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DICHROISM OF BACTERIOCHLOROPHYLL IN CELLS OF THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS PALUSTRIS*

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SUMMARY

1. The dichroism was measured at varied wavelengths of polarized light in "intact" cells of *Rhodopseudomonas palustris* and in films of air-dried lamellar fragments of the bacterium.

2. The values for the dichroism ($\Delta A/A$) of bacteriochlorophyll in lamellae were found to be −1.1 at 590 nm and +0.50 at 800 and 870 nm.

3. Air drying of the lamellar fragments in films did not essentially change the orientation of bacteriochlorophyll from that in intact cells.

INTRODUCTION

In studies of the states of photosynthetic pigments in the photosynthetic apparatus, the measurement of dichroism will provide direct information concerning the orientation of the pigments in the membrane system of the organelle.

The dichroism of chlorophyll *in vivo* has been studied by various investigators, using various methods and test materials[1-9]. One of the fundamental requirements for the measurement of dichroism will be that the organelles are oriented in regular array. A variety of methods has been employed to obtain such orientation of the organelle. One of the methods was to select, under the light-microscope, chloroplasts lying at a given direction in intact cells and to measure the optical properties of the lamellae *in situ*. MENKE[1,2], and MENKE AND MENKE[3] applied this method using Mougeotia and Closterium as test materials and observed a weak dichroism. OLSON *et al.*[4,5] working with Euglena as test material, used photomicrography with polarized light[4] and microspectrophotometry[5] of chloroplasts. GOEDHEER[6] used photomicrography with polarized monochromatic light for the measurements with single sheets of Mougeotia chloroplasts. SAUER AND CALVIN[7] and SAUER[8] working with isolated spinach chloroplasts oriented the chloroplasts by electric field[7] or flow[8]. A method of pressing chloroplasts between thin sheets of metal was used by THOMAS *et al.*[9].

In some cases a more or less significant dichroism has been observed at the absorption band around 695 nm. In other forms of chlorophyll in chloroplasts no significant dichroism has been observed. However, KREUTZ[10] suggested a possibility for

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orientation of chlorophyll molecules in the lamellae, even when dichroism of chlorophyll was apparently absent.

In chromatophores of photosynthetic bacteria, no dichroism was detected by SAUER AND CALVIN[7]. Chromatophores of some photosynthetic bacteria are spherical in shape when suspended in aqueous media[11]. Therefore, no dichroism of any pigment can be expected for the suspension of such chromatophores even when the pigments may have some orientation in the chromatophore structure. Polarization of fluorescence has been observed by GOEDHEER[6] and double circular dichroism has been observed by DRATZ *et al.*[12] in the region of near-infrared absorption of bacteriochlorophyll. These facts indicate the presence of some arrangement of bacteriochlorophyll in chromatophores.

However, some photosynthetic bacteria have lamellar structures in the cells instead of vesicular chromatophores as the photosynthetic apparatus. In *Rhodopseudomonas palustris*[13], electron-microscopic inspection reveals a regular formation of the lamellae in the rod-shaped cells. In a cross section of the cells, they appear as concentric circles. In a longitudinal section, they appear as parallel-packed peels of lamellar sheets similar in form to that of the cell. In this case, an oriented arrangement of the photosynthetic organelle can be obtained by the orientation of the cells in the suspension. The dichroism of the photosynthetic pigments, if there is any, should be detectable in this system.

Another way to provide an orientation of the organelle will be to make a "dry film" of lamellar fragments. In the electron microgram, the lamellar fragments, spread on the mesh and shadowed, show flat images, indicating that most of the lamellar fragments, when spread and dried to prepare the sample for electron microscopy, may be regarded as consisting of sheets of lamellar membrane lying parallel to the plane of the mesh. Thus, when the lamella fragments are spread on a plate and air-dried, most of the surface of the lamellae will be lying parallel to the surface of the glass plate. In such a preparation, the orientation of the pigments should be detected, if there is any.

In this study, the dichroism of bacteriochlorophyll in lamellae of *R. palustris* was detected, using these two methods to make an oriented arrangement of the organelle surface. The orientation of bacteriochlorophyll was estimated on the basis of measurements of dichroism.

MATERIALS AND METHODS

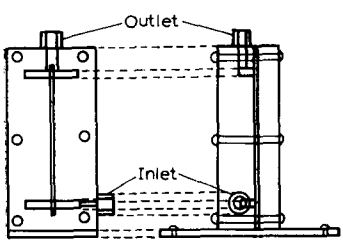
R. palustris, isolated and pure-cultured in this laboratory, was cultured under continuous illumination, in a medium consisting of 0.5 % yeast extracts, 0.3 % peptone and 0.5 % sodium lactate (pH 7.0), at 32° for 3 days[14]. The cells were harvested by centrifugation, and suspended in a 0.5 M phosphate buffer (pH 7.0). The suspension was mixed with a solution of methyl cellulose, to make up the final concentration to 0.3 M phosphate buffer and 0.5 % methyl cellulose, and used as test material for "intact cells".

The lamellar fragments were prepared by disrupting the cells by exposing 50 ml of the cell suspension in 0.5 M phosphate buffer to sonic oscillation (10 kcycles, 100 W, Toyo – riko Ltd., Model 50-5) for 30 min at 4° (cooled by running an ice–water mixture from the outside of the vessel). The resulting suspension was centrifuged at 10000 × *g*

for 15 min to remove the debris and undisrupted cells. The lamellar fragments were collected from the supernatant by centrifugation at 80000 × *g* for 60 min. The lamellar preparation was washed once by centrifugation. The pellet obtained was dispersed in an equal amount of water to make a thick suspension of lamellar fragments. The suspension was spread on a cover-slip and then air-dried at room temperature. The resulting sample was used as the test piece for the "dry film" in most of the experiments.

The absorption measurements were made in a Shimadzu PMS 50 recording spectrophotometer. In the spectrophotometer, photomultipliers (R-236, Hamamatsu TV Ltd.) were used as detectors. As polarizer, a Nicol prism was employed. The polarizer was placed in front of the photomultiplier of the sample side of the spectrophotometer.

The chamber for the flow of the cell suspension consisted of two square pieces of Lucite plate and a sheet of black polyvinyl chloride (0.3 mm thick) was inserted as a spacer between the Lucite plates. In one of the plates, two shallow pits were made, one in the upper part and the other near the bottom, as pools for the flowing fluid. From the pools, canals led to the outlet and inlet of fluid. In the vinyl sheet, a narrow slit, 0.3 mm wide and 40 mm long, was cut to make a path of fluid from one pool to the other. The three component parts, plates and film, were tightly screwed together with bolts and nuts (4 mm in diameter) (Fig. 1). The inlet and the outlet were connected by silicon rubber tubing to one injection syringe each used as reservoirs of the fluid cell suspension. The flow of the suspension was driven by applying appropriate pressure by placing weights on top of one of the injection syringes held in a vertical position. The direction of flow of the "intact cell" suspension for the measurements of dichroism was vertical. For technical reasons, to obtain a steady flow, only the upward flow of the cell suspension was used for the measurements.



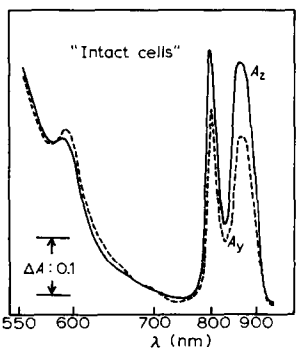


Fig. 1. Flow chamber for measurement of dichroism in a flowing suspension of intact bacterial cells.
Fig. 2. Absorption spectra of intact cell suspension of *R. palustris*, for horizontally and veritcally polarized light. The cell suspension was flowing vertically. Flow rate, 1 ml/1.7 sec (655 cm/sec). ———, vertically polarized light; – – – –, horizontally polarized light.

The flow chamber was placed in the middle of the sample compartment of the spectrophotometer, where the image of the optical slit for the measuring beam is formed. The reference compartment of the spectrophotometer was left empty.

In the measurements of dichroism with "dry film", the test piece was placed in a vertical position in a paraffin oil bath. The bath was placed in the middle of the sample compartment of the spectrophotometer. The measurements were made at four

different angles of measuring light beam to the surface of the test piece (90°, 50°, 40° and 30°), adjusted by turning the coverglass in the paraffin oil bath.

All optical measurements were done at room temperature.

RESULTS

Measurements of dichroism in the flowing suspension of intact cells

The absorbance of vertically and horizontally polarized light (A_z and A_y) were measured. The direction of the measuring light of the spectrophotometer will be taken as the x axis of the co-ordinates. The vertically polarized light is polarized in the direction of the z axis and the horizontally polarized light in the direction of the y axis. In the flow chamber, the longitudial axis of bacterial cells is oriented along the z axis.

A correction for zero absorption was made by substracting the values of absorbance measured using water in place of "intact cell" suspension. To reduce such effects as that of polarization of the incident beam of measuring light and turbidity of the sample, the peak-to-trough difference of absorbance was used for the estimation of the values for dichroism.

A set of the resulting absorption spectra, measured with vertically and horizontally polarized lights, is illustrated in Fig. 2. As will be seen from the figure, A_y was higher than A_z at the absorption band at 590 nm, whereas A_z was higher than A_y in the near-infrared region of the absorption (800 and 870 nm). These findings indicate the presence of dichroism in intact cells.

Estimation of dichroism in the "intact cells"

The absorption parallel to the surface of lamellae is denoted as $A_{||}$ and the one

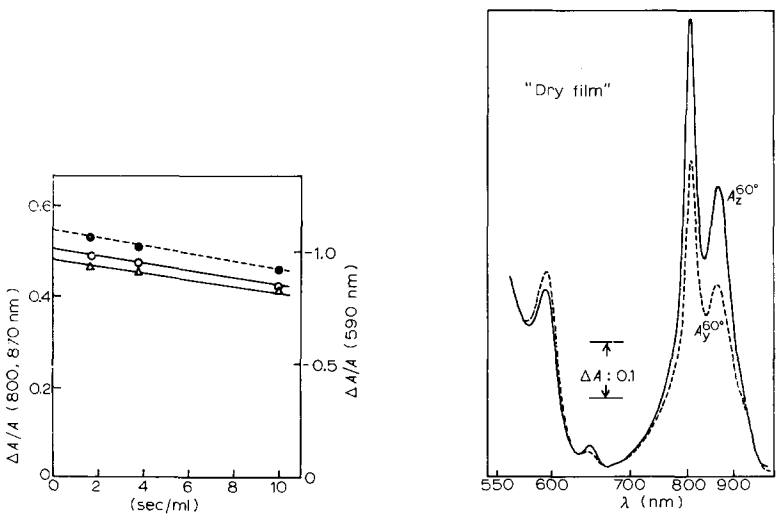


Fig. 3. Relationship between reciprocals of flow rates and values of dichroism ($\Delta A/A$), calculated from the absorbance with respect to vertically and horizontally polarized light, in a vertically flowing suspension of intact cells. ○—○, at 800 nm; △—△, at 870 nm; ●—●, at 580 nm.

Fig. 4. Absorption spectra of dry film of *R. palustris* lamellar fragments, in polarized light. The angle of the dry film to the measuring light beam was 30° ($\theta = 60°$). ———, vertically polarized light; – – – –, horizontally polarized light.

perpendicular to the lamellar surface as $A_\perp$. Under the ideal condition when the cells are completely oriented along the z axis, it will be:

$$A_y = 1/2\ (A_{||} + A_\perp) \tag{1}$$

and neglecting the contribution of the end part of the rod-shaped lamellar thylakoid:

$$A_z = A_{||} \tag{2}$$

From the definition of ΔA and A,

$$\Delta A = A_{||} - A_\perp = 2\ (A_z - A_y) \tag{3}$$

$$A = 1/3\ (2A_{||} + A_\perp) = 1/3\ (A_z + 2A_y) \tag{4}$$

Utilizing Eqns. 3 and 4, values for the dichroism ($\Delta A/A$) were calculated from the values for absorbance, A_z and A_y, determined with respect to the vertically and horizontally polarized light, respectively. The values for $\Delta A/A$ were plotted against the reciprocals of the flow rates of the suspension, and extrapolated to infinite rate of flow (Fig. 3). Thus, the dichroism of bacteriochlorophyll in the lamellae in intact cells was estimated to be −1.09 at 590 nm and +0.50 and +0.48 at the near-infrared absorption bands, 800 and 870 nm, respectively.

Measurements of dichroism in "dry film" of lamellar fragments

The absorbance of vertically and horizontally polarized light (A_z and A_y) were measured, using "dry film" of lamellar fragments from *R. palustris*. The angle between the surface of the test piece and the plane perpendicular to the measuring light beam was denoted as θ. As will be seen from the results illustrated in Fig. 4 ($\theta = 60°$), A_z was higher than A_y in the near-infrared region (800 and 870 nm), whereas at 590 nm A_y was higher than A_z.

Estimation of dichroism in "dry film"

From the observed values of A_z and A_y in the dry film, the values for the dichroism were estimated. It will be evident from the geometrical arrangement of the measurement, that A_z at a given θ (denoted as $A_z{}^\theta$) represents the absorption parallel to the sample surface. However, the thickness of the sample along the axis of the measuring beam will change as a function of the reciprocals of $\cos\theta$. Therefore:

$$A_z{}^\theta = A_{||} \cdot 1/\cos\theta \tag{5}$$

$A_y{}^\theta$ is a combined function of $A_{||} \cdot \cos^2\theta$, $A_\perp \cdot \sin^2\theta$ and also of the thickness factor, $1/\cos\theta$. Thus, for a given θ:

$$\begin{aligned} A_y{}^\theta &= 1/\cos\theta\ (A_{||} \cdot \cos^2\theta + A_\perp \cdot \sin^2\theta) \\ &= A_{||} \cdot \cos\theta + A_\perp (1/\cos\theta - \cos\theta) \end{aligned} \tag{6}$$

Defining the difference $A_z{}^\theta - A_y{}^\theta$ as ΔA^θ,

$$\begin{aligned} \Delta A^\theta &= A_{||} \cdot 1/\cos\theta - A_{||} \cos\theta - A_\perp (1/\cos\theta - \cos\theta) \\ &= (A_{||} - A_\perp) \cdot (1/\cos\theta - \cos\theta) \\ &= \Delta A \cdot (1/\cos\theta - \cos\theta) \end{aligned} \tag{7}$$

Eqns. 5 and 7 mean that there is a linear relationship between $A_z{}^\theta$ and $1/\cos\theta$, as well as between ΔA^θ and $(1/\cos\theta - \cos\theta)$. In Fig. 5, the absorbance $A_z{}^\theta$ and the

difference of absorbance ΔA^{θ}, at 590, 800 and 870 nm, measured at varied positions of the test piece by changing the angle θ to 40°, 50° and 60° are plotted as functions of $1/\cos\theta$ for $A_z{}^{\theta}$ and $(1/\cos\theta - \cos\theta)$ for ΔA^{θ}. According to Eqns. 5 and 7, the values for $A_{\parallel}$ and ΔA will be calculated as the tangents of the straight lines in the plots. The actual values for $A_{\parallel}$ in "dry film" of *R. palustris* lamellar fragments were 0.03 at 590 nm and 0.41, 0.257 at 800 and 870 nm, respectively. The values for ΔA were −0.024, +0.156 and +0.11 at 590, 800 and 870 nm, respectively. From these, the values for the dichroism $\Delta A/A$ were calculated to be −0.65 at 590 nm, +0.44 at 800 nm and + 0.50 at 870 nm. These values for $\Delta A/A$ are practically in agreement with the respective values estimated from the results of the flowing method with "intact cells" (Table I).

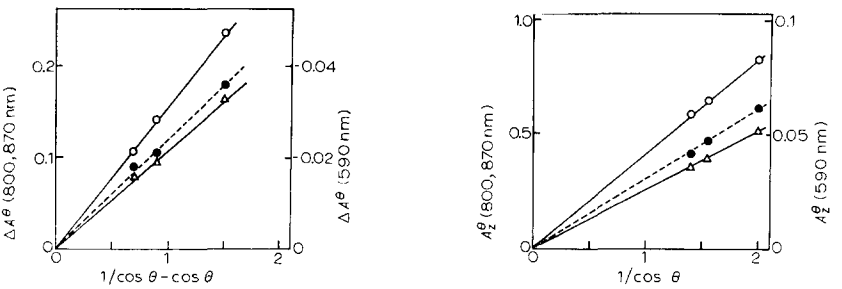


Fig. 5. Relationship between ΔA^{θ} and $(1/\cos\theta - \cos\theta)$, and between A_z and $1/\cos\theta$ at 590, 800 and 870 nm, for varied values of θ (dry film of *R. palustris* lamellar fragments). ○---○, at 800 nm; △—△, at 870 nm; ●---●, at 590 nm.

TABLE I

SUMMARIZED VALUES FOR THE DICHROISM IN INTACT CELLS AND DRY FILM OF LAMELLA FRAGMENTS IN *R. palustris*

Wavelength measured (nm)	*Test material*			
	Intact cells		*Dry film*	
	$\Delta A/A$	D^*	$\Delta A/A$	D^*
590	−1.09	0.37	−0.65	0.55
800	+ 0.50	1.75	+ 0.44	1.62
870	+ 0.48	1.71	+ 0.50	1.75

* D: dichroic ratio, $D = A_{\parallel}/A_{\perp}$.

DISCUSSION

The observed dichroism of bacteriochlorophyll in intact cells and dry film of lamellar fragments will be mostly due to the orientation of the pigment in the lamellar membrane. Theoretically, $\Delta A/A$ may have values ranging from +1.5 to −3.0, depending on the angle of the transition moment to the surface of the membrane and the grade of orientation of the pigment molecules in the test material. When measured in the ways as used in this study, the absorption due to a transition moment parallel to the surface of the membrane will give a value of +1.5, and the absorption due to a

transition moment perpendicular to the surface of the membrane a value of —3.0. The actual values obtained were +0.50 for the infrared region and —1.09 for the 590-nm band. These values indicate that the actual orientation of the transition moments were far from ideal. One of the causes for the small absolute values of dichroism found in the lamellae will be due to an incomplete orientation of the pigment molecules in the membrane and/or an irregularity in the arrangement of the lamellae in the test system.

Actually, there are some factors which may cause an irregularity in the arrangement in the test system. In the electron microgram of a longitudinal section of the cells, some bent or waved portion of lamellae was observed. In the terminal parts of the bacterial cells the lamellae tend to curve inward, and at the polar ends of the cells lamellae are not observed[13]. The portion of lamellae deviating from the ideal shape of straight cylinders will contribute to reduce the absolute values of dichroism. The actual values of these correction factors could not be estimated.

Also in the "dry film" there are factors which may cause a decrease in absolute values of dichroism such that the membrane of the lamellar fragments may not be completely flat to the glass plate on which the lamellar fragments are spread. Actually, the suspension of lamellar fragments was not diluted sufficiently. Therefore, some of the lamellar fragments may be folded up, bent or partly overlapped. These events will also decrease the absolute values of the dichroism.

In view of these considerations, the estimated values for the dichroism of bacteriochlorophyll, will represent the minimum absolute values for the dichroism actually present in the lamellae.

The smaller absolute values of dichroism may also arise from non-uniformity in orientation of the pigment molecules in the lamellar membrane, or may depend on the inclination of the transition moments of the pigment molecules to the surface of the membrane. As a simplest model, if the bacteriochlorophyll molecules are assumed to be arranged uniformly in the lamellae, the orientation of the molecules can be deduced from the values of dichroism.

Employing the relationship between the dichroism and the angle (α) the transition moment makes with the plane of the membrane surface,

$$\Delta A/A = 3/2(3\cos^2\alpha - 1) \tag{8}$$

the angle (α) the transition moment of the near-infrared absorption makes with the surface of lamellae was estimated to be 28°, and α for the transition moment of the 590-nm absorption 41°, in the case of "intact cells". Such combination of orientation angles will be in harmony with the circumstance that the 590-nm absorption transition moment and the near-infrared transition moment in bacteriochlorophyll are perpendicular to each other, if we assume that the plane of the dihydrochlorine ring is inclined at an angle of 53.7° to the plane of the surface of lamellae.

The actual mode of the arrangement will not be so simple as to assume the uniform orientation of bacteriochlorophyll. It will be a mixture of the oriented molecules and the unoriented. Furthermore, there will be a possibility that bacteriochlorophyll molecules are aggregated in the lamellae[15]. In this case, the analysis of orientation of molecules will be more complicated. The analysis based on other models than uniform orientation of molecules was not attempted.

Meanwhile, there may be a possibility that the observed dichroism is not due to an orientation of bacteriochlorophyll molecules, but to artefact(s) due to the optical

apparatus, the preparation of the test material, or the shape, which can be responsible for form dichroism, of the test material.

The possibility that the observed dichroism may be caused by the form dichroism due to the lamellar structure of the photosynthetic organelle of this bacterium[16] was not completely eliminated. However, the wavelength dependence of the dichroism observed in these studies, namely the opposite sign of $\Delta A/A$, positive at 800 and 870 nm and negative at 590 nm, will exclude the possibility that the form dichroism is the sole cause of the observed dichroism, although the values of dichroism may be affected to some extent by form dichroism.

In the measurements with "intact cells", control experiments were performed in which the dichroism of the cell suspension was measured in the flow chamber, but without flowing the suspension. No dichroism was detected. This fact will indicate that the measured dichroism obtained by the flow method is not due to an artefact caused by the optical arrangement or by the wavelength dependence of the polarizer or photomultiplier employed.

Wavelength-dependent scattering of the measuring light of the spectrophotometer by bacterial cells may also have some effect on the measured dichroism when the scattering is anisotropic due to the orientation of the cells. However, in the actual measurements in the present study, the portion of the apparent absorbance (attenuance*) caused by the light scattering by the cells was not significant. The absorbance of the bacterial suspension at 670 nm, which is mainly caused by light scattering by the bacterial cell, was less than one fifth of the absorbance of bacteriochlorophyll at 800 nm. It will be reasonable to conclude that anisotropic light scattering by bacterial cells had no significant influence on the measured dichroism.

In the measurements with "dry film", there may be an artefact which was caused by the arrangement of these measurements. The glass plate bearing the dry film of lamellar fragments, in paraffin oil, will transmit partially plane polarized light. This effect may be considered in the calculation of values of the dichroism. However, in this study the peak-trough differences of absorbance were used for calculation; therefore, this effect may be eliminated from the estimated values of the dichroism. Actually, to test the effect, another experimental arrangement, in which a similar paraffin oil bath and glass plate, bearing a dry film of methanol-extracted lamellar fragments in place of the lamellar fragments, was placed in the reference side of spectrophotometer, was used. No difference between the values of dichroism (at $\theta = 60°$) was detected in these two kinds of arrangements. This fact will indicate that this kind of artefact was negligible in the estimation of values of dichroism in these studies.

The possibility of another artefact, that the orientation of bacteriochlorophyll molecules in the lamellae may change by sonication, was tested, using two kinds of "dry film" material. In each kind of test piece, lamellar fragment preparations prepared by disrupting the cells, the one by means of a French pressure cell and the other by grinding in a mortar with alumina powder, were mounted. The measured values of dichroism ($\Delta A/A$) were -0.64 at 590 nm and $+0.51$ at 800 and 870 nm in the preparation from the French pressure cell; -0.61 at 590 nm and $+0.54$ at 800 and 870 nm in the preparation from mortar-grinding lamellar fragments. The values, with respect to wavelength, were practically identical and showed that sonication did not change the arrangement of bacteriochlorophyll molecules in the lamellae.

* Proposed by SHIBATA[17].

As a control in the measurements with "dry film", a thin layer of a water suspension of lamellar fragments, placed between two sheets of cover glass for microscopy, was used in place of dry film. No significant dichroism was detected, indicating that the dichroism observed in "dry film" was not due to optical artefacts.

The values for the dichroism of bacteriochlorophyll in lamellae were essentially similar in dry film and intact cells, although the size and shape of the test material differed widely. This fact will exclude the possibility that the observed dichroism was due to the artefacts caused by shape and size of the material used. Also, the possibility that the observed dichroism in dry film was caused by air drying of the material, was excluded.

In chloroplasts, it has been inferred from the results of studies of dichroism that a part of the chlorophyll molecules (the 695-nm form) is oriented to make the transition moment of absorption at longer wavelengths nearly parallel to the plane of the lamellae. The conlusion of the present study concerning the arrangement of bacteriochlorophyll in lamellae of *R. palustris* is in accord with what has been reported with respect to the chlorophyll 695-nm form in green plants. However, the dichroism is much more significant in the case of lamellae of the photosynthetic bacterium. Moreover, the dichroism was detected in various forms of bacteriochlorophyll, including B_{800} and B_{870}.

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