Exciton Interaction in a Bacteriochlorophyll—Protein from *Chloropseudomonas ethylica*. Absorption and Circular Dichroism at 77°K†

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ABSTRACT: A water-soluble 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 multiple components in the circular dichroism spectrum in this region. Four absorption and five circular dichroism components are observed, indicating the interaction of at least five bacteriochlorophyll *a* molecules in the complex. The total exciton band splitting is 490 cm^{-1}. The circular dichroism of a second electronic transition at 603 nm exhibits three resolved exciton components. The separation of bacteriochlorophyll *a* molecules is estimated to 12–15 Å in one subunit of the protein.

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)[1] 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 paper we present the results of optical studies on the BChl-P. Absorption and circular dichroism (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 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).

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 mM^{-1} 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

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[1] Abbreviations used are: BChl-P, bacteriochlorophyll–protein; *Cps. ethylica*, *Chloropseudomonas ethylica*; BChl *a*, bacteriochlorophyll *a*.

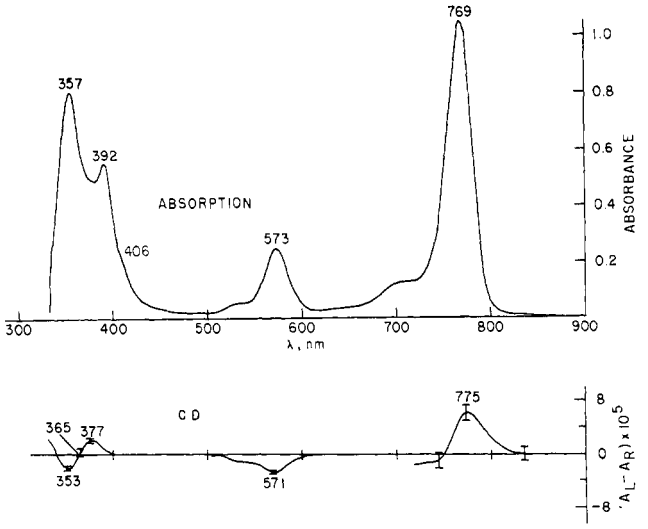

FIGURE 1: Absorption and CD spectra of BChl *a* in ether at room temperature; concn 1×10^{-5} M and path length 1 cm (L. Coyne, 1968, unpublished data).

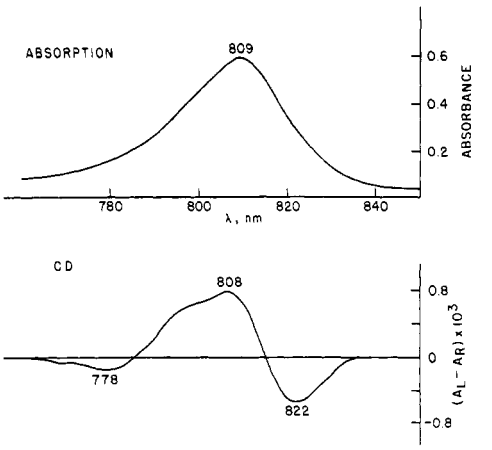

FIGURE 2: Absorption and CD spectra of Q_y band of BChl-P at room temperature in potassium glycerophosphate, Tris buffer (pH 7.5, 0.2 M NaCl), glycerol; BChl *a* concentration 1.3×10^{-5} M and path length 3 mm.

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 1, 2, and 3 were recorded on an instrument built in this laboratory (Dratz, 1966). The CD spectrum in Figure 4 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.

Results

The CD and absorption spectra of BChl *a* in its monomeric form in ether are shown in Figure 1. 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).

In Figures 2 and 3 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 2 is shifted to longer wavelength (809 nm) compared to that of BChl *a* in solution (Figure 1), 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 com-

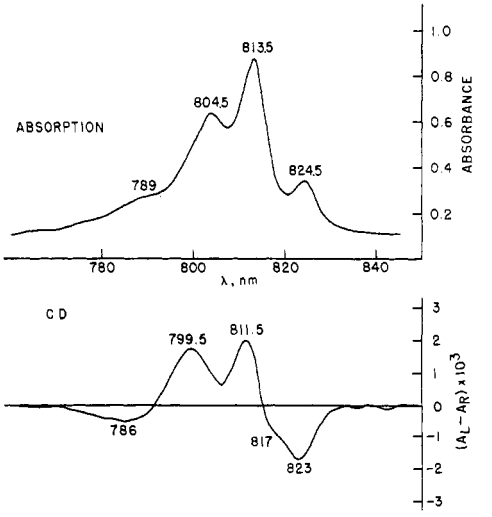

FIGURE 3: Absorption and CD spectra of Q_y band of BChl-P at 77°K in potassium glycerophosphate, Tris buffer (pH 7.5, 0.2 M NaCl), glycerol; BChl *a* concentration 1.3×10^{-5} M (uncorrected for solvent shrinkage) and path length 3 mm.

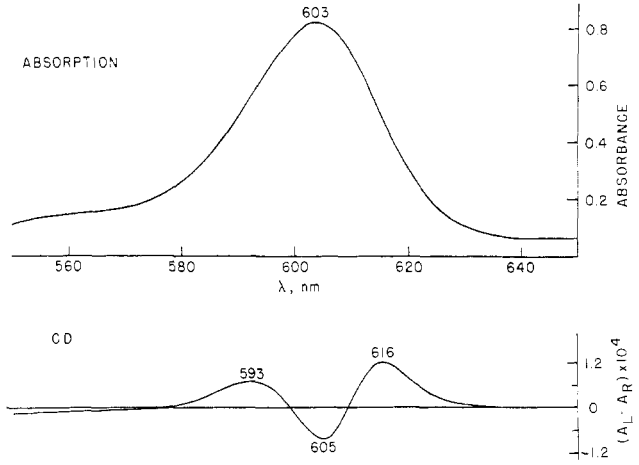

FIGURE 4: Absorption and CD spectra of Q_x band of BChl-P at room temperature in glycerol, Tris buffer (pH 7.5, 0.2 M NaCl); BChl *a* concentration 5.2×10^{-5} M and path length 5.7 mm.

ponents are clearly visible (at 822, 808, and 778 nm). A similar spectrum has previously been reported (Kim and Ke, 1970). At 77°K (Figure 3) 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 4). The single small negative CD peak of BChl *a* in ether (Figure 1) 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

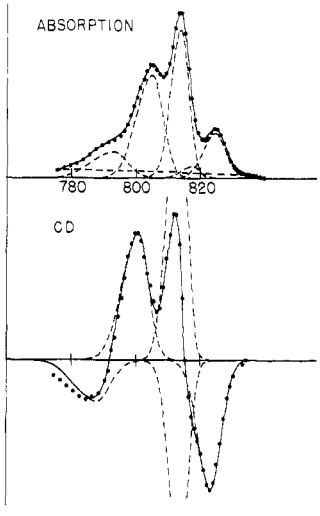

FIGURE 5: The absorption and CD of the Q_y band of the BChl-P at 77°K resolved into asymmetric gaussians. See text and Table I. Experimental data, ●; gaussian component, - - -; sum of gaussians, ——.

TABLE I: 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 5) and Data on the Q_y Band of BChl *a* in Acetone at 299 and 180°K.

λ (nm)		Dipole Strength[a]	Rotational Strength[a] (debye magneton)	Full-Width at Half-Maximum (nm)	Skew[b]
Abs	CD	(debye²)			
		BChl-P at 77°K			
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
		BChl *a* in acetone at 299°K[c]			
770	772	49	0.06	44.0	0.69
		BChl *a* in acetone at 180°K[c]			
771		50		36.5	0.74

[a] Per molecule of BChl *a*. [b] Half-width of long-wavelength side of gaussian = (skew) × (half-width of short-wavelength side). [c] J. R. Lindsay Smith and K. Sauer, 1966, unpublished data.

−120° the CD shows no evidence of additional peaks. 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 within 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 (Figure 3) were computer resolved into component peaks (Figure 5 and Table I). Since the absorption peak of monomeric solution BChl *a* is asymmetric (the half-width of the short-wavelength side of the peak 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 5 was obtained. The long sloping line in the upper half on Figure 5, 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 short wavelength region of the absorption spectrum. The CD spectrum also

exhibits its poorest fit in the short wavelength region. As seen in Table I, 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).

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 (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 probabilty; *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 signs and rotational strengths of the CD band components resulting from exciton interaction are determined by the specific geometric arrangement of the interacting molecules, rather than by the inherent asymmetry of the individual molecules. The exciton contribution to the rotational strength is conservative (Tinoco, 1962), meaning that its contribution to the rotational strength should sum to zero over the exciton band. Thus, it is characteristic of exciton interactions that the different components within the one electronic band will have both positive and negative signs.

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 1) and asymmetric interactions of each of the BChl *a* molecules with transitions in the protein matrix and with nondegenerate 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 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 3. The CD for both the Q_y and Q_x bands is greatly enhanced as compared with the BChl *a* solution monomers (Figure 1) 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 3) 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 3 and listed in Table I.

Equations for calculating exciton spectral properties have been developed by Tinoco (1963). The wave function for an exciton state is written as a linear combination of states in which the excitation is localized on each of the molecules of the aggregate. These linear-combination wave functions and the energy of the transition to each of the exciton states are obtained as the eigenvalues and eigenvectors of the perturbation matrix whose elements are the transition interaction energies between each of the pairs of like molecules. The interaction energies have roughly an inverse cube dependence on distance. Using this procedure, calculations were undertaken to see if the distances between BChl *a* molecules could be estimated. In these calculations the elements of the perturbation matrix were obtained by using electric transition monopoles located at each of the conjugated atoms of the porphyrin ring (Philipson *et al.*, 1971), rather than by the more common point dipole approach. This method better takes into account the effect of delocalization on the interaction potential.

For these model calculations the centers of the five BChl *a* molecules were arbitarily 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 Å and its molecular volume is

193,000 Å^3 (Olson and Thornber, 1971). The calculated diameter of one subunit (assuming a spherical shape) of the protein is, therefore, 45 Å. These dimensions for the subunit will suffice to hold five molecules of BChl *a* with centers 12–15 Å 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 (Simpson and Peterson, 1957); this case is designated weak exciton coupling by Förster (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 3), it was necessary to use two large gaussian curves of opposite sign almost on top of one another. These are the components in Figure 5 (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 3) 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 I) 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, 1966[2]). 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. By the Franck–Condon principle, this will mean that the O–O 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 (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 *Rhodopseudomonas spheroides* (Sauer *et al.*, 1968) and *Rhodospirillum rubrum* (Philipson and Sato, 1972[3]) 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[4]) discussed in this paper. Thus, a CD study can reveal more detailed similarities than would be possible through biochemical techniques.

Acknowledgments

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References

Clayton, R. K. (1966), *Brookhaven Symp. Biol. 19*, 49.

Davydov, A. S. (1948), *Zh. Eksp. Teor. Fiz. 18*, 210.

Dratz, E. A. (1966), Ph.D. Thesis, University of California, Berkeley, Calif.

Förster, Th. (1965), *in* Modern Quantum Chemistry, Vol. 3, Sinanoglu, O., Ed., New York, N. Y., Academic Press, p 93.

Ghosh, A. K., Broker, T. R., and Olson, J. M. (1968), *Biochim. Biophys. Acta 162*, 402.

Goedheer, J. C. (1966), *in* The Chlorophylls, Vernon, L. P., and Seely, G. R., Ed., New York, N. Y., Academic Press, p 147.

Gouterman, M. (1961), *J. Mol. Spectrosc. 6*, 138.

Houssier, C., and Sauer, K. (1970), *J. Amer. Chem. Soc. 92*, 779.

[2] J. Lindsay Smith and K. Sauer, unpublished data.

[3] K. D. Philipson and V. L. Sato, unpublished data.

[4] J. M. Olson, K. D. Philipson, and K. Sauer, manuscript in preparation.

Hsu, M. C., and Woody, R. W. (1971a), *J. Amer. Chem. Soc. 93*, 3515.
Hsu, M. C., and Woody, R. W. (1971b), *Biopolymers 10*, 1421.
Kasha, M. (1963), *Radiat. Res. 20*, 55.
Kasha, M., Rawls, H. R., and El-Bayoumi, M. A. (1965), *Pure Appl. Chem. 11*, 371.
Kim, Y. D., and Ke, B. (1970), *Arch. Biochem. Biophys. 140*, 341.
Kreutz, W. (1970), *Advan. Bot. Res. 3*, 53.
Labaw, L. W., and Olson, R. A. (1970), *J. Ultrastructure Res. 31*, 456.
Olson, J. M. (1966), *in* The Chlorophylls, Vernon, L. P., and Seely, G. R., Ed., New York, N. Y., Academic Press, p 413.
Olson, J. M. (1971), *Methods Enzymol. 23*, 636.
Olson, J. M., and Thornber, J. P. (1971), *Photochem. Photobiol. 14*, 329.
Philipson, K. D., Tsai, S. C., and Sauer, K. (1971), *J. Phys. Chem. 75*, 1440.
Rhodes, W. (1961), *J. Amer. Chem. Soc. 83*, 3609.
Robinson, G. W. (1966), *Brookhaven Symp. Biol. 19*, 16.
Sauer, K., Dratz, E. A., and Coyne, L. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 17.
Sauer, K., Lindsay Smith, J. R., and Schultz, A. J. (1966), *J. Amer. Chem. Soc. 68*, 2681.
Schultz, A. J., and Sauer, K. (1972), *Biochim. Biophys. Acta* (in press).
Seely, G. R., and Jensen, R. G. (1965), *Spectrochim. Acta 21*, 1835.
Simpson, W. T., and Peterson, D. L. (1957), *J. Chem. Phys. 26*, 588.
Thornber, J. P., and Olson, J. M. (1968), *Biochemistry 7*, 2242.
Tinoco, Jr., I. (1962), *Advan. Chem. Phys. 4*, 113.
Tinoco, Jr., I. (1963), *Radiat. Res. 20*, 133.
Vredenberg, W. J. (1965), Ph.D. Thesis, University of Leiden, Leiden, The Netherlands.
Vredenberg, W. J., and Amesz, J. (1966), *Brookhaven Symp. Biol. 19*, 62.
Weier, T. E., Stocking, C. R., and Shumway, L. K. (1966), *Brookhaven Symp. Biol. 19*, 353.

Determination of the Structures of Cutin Monomers by a Novel Depolymerization Procedure and Combined Gas Chromatography and Mass Spectrometry†

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ABSTRACT: A convenient novel technique for the structural analysis of the hydroxy fatty acid polymer cutin, the major component of plant cuticle, is described. Treatment of cutin powder with $LiAlH_4$ in tetrahydrofuran gave high yields (80–95%) of ether-soluble reduced monomers which were quite stable and suitable for direct analysis by a combination of gas–liquid chromatography and mass spectrometry (glc-ms). For structural studies this mixture was analyzed by silica gel G and argentation thin-layer chromatography in conjunction with glc-ms analysis. The $LiAlH_4$-susceptible functions of cutin were identified by a similar analysis of the $LiAlD_4$-cleavage products of cutin. The diagnostic fragmentations exhibited by the trimethylsilyl ethers of the individual components together with their deuterium labeling pattern enabled rapid identification of the cutin monomers. This method was applied to cutin isolated from the fruits of peach (*Amygdalus persica*), pear (*Pyrus communis*), papaya (*Malabar papaiarnarum*), apple (*Malus pumila*), and grape (*Vitis vinifera*) and the leaves of *Senecio odoris* and apple. All the cutins examined contained a similar complement of minor components (usually <2%) consisting of fatty acids, alcohols, and α,ω-diols, as well as monohydroxy fatty acids which typically constituted 8–17% of the cutin. However, there were striking differences in the structure of the more polar cutin acids, and on this basis two types of cutin were recognized. One type, typified by papaya, contained dihydroxypalmitic acid as the major component (>74%) and the other contained various proportions of 9,10-epoxy-18-hydroxystearic acid (6.5–32%), 9,10,18-trihydroxystearic acid (5–25%), and their Δ^{12}-monounsaturated counterparts (14–30 and 2–6%, respectively) in addition to dihydroxypalmitic acid (13–45%). In the dihydroxypalmitic acid fraction both 9,16 and 10,16 isomers were present; in papaya the former isomer predominated and in the other cutins examined the latter isomer was dominant. The presence of positional isomers in the dihydroxy-C_{16} acid fraction and the absence of unsaturated C_{16} acids from cutin are consistent with a direct hydroxylation mechanism for the introduction of the in-chain hydroxyl group. On the other hand, the occurrence of unsaturated ω-hydroxy-C_{18} acid, 9,10-epoxy-18-hydroxy-C_{18} acid together with 9,10,18-trihydroxy-C_{18} acid suggests that oleic acid and linoleic acid undergo hydroxylation at C-18, epoxidation at Δ^9, followed by hydration of the epoxide to yield these cutin components.

The plant cuticle is comprised of cutin, a lipid polymer, which is a relatively rigid meshwork of interesterified hydroxy fatty acids, embedded in a layer of waxy material. The structures of the cutin and the waxes largely determine the physical and chemical properties of the plant cuticle, which plays an important role in the interaction between the plant and its en-

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