengths that has been previously reported to accompany P700 photooxidation (Vernon et al., 1969). We interpret this feature to represent the simultaneous disappearance of a short wavelength exciton component of P700, located at approx. 683 nm, and the appearance of a band at approx. 686 nm owing to the remaining unoxidized Chl molecule in P700⁺. This process can be summarized by the equation



The apparent bandwidths (5.5 nm) associated with the shift centered at 684.5 nm seem anomalously narrow when compared with the 16.5 nm band-

width of the trough at 697.5 nm. This confirms the proposal that a rather small difference (2-3 nm) exists between the two components involved in the "band shift". This apparent narrowing associated with the canceling of overlapping features is also evident in the analysis of the CD spectrum (Figure 11, lower curve).

Although the exciton model is in agreement with the suggestion of other investigators (Murata and Takamiya, 1969; Döring et al., 1968; Vernon et al., 1969) that at least two Chl molecules are involved in the reaction center, it differs significantly in several respects. Unlike these previous models, which have not specified the nature of the relationship among the associated Chl's, our proposal involves strong electronic coupling of the Chl's within the reaction center. Thus, both the 680 nm and 700 nm absorbance changes must be considered characteristic of the aggregate of chromophores; the 680 nm change cannot be assigned to a separate pigment molecule which is only loosely associated with an oxidizable Chl. Studies of the in vivo and in vitro EPR linewidths have been interpreted in terms of strongly coupled Chl molecules in the reaction centers of both higher plants (Norris et al., 1971) and bacteria (McElroy et al., 1972). The optical results presented here are consistent with and complementary to these proposals.

Although the spectroscopic evidence presented here does not exclude the possibility that conformational changes of reaction center proteins may cause the observed optical effects, we feel that the size and shape of the difference CD spectrum are most simply explained by the presence of exciton interaction.

The model of a Photosystem I reaction center containing two Chl's should be regarded as a simplest hypothesis. Because of the presence of

a large amount of antenna Chl, we do not know the absorption and CD properties of either the reduced or the oxidized reaction center pigments. This is by contrast with the purple bacterial reaction center of R. spheroides (Sauer et al., 1968), where the CD spectrum of the reduced form provides strong evidence for the participation of at least three BChl molecules. For purple bacterial reaction centers the light-minus-dark difference CD spectra (following section) have rather similar shapes to that observed for the HP700 preparation, except that the signs of the long- and short-wavelength components are reversed. Thus, while it is possible that the HP700 preparation reaction centers contain three instead of two participating Chl molecules, the sign reversal demonstrates that the geometries of the bacterial and higher plant reaction centers are substantially different from one another. A comparison of CD difference spectra of reaction centers from the different classes of photosynthetic organisms can be found in section IV.D.

B. Purple Bacteria

Associated with the reaction centers in purple photosynthetic bacteria there are normally 40-100 antenna BChl molecules, which function to transfer light excitation energy to the reaction center. Several purified or enriched reaction center preparations have been made from these bacteria (Reed and Clayton, 1968; Reed, 1969; Gingras and Jolchine, 1969; Thornber et al., 1969; Thornber, 1970; Smith et al., 1972). This is in contrast to green photosynthetic bacteria and higher plants from which purified reaction centers have not yet been isolated. Only samples enriched in photoactive pigments have been obtained from these two classes

of photosynthetic organisms (Fowler et al., 1971; Olson et al., 1972; Kok, 1961; Vernon et al., 1969; Sane and Park, 1970).

Despite much effort, little is known about the detailed structure or mechanism of action of the photoactive BChl-proteins. In vivo the reaction centers of purple bacteria are membrane-bound within the chromatophore and isolation procedures require the use of a detergent to release and solubilize the reaction center complexes. The presence of detergent in the reaction center preparations is a hindrance to many biochemical analyses such as molecular weight determinations. Even the number of pigment molecules present per protein remains unresolved. Recent measurements (Feher, 1971; Clayton et al., 1972) indicate that reaction centers from Rhodospseudomonas spheroides contain two molecules of bacteriopheophytin (BPh) and either three, four, or five molecules of BChl. Whether the BPh plays an important role in the electron transfer reactions remains unknown. Molecular weight approximations of reaction centers from R. spheroides (Feher, 1971), R. viridis (Thornber and Olson, 1971), and R. rubrum (Smith et al., 1972) give estimates ranging from 37,000 to 110,000 daltons.

The CD of reaction centers from R. spheroides in both the oxidized and reduced states has previously been analyzed by Sauer et al. (1968). They concluded, on the basis of large changes in the CD spectrum upon oxidation, that the reduced reaction center contains three or more closely coupled BChl molecules which exhibit exciton interaction. In the oxidized state one of these molecules has been converted to a BChl⁺ radical and the CD evidence for exciton interaction has disappeared. A model for the structure of the reaction center was proposed in which

the oxidizable BChl was located between the other BChl molecules. Thus, in the oxidized form, the remaining BChl molecules are separated by a $BChl^+$, the distance between BChl molecules has increased, and resonance interaction can be significantly attenuated. More recent research (Philipson and Sauer, 1972; Schultz and Sauer, 1972; Philipson et al., 1972) has confirmed the existence of exciton interaction in photosynthetic materials. EPR measurements (Norris et al., 1971; McElroy et al., 1972) are consistent with the concept of interacting reaction center molecules.

In this section we examine absorption and CD spectra of reaction centers from R. viridis and R. rubrum and of a preparation enriched in reaction centers from Chromatium (Fraction A) (Thornber, 1970). These spectra are compared with one another and with the previous results from R. spheroides. The reaction centers from purple bacteria appear spectrally very similar, except for those from R. viridis which show some marked dissimilarities, especially in the low temperature absorption spectrum. No CD evidence of interaction between reaction center BPh and BChl is detected, indicating that the BPh probably does not actively participate in the electron transfer reactions.

1. Materials and Methods

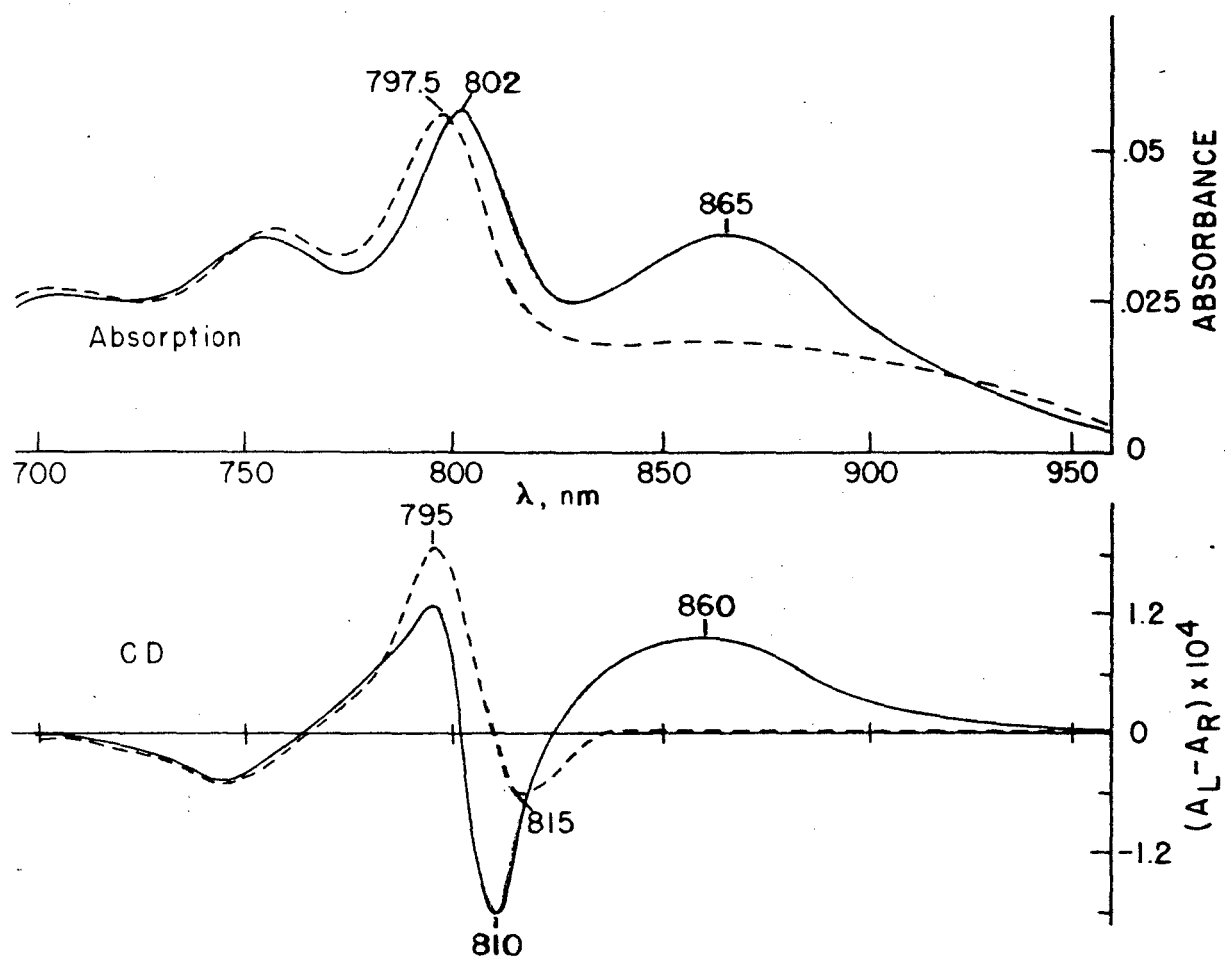
The reaction centers from R. viridis and Chromatium Fraction A were prepared in the laboratory of J. M. Olson, using the methods of Thornber (1970, 1971). Key steps in the isolation procedures involve the use of the detergent, sodium dodecyl sulfate (SDS), for solubilization, and hydroxylapatite chromatography. The R. rubrum reaction centers were prepared by W. R. Smith in the laboratory of C. Sybesma (Smith et al., 1972) using SDS and sucrose density gradient centrifugation. All samples

were stored in the freeze-dried state and were resuspended in the appropriate buffer just prior to the experiments.

Absorption spectra were recorded using a Cary 14R spectrophotometer and CD spectra were recorded using a Durrum-Jasco J-20 spectropolarimeter with sensitivity extended to 1000 nm. Light-minus-dark difference spectra were obtained in both instruments using side illumination at 530 nm by a projector equipped with a 150 W tungsten lamp. The wavelength of illumination was defined by an interference filter and a Corning 4-94 filter. The incident light intensity was measured to be 1.5×10^5 ergs $\text{cm}^{-2} \text{sec}^{-1}$. The photomultipliers were shielded from stray light with a Corning 2-64 filter.

2. Results

The room temperature absorption and CD spectra for a dilute suspension of R. rubrum reaction centers in both the chemically reduced and chemically oxidized states are shown in Figure 12. The spectra result from the long wavelength, non-degenerate, Q_y transitions of BChl and BPh a. Three distinct peaks are seen in the reduced absorption spectrum. Those at 802 and 865 nm are attributed to BChl a transitions and the peak at 755 nm is usually assigned to BPh a (but see Feher, 1971). The 802 and 865 nm components are commonly referred to as P800 and P870 respectively. Upon oxidation there is a bleaching of the 865 nm absorption band and a blue shift of the 802 nm component. These spectral effects, caused by oxidation (whether induced by light or chemicals), are qualitatively similar in all purple bacteria reaction center preparations and produce a characteristic oxidized-minus-reduced absorption difference spectrum (e.g., Figure 16b).



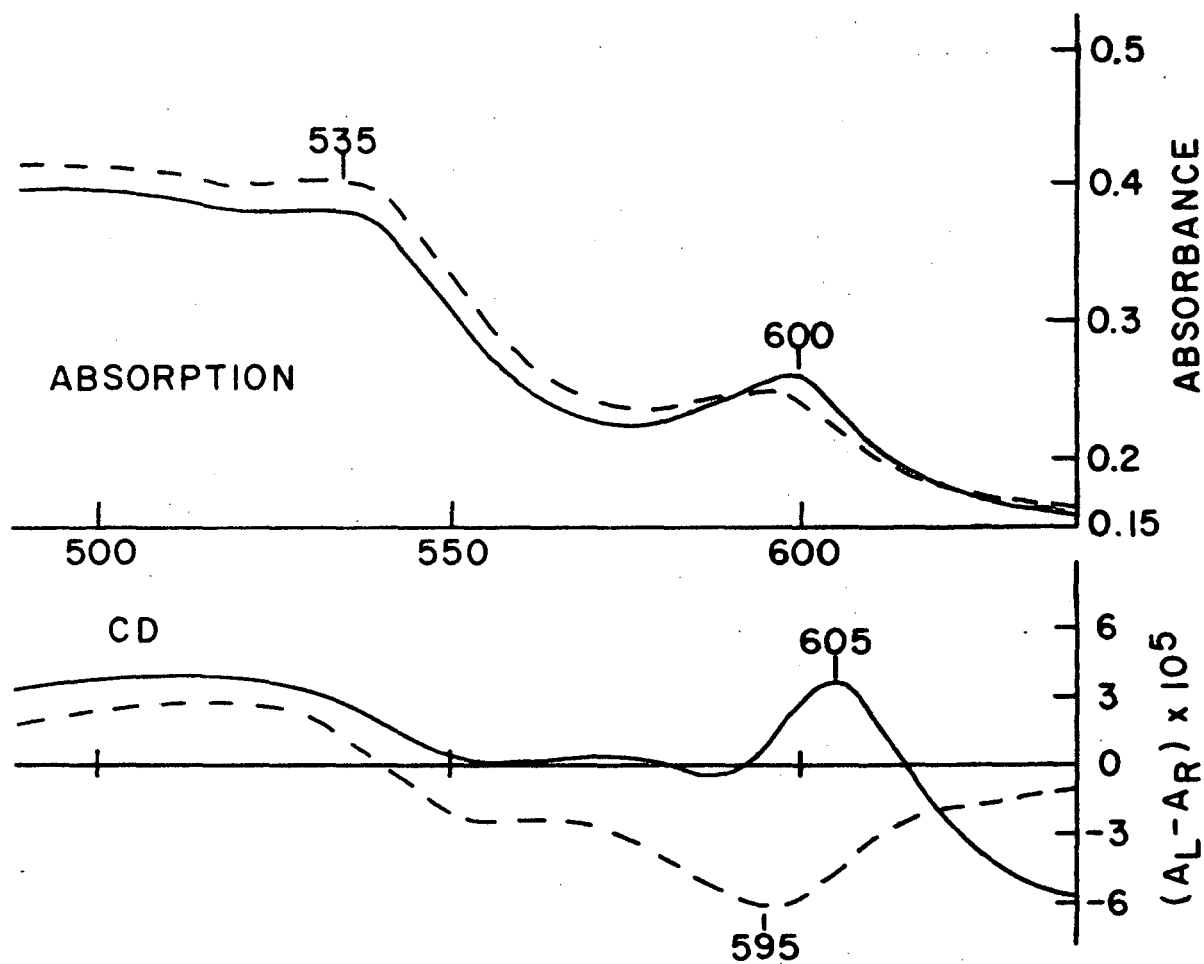
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Figure 12. Absorption and CD spectra of *R. rubrum* reaction centers. Reduced (—) and oxidized (-----). $T = 24^{\circ}\text{C}$, 1 cm pathlength, 50 mM Tris buffer (pH 8.0). Solid sodium ascorbate added to reduced sample and potassium ferricyanide crystals added to oxidized sample.

The CD spectrum (Figure 12) for this sample shows components at 860(+), 810(-), 795(+), and 745(-) nm. (The symbols (+) and (-) after the wavelength values indicate the signs of the CD components.) Oxidation causes the 860 nm peak to disappear, the 810 nm trough largely to disappear, and an increase in magnitude of the 795 nm peak. The CD (in the region of BChl a absorption) has thus changed from a three to a two component spectrum. The spectra shown in Figure 12 for R. rubrum reaction centers are very similar, apart from some wavelength differences in the oxidized CD spectrum, to those previously described for R. spheroides reaction centers (Sauer et al., 1968).

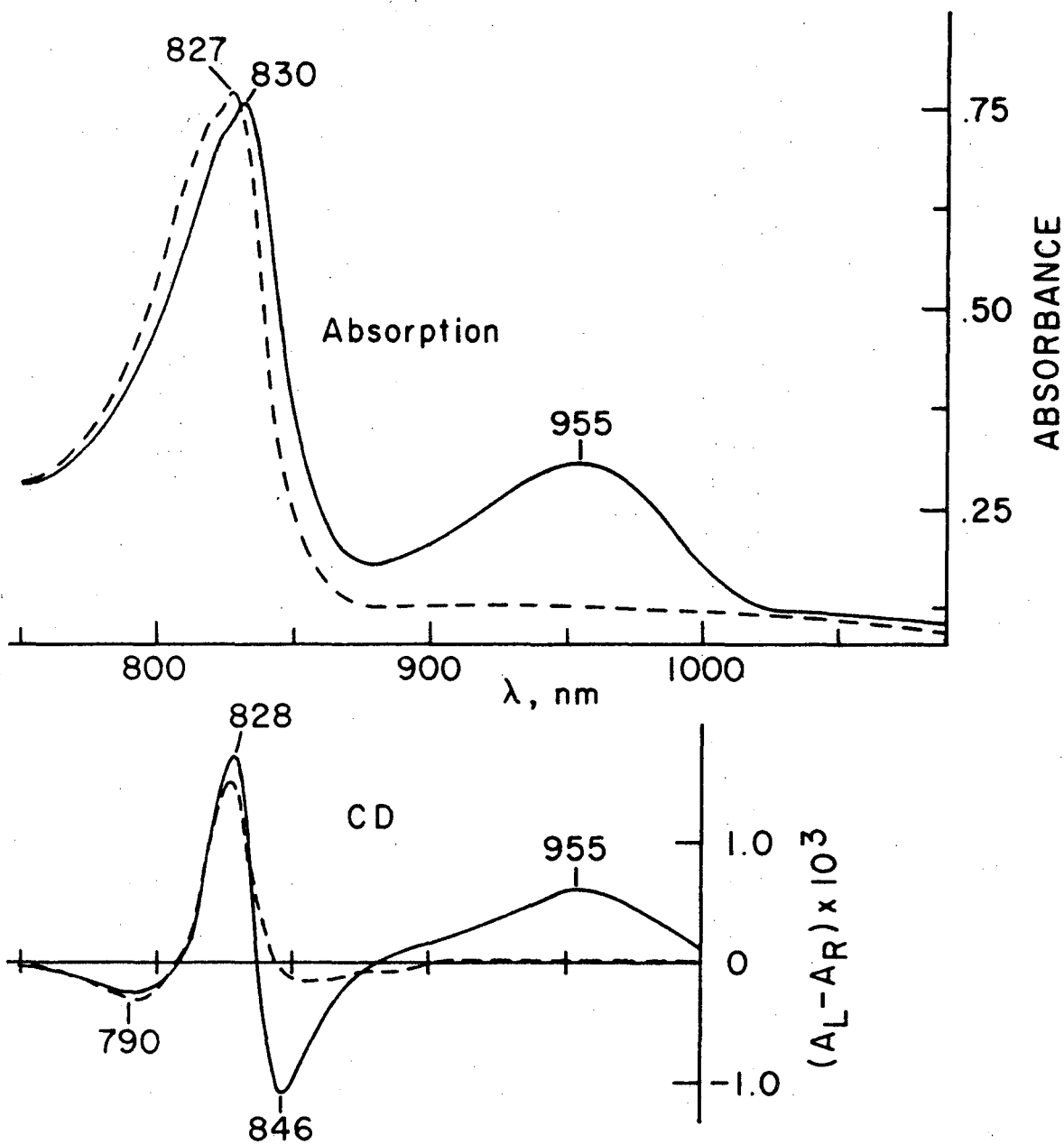
The reduced and oxidized absorption and CD spectra for the 600 and 535 nm (Q_x) transitions of BChl a and BPh a, respectively, are shown in Figure 13 for the R. rubrum reaction centers. In these spectra the only significant changes owing to chemical oxidation occur in the region of the BChl a transition. Here, the CD is significantly affected by the redox state of the photoactive complex, going from a positive component at 605 nm to a negative one at 595 nm upon ferricyanide addition. The presence of scattering is responsible for a portion of the absorption in this region, and it is possible that the negatively displaced baseline at 650 nm in the CD spectrum of the reduced reaction centers is due to a scattering artifact.

The absorption and CD results (Figure 14) for R. viridis reaction centers at room temperature qualitatively resemble those for R. rubrum. The major difference is the much longer wavelengths at which the chromophores absorb. Here the photoactive pigments are P830 and P960 by contrast with P800 and P860 in R. rubrum. Part of this wavelength shift is explained by the presence in R. viridis of BChl b rather than the more



XBL727-4711

Figure 13. Absorption and CD spectra of *R. rubrum* reaction centers. Reduced (—) and oxidized (-----). $T = 24^{\circ}\text{C}$, 1 cm pathlength, 50 mM Tris buffer (pH 8.0), $A_{802} = 0.52$. Sodium ascorbate added to reduced sample and potassium ferricyanide crystals added to oxidized sample.



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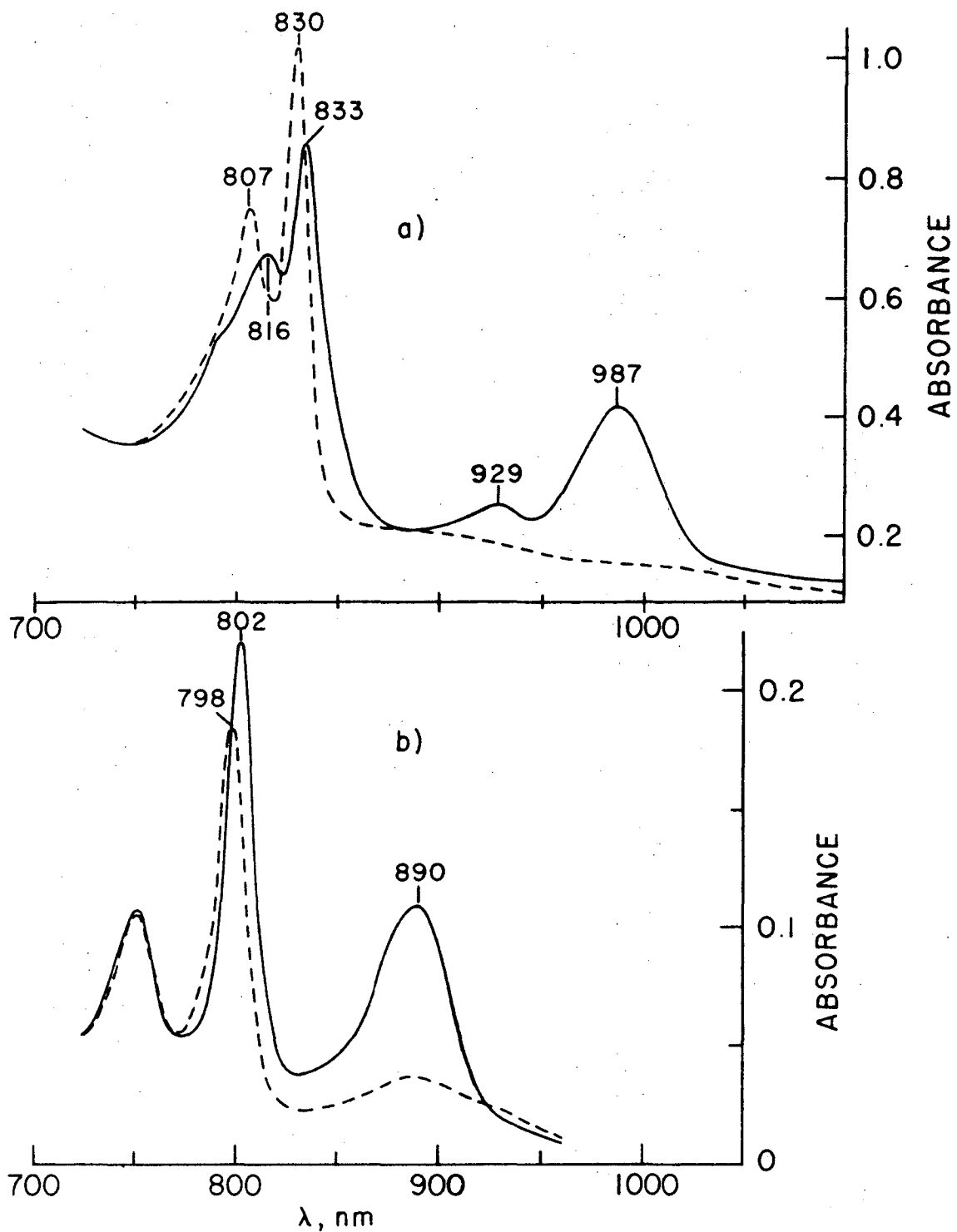
Figure 14. Absorption and CD spectra of *R. viridis* reaction centers. Reduced (—) and oxidized (-----). $T = 24^\circ\text{C}$, 1 cm pathlength, 50 mM Tris buffer (pH 8.0). Samples reduced with solid sodium ascorbate and oxidized with crystalline potassium ferricyanide.

common BChl a; in acetone BChl a and BChl b absorb at 771 and 794 nm respectively (Eimhjellen et al., 1963). The structure of this molecule (Brockmann and Kleber, 1970; Baumgarten, 1971), although not completely known, is closely related to that of BChl a. Also, in this reaction center, there is no clearly resolved absorption peak due to BPh b. The 790(-) nm component in the CD (Figure 14) implies, however, that this pigment is present; the analogous component, at 745(-) nm, in R. rubrum is clearly associated with the BPh a absorption peak.

Significant differences in the absorption properties of R. viridis and R. rubrum reaction centers are found at 77°K (Figure 15). In R. viridis (Figure 15a) the low temperature absorption spectrum reveals much new structure. In the reduced state five components are resolved. P960 is split into two components absorbing at 929 and 987 nm and P830 shows peaks at 833 and 816 nm and a shoulder (probably due to the BPh b) at 790 nm. Chemical oxidation causes bleaching of both long wavelength bands and leaves two resolved components at the shorter wavelengths.

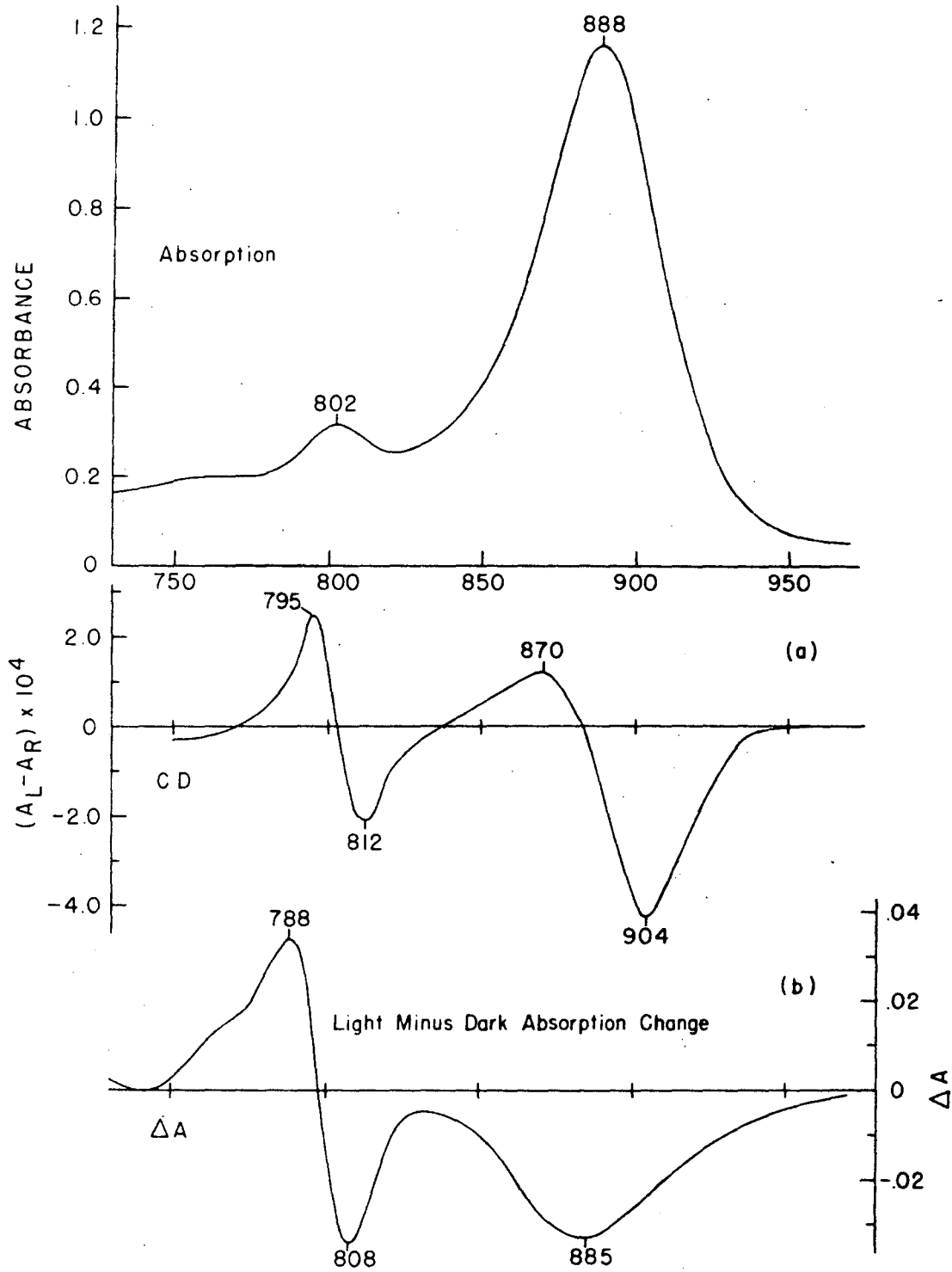
The R. rubrum reaction centers (Figure 15b) show no new structure at liquid nitrogen temperature. The 865 nm band is shifted to 890 nm, and P800, in both reduced and oxidized states, is narrowed but does not reveal any new components. Feher (1971) has reported that second derivative spectroscopy shows two components in the reduced P800 of R. spheroides reaction centers at 77°K. The small peak at 890 nm in the chemically oxidized spectrum is probably due to incomplete oxidation.

Chromatium Fraction A is a BChl-protein with a molecular weight of about 500,000 and with one photo-oxidizable (P880) molecule per approximately 45 BChl a molecules (Thornber, 1970). Since the absorption and CD (Figure 16a) spectra in this case are mostly a function of the bulk



XBL727-4687

Figure 15. Low temperature absorption spectra of *R. viridis* (a) and *R. rubrum* (b) reaction centers. Reduced (—) and oxidized (-----). $T = 77^\circ\text{K}$, 3 mm pathlength, glycerol:50 mM Tris (pH 8.0)(1:1, v/v). Samples reduced with sodium ascorbate and oxidized with potassium ferricyanide.



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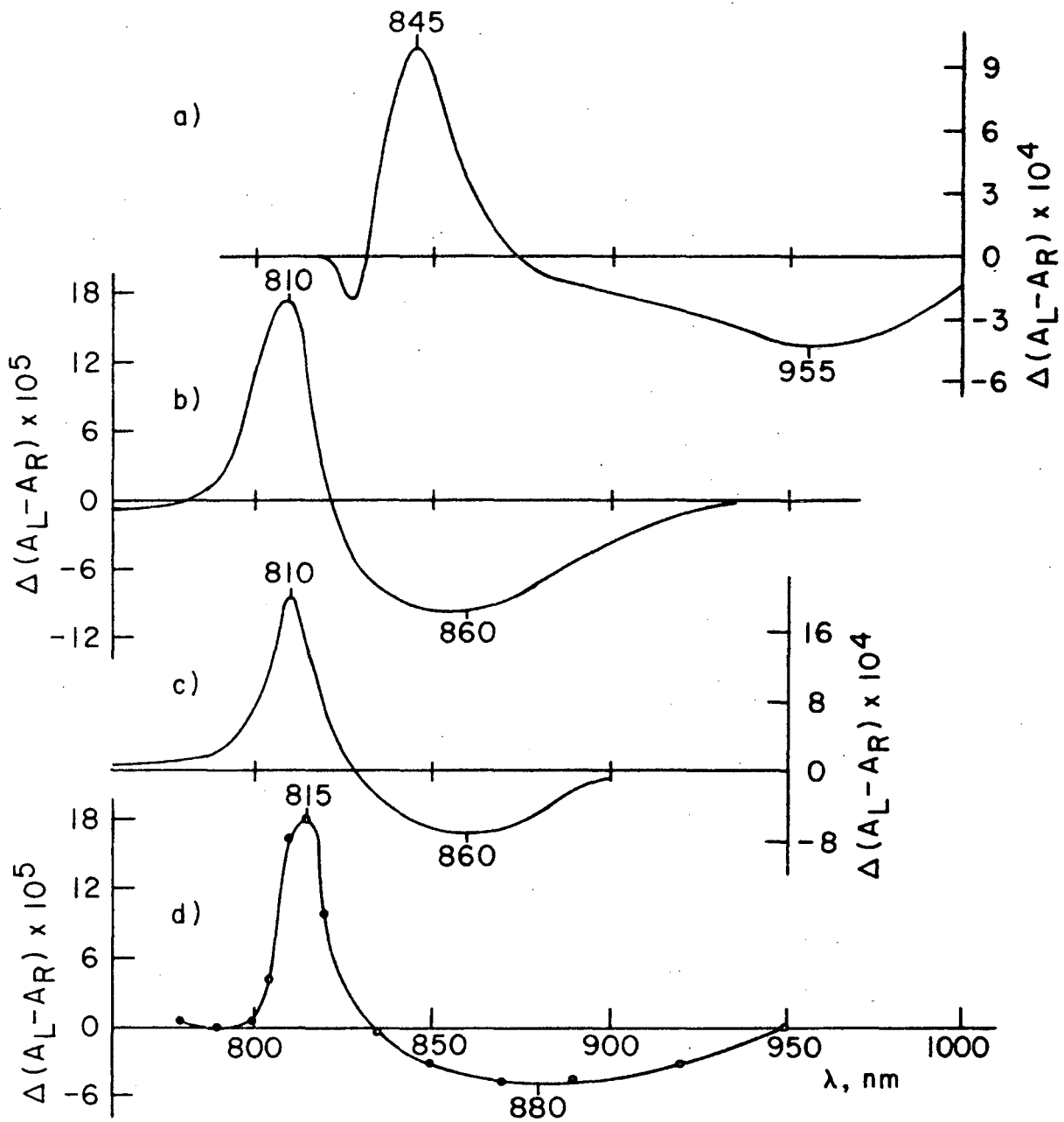
Figure 16. Spectra of Chromatium strain D, Fraction A. a) Absorption and CD. b) Light-minus-dark difference absorption. T.= 24°C, 1 cm pathlength, 50 mM Tris (pH 8.0), 3.3 μ M phenazine methosulfate. Activation wavelength, 530 nm.

BChl molecules rather than of the reaction center itself, more information about the reaction center is obtainable from the light-minus-dark absorption (Figure 16b) and CD (Figure 17d) difference spectra. The absorption difference spectrum (Figure 16b) has the familiar shape of a purple bacterial reaction center with components P800 and P880. The CD difference spectrum (Figure 17d) for this enriched fraction can be compared with difference spectra calculated from the data for the purified reaction centers of *R. viridis*, *R. rubrum*, and *R. spheroides* (Sauer *et al.*, 1968) (Figure 17a,b,c). Again the Chromatium reaction center appears to be quite similar to other purple bacteria reaction centers.

3. Discussion

The CD spectrum of a BChl-protein is a sensitive measure of BChl-BChl interaction within the protein. A perturbation of the protein which affects the spatial arrangement of BChl molecules with respect to one another can result in large changes in the CD. Furthermore, if the interaction is strong enough, *i.e.*, an exciton interaction (Kasha *et al.*, 1965), as it appears to be in many photosynthetic materials (Philipson and Sauer, 1972; Schultz and Sauer, 1972; Philipson *et al.*, 1972), theory predicts a characteristic CD spectrum with multiple components of both positive and negative sign (Tinoco, 1963). For non-degenerate transitions the number of resolved components will always be less than or equal to the number of interacting molecules. Pure BChl in solution exhibits a small CD with a single positive component in the long wavelength region.

Since CD is more sensitive than absorption spectroscopy to protein conformation and intermolecular interaction, a comparison of the CD spectra of reaction centers from different organisms can reveal similarities or differences between the BChl-protein complexes that may be



XBL727-4688

Figure 17. Oxidized-minus-reduced difference CD spectra. a) R. viridis reaction centers, calculated from data in Figure 3. b) R. rubrum reaction centers, calculated from data in Figure 1. c) R. spheroides reaction centers, calculated from data in Sauer et al. (1968), Figures 2 and 3, normalized to equivalent concentrations. d) Chromatium Fraction A, light-minus-dark CD spectrum. Activation wavelength, 530 nm.

undetectable in absorption experiments. We find that R. spheroides and R. rubrum reaction centers are spectrally extremely similar and may even have identical configurations.

The techniques of absorption and CD difference spectroscopy show that the reaction center in Chromatium Fraction A also has much in common with the R. spheroides reaction center, the main difference being the shift of the long-wavelength transition from 865 to 885 nm. The source of this is probably a slightly altered arrangement of reaction center BChl a molecules, possibly due to the presence of the antenna BChl a in this preparation.

R. viridis reaction centers exhibit unique spectral properties, especially at liquid nitrogen temperature where five resolved components are found in the reduced absorption spectrum. The five component spectrum implies at least five reaction center pigment molecules (both BChl b and BPh b). From the shape of its absorption and CD spectra (Figure 14) and from its spectral behavior upon oxidation (Figures 14 and 17a) it is obvious that the reaction centers from the BChl b-containing R. viridis have much in common with those from other purple bacteria. It is thus possible that more components are present, but unresolved, in the reaction centers from the other purple bacteria. The fact that two components at 929 and 987 nm (Figure 15a) are bleached in the oxidized states raises the possibility that R. viridis contains two different reaction centers. If this is correct, it remains to be determined whether both reaction centers reside simultaneously on the same protein or whether they are separate proteins which can be isolated from one another. Experiments must also determine whether both photoactive components, P930 and P990, play identical roles in the electron transport pathways. Anomalous titration curves

(Thorner and Olson, 1971) for R. viridis reaction centers have already pointed to the presence of reaction centers with different midpoint potentials.

BPh a, originally thought to be a contaminant (Clayton, 1966c) in reaction center preparations, has been found in stoichiometric amounts in more recent preparations (Clayton et al., 1972) and is now considered to be an intrinsic part of reaction centers. What function, if any, these molecules play is unknown. The 745(-) (Figure 1) and 790(-) nm (Figure 14) CD components of the R. rubrum and R. viridis reaction centers, attributable to BPh, undergo no significant changes upon oxidation, implying that the BPh molecules are not interacting strongly with the oxidizable BChl molecules. This conclusion is further supported by the spectra of the R. rubrum reaction centers in the Q_x region (Figure 13). The 535 and 600 nm absorption peaks are well separated and can be assigned with confidence to BPh and BChl respectively. In the CD spectrum the component due to BChl a changes dramatically with oxidation, while the BPh a spectrum remains essentially unchanged.

C. Green Bacteria

The photosynthetic unit of green bacteria is unusually large. There are approximately 80-100 BChl a molecules and about 1000 Chlorobium Chl molecules per reaction center BChl, P840 (Fowler et al., 1971). The majority of the BChl is associated with the BChl-protein complex discussed in Chapter III. In vivo, the Chlorobium Chl functions to transfer electronic excitation energy to the BChl-protein complex which in turn transfers this energy to the reaction center to produce the P840 spectral change (Sybesma and Olson, 1963; Sybesma and Vredenberg, 1963).

The photosynthetic apparatus of green bacteria is located within egg-shaped vesicles surrounded by a single-layered membrane only 20-30 Å thick (Cruden and Stanier, 1970). This is unique among photosynthetic organisms in that the photosynthetic apparatus is not made up of a lamellar unit-membrane system. Apparently the forces holding the vesicles of green bacteria intact are also unique since strong treatment is not necessary, as in other organisms, to fractionate the photosynthetic unit. Thus, it is possible to obtain two types of water-soluble BChl a containing complexes from these organisms without the use of detergent.

The first of these is typified by the well characterized BChl-protein from Chloropseudomonas ethylica (Chapter III; Olson, 1966). This homogeneous protein fraction contains 20 BChl molecules per protein macromolecule with a molecular weight of 152,000 dalton (Olson, 1971). A BChl-protein very similar to that from Cps. ethylica has also been isolated from the green bacterium Chlorobium thiosulfatophilum (Olson and Romano, 1962; Olson et al., 1972).

A second type of complex, first isolated by Fowler et al. (1971), from Cps. ethylica, contains 80-120 BChl a molecules and has a molecular weight around 1.5×10^6 daltons. This "heavy" fraction is photochemically active with light-induced absorption changes of cytochrome 553 and of the reaction center pigment, P840. The absorption properties of this BChl-Reaction-Center (BChl-RC) complex at room temperature are very similar to those of the BChl-protein complex in the long wavelength, Q_y , region of the spectrum. The only notable difference is the presence of a small, long wavelength shoulder on the absorption peak of the BChl-RC complex.

In this study we have isolated a very similar BChl-RC complex from C. thiosulfatophilum and observed its optical properties (Olson et al., 1972). Using the CD and a low temperature absorption spectrum we are able to compare structural properties of the BChl-RC complex with those of the BChl-protein (see Chapter III). The light-minus-dark CD and absorption spectra are also studied in an attempt to learn something of the nature of the BChl molecules which make up the reaction center component of this complex.

1. Materials and Methods

Crude preparations of BChl-RC complex were prepared from C. thiosulfatophilum by a modification of the procedure devised by Fowler et al. (1971) for Cps. ethylica. Frozen cells (approx. 100 g) of C. thiosulfatophilum were thawed and then resuspended (homogenized) in 200 ml of 10 mM Na/K phosphate (pH 7.5). The suspension was centrifuged at 10,000 x g for 10 min to sediment the cells, and the supernatant (wash) was discarded. In this manner the cells were washed 2 or 3 times to get rid of the alum used to harvest the cells. The washed cells were again resuspended in 10 mM phosphate. Solid Na ascorbate was added to a concentration of 10 mM, and the cell suspension was passed through a French pressure cell at 20,000 psi 2 or 3 times to break the cells and also to break up the vesicles. The effluent from the pressure cell was centrifuged for 90 min at 40,000 x g to sediment unbroken cells and intact vesicles. The supernatant, which is enriched in BChl relative to Chlorobium Chl, was then centrifuged on a layer of 40% sucrose (10 ml) in a Spinco swinging bucket rotor SW27 for 22 hours at 27,000 rpm to concentrate the BChl-RC complex in the 40% sucrose. Fractions (1 ml) were collected from the bottom of

the centrifuge tubes and examined in the spectrophotometer to determine the ratio of BChl (810 nm) to aggregated Chlorobium Chl (745 nm) in each fraction. The first fractions containing the highest ratio of BChl to aggregated Chlorobium Chl were pooled and stored in the freezer. In the first preparation of crude BChl-RC complex the ratio of A_{745} (Chlorobium Chl) to A_{810} (BChl) was 0.73; in the second preparation the ratio was negligibly small. All preparations contained large amounts of Chlorobium pheophytin which has an absorption peak at 670 nm. The presence of Chlorobium pheophytin, however, did not interfere with either absorption or CD measurements in the region of the Q_y band of BChl.

Absorption spectra were recorded on a Cary 14R spectrophotometer and CD spectra were recorded on a Jasco-Durrum spectropolarimeter Model J-20, with extended red sensitivity. For low temperature absorption spectra ($T = 100^\circ\text{K}$), samples were mixed with glycerol to give a 50-50 mixture by volume. The pathlength was either 2.5 mm or 3 mm in a sandwich type cell constructed of copper with plexiglass windows. The sample in the cell was maintained at approximately 100°K in a copper block in contact with liquid N_2 .

Absorption difference spectra (light-minus-dark) in the long wavelength region were obtained in the Cary 14R using side illumination at 530 nm by a 150 W tungsten lamp through an interference filter and a Corning 4-94 filter, with the photomultiplier protected by a 2-64 filter. A similar illumination was used for the light-minus-dark CD spectra, but measurements were made at a series of fixed wavelengths owing to the small magnitude of the change induced in the CD spectra.

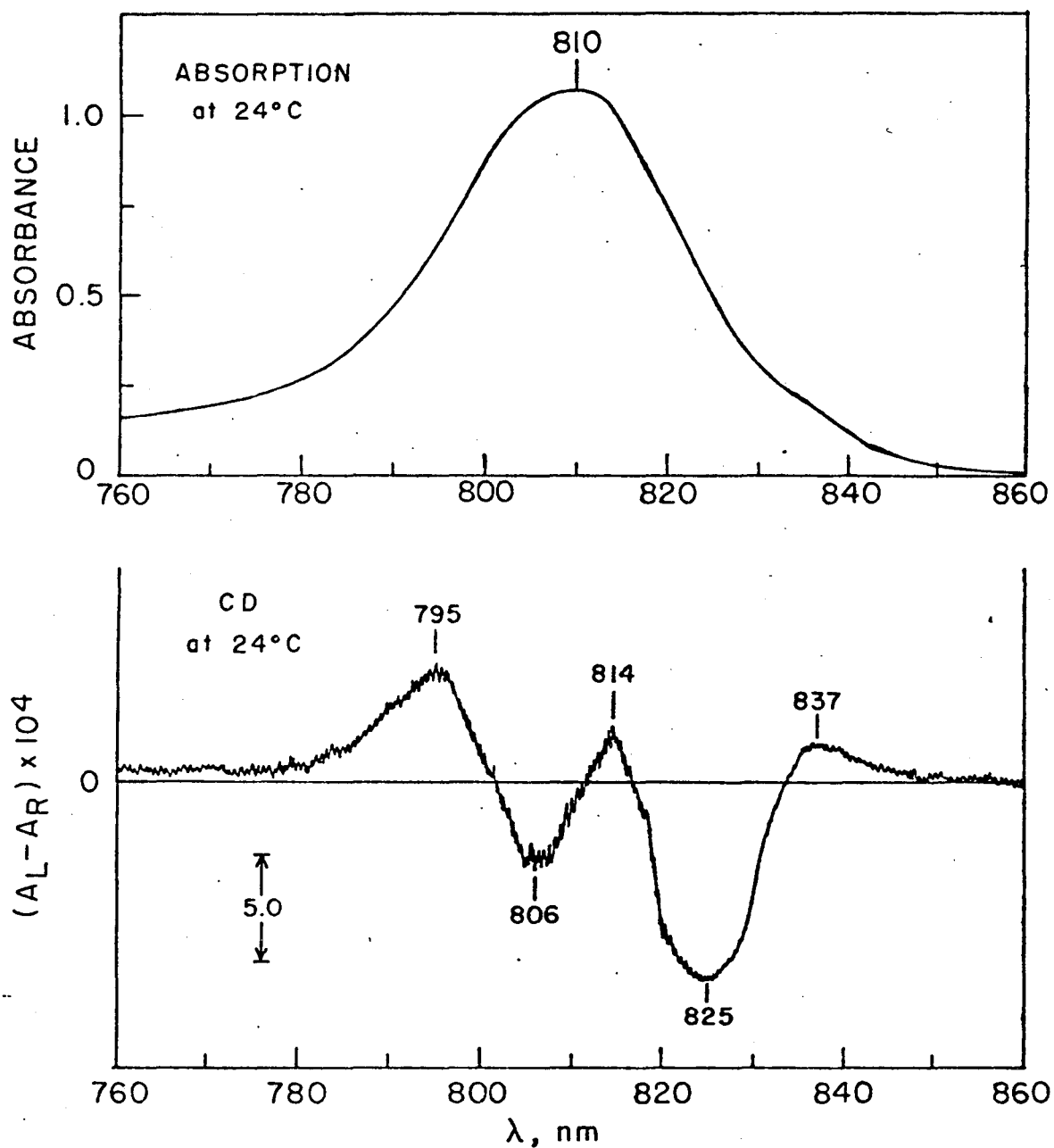
2. Results

The absorption spectrum of the Q_y band for the BChl-RC complex from C. thiosulfatophilum at room temperature (Figure 18, upper curve) is very similar to the spectrum from the BChl-RC complex from Cps. ethylica [see Figure 2 in Fowler et al. (1971)]. Both exhibit an absorption maximum at 810 nm with a shoulder at about 835 nm that is not present in the spectra of the purified BChl-proteins.

The room temperature CD spectrum of the Q_y band of the C. thiosulfatophilum complex shown in Figure 18 (lower curve) implies the presence of multiple exciton components in this region. Distinct differences are seen from the CD spectrum of the BChl-protein (Figure 5) from Cps. ethylica (the optical properties of the BChl-protein from Cps. ethylica are very similar to those of the corresponding BChl-protein from C. thiosulfatophilum), particularly the presence of a trough at 806 nm and a small peak at 837 nm in the CD spectrum of the BChl-RC complex. The low temperature absorption spectrum (Figure 19) of the complex also exhibits clear evidence to support the presence of exciton components. Taken together, the room temperature CD and the low temperature absorption spectra imply the existence of at least five distinct spectral components in the BChl-RC complex from C. thiosulfatophilum. These are summarized in Table 6.

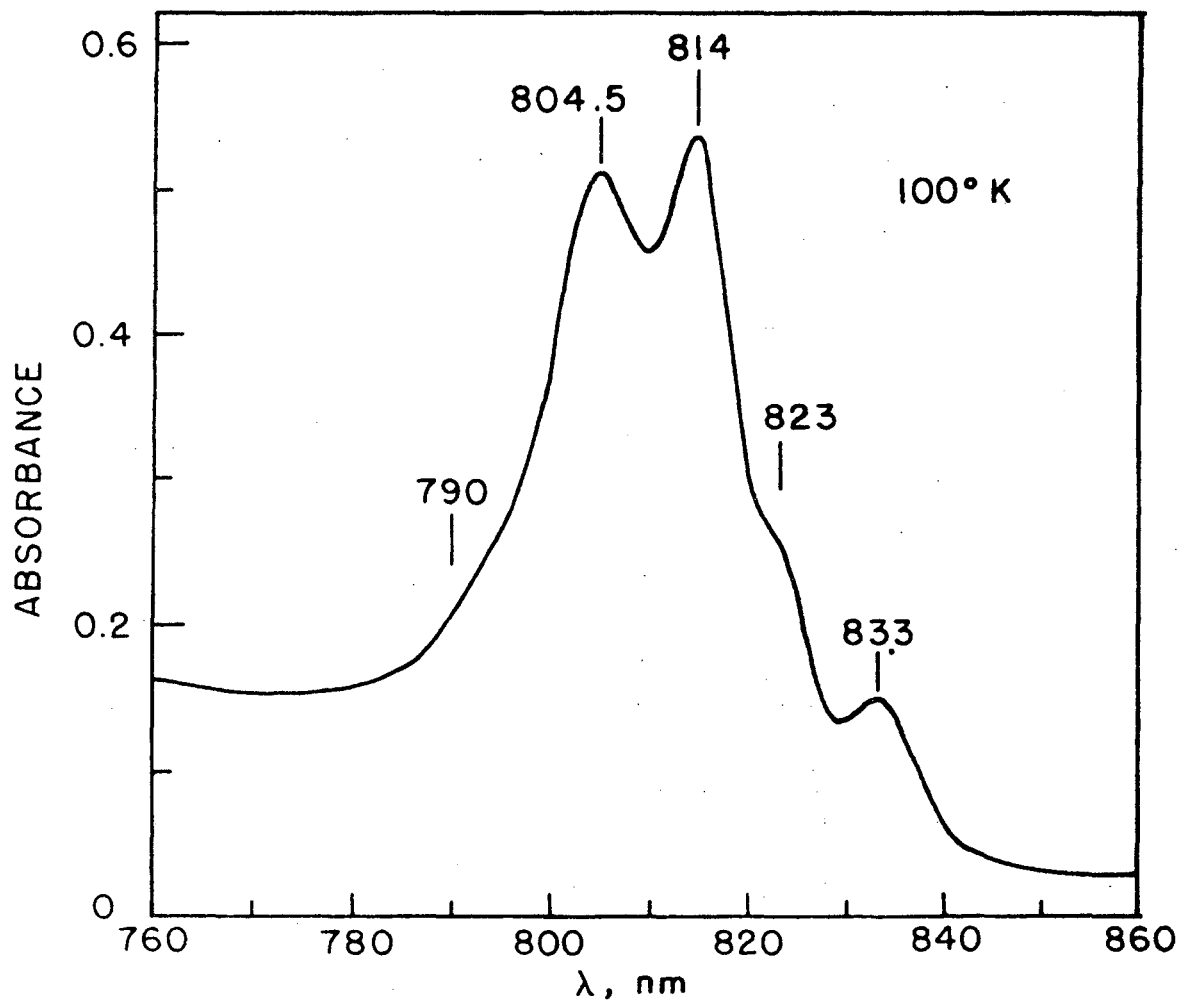
Evidence for photochemical activity in the BChl-RC complex from C. thiosulfatophilum was demonstrated by measuring light induced absorbance changes. The light-minus-dark absorption spectrum in the region of the Q_y band of BChl are presented in Figure 20 (upper curve). The light induced decrease in absorption is maximal at 830 and 840 nm. This spectrum has a very similar shape to that observed by Fowler et al. (1971)

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XBL725-4657

Figure 18. Absorption (upper curve) and CD (lower curve) spectra of BChl-RC complex from *C. thiosulfatophilum*. Pathlength, 1.0 cm; temperature, 24°C. Sample prepared by diluting the centrifuge fraction (in 40% sucrose) eightfold with 10 mM phosphate (pH 7.5), 2 mM KCl solution.



XBL725-4655

Figure 19. Low temperature absorption spectrum of BChl-RC complex from *C. thiosulfatophilum*. Sample dissolved in a glycerol, 10 mM Tris (pH 7.5) + 0.2 M NaCl (1:1 v/v) matrix; spectrum measured at approximately 100°K; pathlength, 2.5 mm.

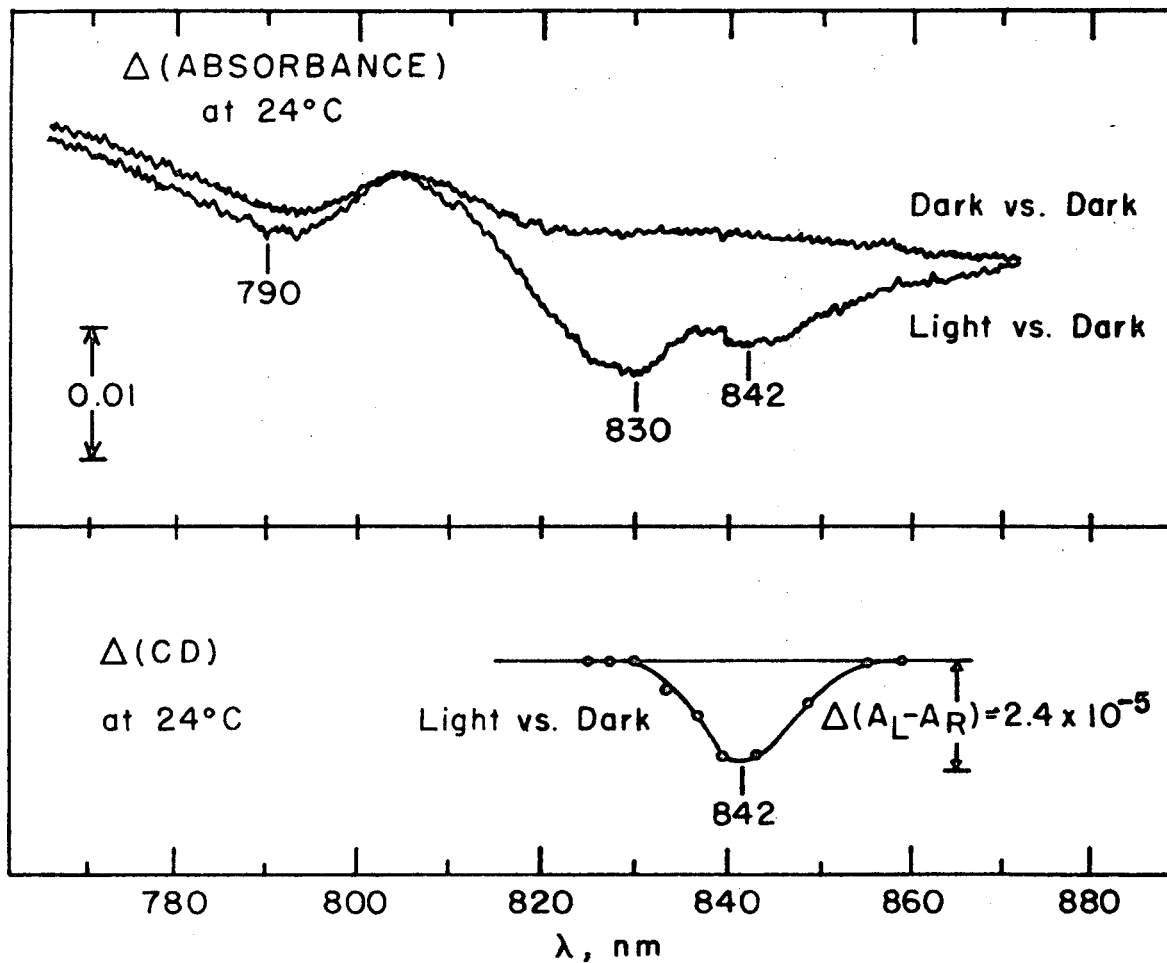
Table 6. Components of Exciton Splitting of Q_y Band of BChl-RC Complex
From C. thiosulfatophilum

| | | | | | | |
|----------------------------------|----------------|-------|-------|-----|-------|-----|
| Absorption spectrum, 100°K | λ , nm | (790) | 804.5 | 814 | (823) | 833 |
| CD spectrum, 24°C | λ , nm | 795 | 806 | 814 | 825 | 837 |
| | Sign | + | - | + | - | + |

(Shoulders indicated as approximate wavelengths.)

using the Cps. ethylica BChl-RC. The chemically oxidized-minus-reduced (ferricyanide vs. ascorbate) absorption spectrum for the BChl-PC complex from C. thiosulfatophilum was essentially identical to the light induced absorption spectrum shown in Figure 20 (upper curve). Unlike the measurements of Fowler et al. (1971) on the Cps. ethylica BChl-RC, we did not observe a relatively greater oxidized-minus-reduced absorption change at 790 nm as compared with the change observed in the light induced difference spectrum.

It was possible to observe very small photoinduced CD changes by illuminating the ascorbate-reduced sample from the side with short wavelength actinic light. The CD changes were obtained as dark-light-dark signals while the instrument monochromator was fixed at a particular wavelength in the region of interest. From a series of measurements carried out in this fashion, the data plotted in Figure 20 (lower curve) were observed. A decrease in the CD centered at 842 nm is observed; this decrease is reversed upon turning off the actinic light. No measurable CD changes were observed at 830 nm. The magnitude of the CD change is $\Delta(A_L - A_R)_{842} = 2.4 \times 10^{-5}$ for a sample concentration giving $A_{810} = 1.07$.



XBL725-4654

Figure 20. Light-induced absorbance (upper curves) and CD (lower curves) changes of the BChl-RC complex from *C. thiosulfatophilum* at room temperature. Samples used exhibited $A_{809} = 1.07$; pathlengths 1.0 cm. A small amount of solid Na ascorbate was added prior to the measurements. See text for additional experimental details.

3. Discussion

On the basis of the absorption (Figure 18) and light-induced absorption difference spectra (Figure 20) it can be seen that the BChl-RC complex isolated from C. thiosulfatophilum is very similar to the "heavy fraction" from Cps. ethylica described by Fowler et al. (1971). This is made clear from a comparison of Figures 18 and 20 above, and Figures 2 and 3 in Fowler et al. (1971). Thus, both green bacteria appear to have identical structural apparatus for performing initial photochemistry at the reaction center.

A comparison of the optical properties of the BChl-RC from C. thiosulfatophilum with those of the non-photoactive BChl-protein (Chapter III) from Cps. ethylica reveals both marked similarities and differences. (Both the absorption and CD properties are very much alike for the BChl-proteins from C. thiosulfatophilum and Cps. ethylica.) CD and low temperature absorption spectra (compare Figures 19 and 20 with Figures 5 and 6) of the BChl-P and the BChl-RC both exhibit multiple components implying exciton interactions. The CD spectrum, however, is qualitatively different for the BChl-RC with peaks and troughs in altered positions. We had anticipated greater similarity in the spectral properties, thinking that the BChl-RC consisted of perhaps four aggregated BChl-proteins, containing 80 BChl's plus a few reaction center BChl molecules absorbing to longer wavelengths. In light of the CD evidence, however, this model must be modified. The BChl-RC complex may contain three BChl-proteins plus a different BChl-protein with an altered molecular structure. Like the normal BChl-protein it may also contain 20 BChl molecules a few of which, because of a different local environment, make up the reaction center of the complex. At the moment, this is just

hypothesis, but it can serve as a working model for future research on the photosynthetic apparatus of green bacteria.

The light-minus-dark difference absorption spectrum (Figure 20), with its two negative components, implies the interaction of at least two BChl molecules within the reaction centers of green bacteria, although the difference CD spectrum (Figure 20) reveals but a single small component. These experiments were done on BChl-RC samples containing only one photoactive BChl per about one hundred BChl molecules. At this concentration it is difficult to obtain much spectral evidence on the nature of the reaction center and preparations are needed which are more highly enriched in the photoactive pigment, P840.

D. Comparison of the Light Induced CD Changes of Reaction Centers from Different Classes of Photosynthetic Organisms

The light-minus-dark CD difference spectra due to changes in the reaction centers of higher plants, purple bacteria, and green bacteria have been examined and are discussed above. The Photosystem I reaction center from spinach chloroplasts appears to have at least two Chl a molecules coupled by exciton interaction. The light-minus-dark difference CD spectrum for the enriched P700 preparation has two components of opposite sign, similar to what is found for purple bacterial reaction centers. The signs of the long and short wavelength components for the chloroplast preparations are reversed relative to those from bacteria, however. Although the spinach and purple bacterial reaction centers may resemble one another in having strongly interacting chromophores, the detailed arrangement of pigments within these different reaction centers is significantly different.

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The reaction centers from green bacteria appear to be fundamentally different from those in purple bacteria or higher plants. Although the difference absorption spectrum for the green bacteria has two negative components, the difference CD spectrum shows only a single negative component. Thus, the CD studies of reaction centers from three major classes of photosynthetic organisms reveal significant differences among the different classes of reaction centers on this sensitive level of examination.

V. CIRCULAR DICHROISM AND FUTURE PHOTOSYNTHETIC RESEARCH

It is hoped that the preceding discussions have made clear the usefulness of CD as a probe of photosynthetic materials. CD has provided detailed information about the nature of interactions within Chl-proteins which serve as reaction centers and as light harvestors. Exciton interaction has been identified as the mechanism of energy transfer among the BChl's of the BChl-protein from Cps. ethylica, and an estimate has been made of the intermolecular distances among these BChl's. The photoactive pigments of reaction centers have been shown to consist of strongly interacting Chl molecules. Knowledge about photosynthetic materials of this nature has previously been lacking. The question exists as to what future use CD will be in helping to elucidate further the photosynthetic apparatus. Some areas amenable to future study are outlined here.

(1) Since the optical activity of a Chl-protein may be primarily a function of the specific geometrical arrangement of interacting Chl's with respect to one another, the question arises as to whether one can calculate this structure from the experimentally obtained CD spectrum. Using the low temperature CD and absorption spectra of the BChl-protein from Cps. ethylica (Chapter III), calculations of this sort were attempted. The goal was a determination of the arrangement of the five BChl molecules within a protein subunit. Unfortunately, the

calculations were too complicated and this overambitious project was discontinued. Before such a calculation becomes feasible on a Chl-protein some restrictions are needed on the possible molecular arrangements to be expected. In this manner, an approximate Chl a dimer structure was calculated by Dratz (1966). Use was made in these calculations of restrictions on possible structures provided by NMR experiments and studies on the chemical nature of dimers. As smaller Chl-proteins are isolated and further characterized it is hoped that information will be obtained, perhaps from NMR, which will suggest models for the Chl arrangements. At that time theoretical CD calculations may provide insight into the correct three-dimensional arrangement of Chl-protein structures.

(2) Although the CD spectra of Chl-proteins strongly suggest the presence of exciton interactions within the protein, this assignment is often equivocal. There are effects, other than exciton interaction, which can cause changes in the CD spectrum. Examples are the effects of the protein environment and of interactions with nondegenerate transitions in other Chl molecules. The magnitude of CD change from these sources is difficult to estimate. Theoretical calculations such as those done on hemoglobin and myoglobin by Hsu and Woody (1971a) and further experiments should shed some light on this problem.

(3) CD spectra have set a lower limit of three BChl molecules in the reaction centers of purple bacteria (Chapter IV, B). Chemical analyses of these reaction centers for BChl content (Clayton *et al.*, 1972), as was mentioned, have been contradictory. Low temperature CD spectroscopy may be able to resolve the presence, or absence, of more

than three reaction center BChl components. This experiment is feasible for the near future despite some technical problems in the low temperature method. This technique should also be useful in analyzing the anomalous reaction center from R. viridis (Chapter IV, B).

(4) As photosynthetic membranes are fractionated by various procedures, the question arises of whether artifacts are being introduced to the system of interest. In particular, there is concern over whether the conformational properties of component Chl-proteins are changed by current isolation techniques. CD should prove useful in probing this question. This could be done by studying CD spectra taken of the various fractions obtained in the isolation procedures. The question of interest would be whether the CD of an intact photosynthetic apparatus could be accounted for in terms of the CD of its parts. This approach and related studies may supply fruitful and unexpected results.

(5) The technique of difference CD spectroscopy described here (Chapter IV) is potentially useful for studying other photoactive components of photosynthetic systems besides reaction center Chl molecules. Electron transport components such as cytochromes and ferredoxin exhibit light-induced absorption changes. A study of the in vivo CD signals of cytochromes might supply data on the conformational state of these components as a function of redox potential. Light-induced CD changes from in vivo ferredoxin might be used to help establish whether ferredoxin serves as the primary acceptor of Photosystem I in chloroplasts. The information available from the in vivo light-induced CD changes of these components has so far been untapped.

APPENDIX

The computer program for curve resolving, using Gauss's method of non-linear least squares, did simultaneous fitting of the experimental absorption and CD spectra. That is, the program was set up so that corresponding components in the absorption and CD resolved spectra could be required to have equal half-width, position, and skew. It was found that asymmetric gaussian curves gave better fits than normal gaussians where an asymmetric gaussian curve is defined as one in which the half-width of the long-wavelength side is different than the half-width of the short-wavelength side. The skew is a measure of this asymmetry. For the resolved spectra in Figure 8 the half-width and skew of corresponding absorption and CD components were required to be equal.

In resolving the components of a CD spectrum one is often unsure of the relationship of the final resolved components to reality. Often several different solutions fit equally well. The advantage of simultaneous fitting of absorption and CD spectra is that the additional requirements eliminate many possible solutions. It is still difficult, however, to be certain of the uniqueness of the resultant resolved spectra.

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