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THE ORIENTATION OF THE MAGNETIC AXES OF MEMBRANE-BOUND IRON-SULFUR CLUSTERS AND A CYTOCHROME *b*-559 IN THE GREEN HALOPHILIC ALGA *DUNALIELLA PARVA*

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Photosynthetic membrane fragments separated from whole cells of the green alga *Dunaliella parva*, were oriented by incorporation into multilayers on thin Mylar films. These partially dehydrated films were then examined by EPR spectroscopy for evidence of orientation of paramagnetic components. Five previously identified paramagnetic components, the reduced states of iron-sulfur clusters A and B, the intermediate acceptor X^-, the reduced Rieske iron-sulfur cluster, and oxidized cytochrome *b*-559, displayed EPR signals showing orientation. In addition, several previously unknown paramagnetic components were also observed to be oriented. Four components, previously characterized in spinach chloroplast preparations, the iron-sulfur clusters A and B, the intermediate acceptor X^-, and cytochrome *b*-559, were shown to be similar in the green alga, *D. parva*. The orientations of iron-sulfur clusters A and B, however, were determined unambiguously in this preparation; this was not possible in previous work with spinach. The heme plane orientation of cytochrome *b*-559 was found to be perpendicular to the membrane plane in agreement with the results in spinach preparations. A new photoinduced EPR signal with *g* values of 1.88, 1.97 and 2.12 was seen only in the oriented preparations and was indicative of a reduced iron-sulfur cluster with an orientation different from that of iron-sulfur cluster A or B. This suggests the existence of a previously unidentified acceptor in Photosystem I of green plants. These studies clearly show that the orientation of these components in bioenergetic membranes are conserved over a large span of evolutionary development and are, therefore, an important aspect of the mechanism of electron transfer.

Introduction

There are three advantages in studying oriented photosynthetic membranes or membrane fragments by EPR spectroscopy. First, orientation of the paramagnetic moments can result in enhanced detectability EPR intensity is proportional to the number of spins interacting with the microwave radiation field. However, in the usual 'powder spectrum' case, at any magnetic field value (H_0), only a small fraction of the spins satisfy the EPR resonance condition In oriented samples at an optimal angle, the same number of spins are distributed over a smaller range of H_0 values, therefore, the resonance condition is satisfied for a larger number of spins for a given H_0 and, consequently, the EPR signal-to-noise ratio is increased when compared to the usual powder spectrum case Second, a determination of the angular dependence of the EPR signal intensity leads to

Abbreviations DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone, DDQ, 2,3-dicyano-5,6-dichloro-*p*-benzoquinone

assignment of measured g values belonging to the same paramagnetic center, since related g value components must be orthogonal; i.e., the squared direction cosines of the angles between the measured g value maximum intensities and the membrane normal must sum to unity for related components Third, the measured orientation for a set of g values derived from a particular paramagnetic center can lead to structural information about that center and its spatial orientation within the photosynthetic membrane when convincing models exist for the electronic configuration of the EPR chromophore

Detailed spectroscopic studies have been reported for the orientation of cytochromes, iron-sulfur clusters, and other components in both mitochondrial and photosynthetic (bacterial) systems [1–5]. Other investigations in plant photosynthetic systems have indicated the orientation of iron-sulfur clusters A and B , and the Rieske iron-sulfur cluster, as well as of several cytochromes involved in the dark electron-transport region of the green-plant photosynthetic 'Z-scheme' [6–8] Studies presented here provide orientation data yielding the orientation of the magnetic axes of iron-sulfur centers A and B, the Rieske center, the intermediate photoacceptor of Photosystem I (X^-), cytochrome *b*-559, and for several previously unknown paramagnetic components in the photosynthetic electron-transport systems of the green alga, *Dunaliella parva* In addition, in the case of cytochrome *b*-559, we determined that the heme plane is perpendicular to the membrane plane.

A comparison between these electron-transferring components in the green alga, *D parva*, and those in spinach and analogous components in bacteria illustrates remarkable similarities in the orientation of these components which span the evolutionary scale from bacteria to higher plants. This similarity provided additional evidence for the influence of specific orientations in mechanism of electron transfer

Materials and Methods

D parva membrane fragments were separated from cultures which were a gift from Professor M. Avron (Weizmann Institute, Israel) and prepared as previously reported [9]. Broken chloroplasts were then oriented by layering a concentrated suspension onto collodion-coated Mylar strips [2,10]. Low-temperature EPR spectra were obtained with cryogenic sample temperatures from 5 to 10 K, monitored by a Cu (90% Au/10% Fe) thermocouple, using a TE_{011}-mode cylindrical microwave cavity with a loaded $Q = 5000$ Careful attention was given to matching the EPR microwave cavity through 50 dB of isolation to a Gunn-diode microwave source supplying 10 mW First-derivative EPR spectra were recorded using 100 kHz field modulation and phase-sensitive homodyne detection at the modulation frequency Field modulation amplitudes were 1.25 mT for all measurements except that 1 6 mT was used to obtain the cytochrome data. In order to place enough material in the sensitive region of the EPR microwave cavity, several layers of Mylar coated with oriented photosynthetic membranes were placed in a quartz EPR tube (3 mm inner diameter) for spectroscopic examination Up to ten scans of 'signal averaging' were employed to obtain the data presented.

Polar plots of EPR signal amplitude as a function of the angle θ between the layered membranes and the spectrometer magnetic field, H_0, taken at specific g values, were generated by one of several methods. Dark-adapted preparations were measured at 10° intervals through a 360° circle and then the experimental values for θ and $\theta + 180°$ were averaged and plotted as data for both angles. θ is defined as the angle between H_0 and the normal to the membrane plane. Light minus dark difference spectra were obtained by illuminating the dark-adapted sample for one minute with broad-band (640–800 nm) light and then recording postillumination EPR spectra at 10° intervals over 360° as in the previous procedure for the dark-adapted samples EPR spectra of samples illuminated with white light while freezing from room temperature to 77 K in the presence of dithionite were recorded at 10° intervals over 180°; and then replotted for the range from 190 to 360° Cytochrome EPR spectra in the low-field, $g = 6$ region were taken at 10° intervals from 0 to 90°.

DBMIB was a kind gift from Professor R Malkin

Results

Figs. 1 and 2 present the EPR spectra of dark-adapted, oriented membrane fragments from *D parva* and the associated polar-angle plots of observed g values From the spectra taken at 10 K, five EPR g values may be discerned at $g = 2\ 23$, 2 023, 2.00, 1.895 and 1.735. The signals at $g = 2\ 23$ and 1 735 exhibite a strong orientation perpendicular to the membrane. These signals are indicative of an oxidized low-spin cytochrome, probably cytochrome *b*-559. The isotropic signal at $g = 2.00$ is due to the oxidized electron donor of Photosystem I, P-700$^+$. The reduced Rieske iron-sulfur center is responsible for the signals with g values at 2.023 and 1.895, the signal at $g = 2.023$ exhibits an orientation parallel to the membrane plane In contrast to the result in spinach photosynthetic membranes where the $g = 1.895$ signal has a maximum in intensity perpendicular to the membrane plane [7], the Rieske center signal here does not show an orientation dependence.

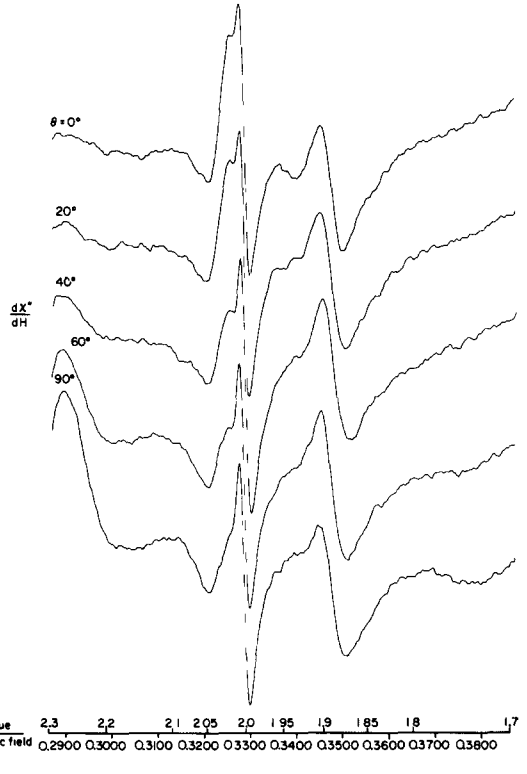

Fig 1 EPR spectra of the $g = 2$ region of dark-adapted, oriented *D parva* membrane fragments The reductant ascorbate was included in the wash before the dehydration-orientation process was carried out EPR spectroscopic conditions frequency, 9 200 s^{-1}, microwave power, 10 mW, sample temperature, 10 K, modulation aplitude, 1 25 mT

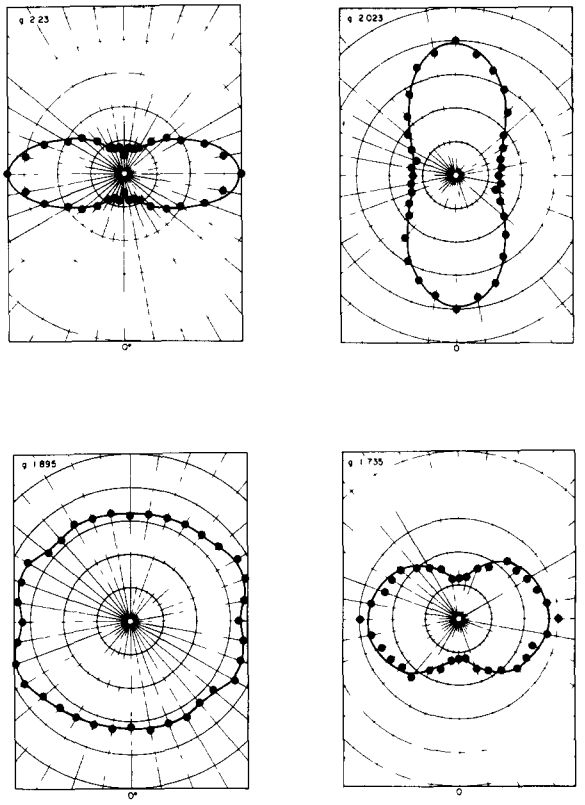

Fig 2 Polar-angle plots for the observed g values of Fig 1 θ (°) refers to the angle between the layered membranes and the EPR spectrometer magnetic field (H_0) The plots are generated by plotting the EPR first-derivative signal intensity as a function of θ for a particular g value

Figs. 3 and 4 show the EPR spectra and associated polar-angle plots for *D parva* membrane fragments incubated with the plastoquinone analog DBMIB before undergoing orientation and dark adaptation In comparison to the data shown in Figs. 1 and 2, the signal at a g value of 1.735 disappears, the signals at $g = 2.23$ and 2.023 decrease in amplitude, and signals now appear with g values of 2.0 and 1.945. The $g = 2.0$ signal has a line width of 5.0 mT, exhibits a partial microwave power saturation at 10 mW, and is oriented parallel to the membrane plane The signal at $g = 1.945$ also displays orientation parallel to the membrane

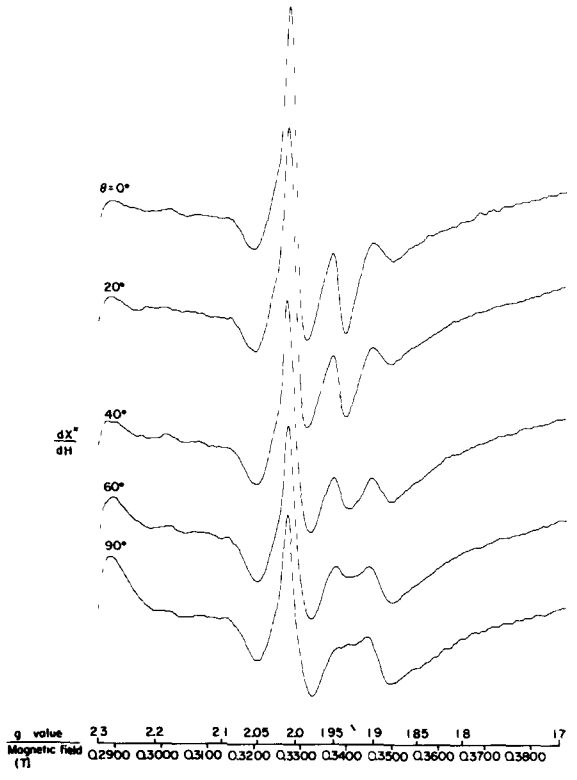

Fig 3 EPR spectra of the $g = 2$ region of dark-adapted, oriented *D parva* membrane fragments Ascorbate and 50 μM DBMIB were included in the sample before the dehydration-orientation process EPR spectroscopic conditions same as in Fig 1

plane. Most interesting, however, is that an orientation (parallel to the membrane plane) now appears for the previously observed component with a g value of 1.895, a component unoriented if DMBIM is not added to the preparation

Figs 5 and 7 illustrates the EPR spectra of

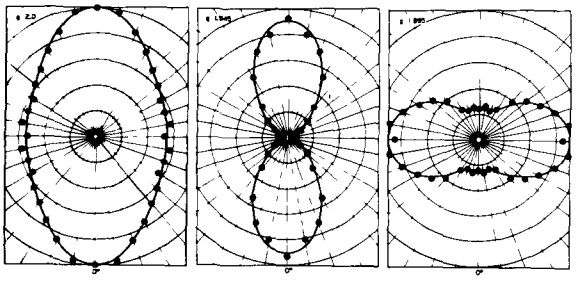

Fig 4 Polar-angle plots for the observed EPR g values shown in Fig 3 Plots generated as in Fig 2

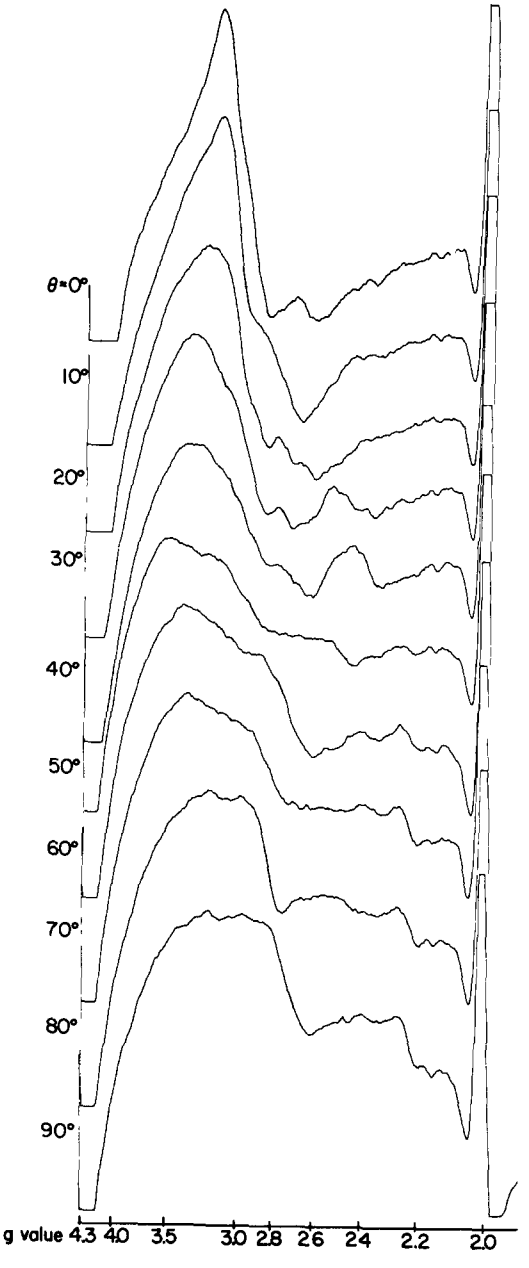

Fig 5 EPR spectra of the $g = 2$–4 3 region of dark-adapted, oriented *D parva* membrane fragments 50 μM of the oxidant DDQ was added to the preparations before the dehydration-orientation process EPR spectroscopic conditions. same as in Fig 1 except that the modulation amplitude was changed to 1 6 mT

dark-adapted preparations over magnetic field ranges corresponding to g values from 2 to 4.3 and 4.3 to 12, respectively. The spectra are obtained

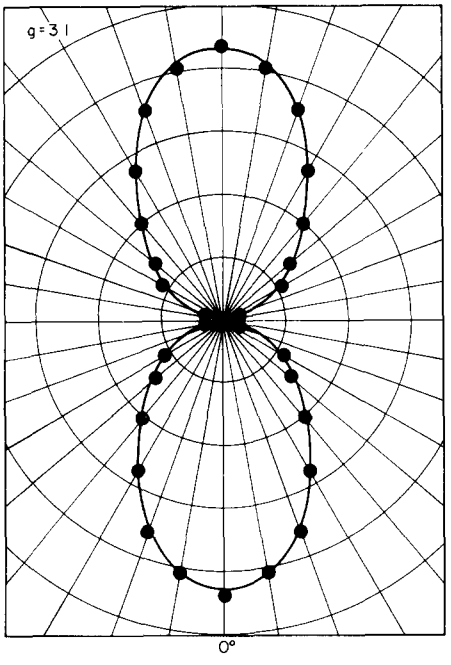

Fig 6 Polar-angle plot of the $g = 3\,1$ signal observed in Fig 5 Plot generated as in Fig 2

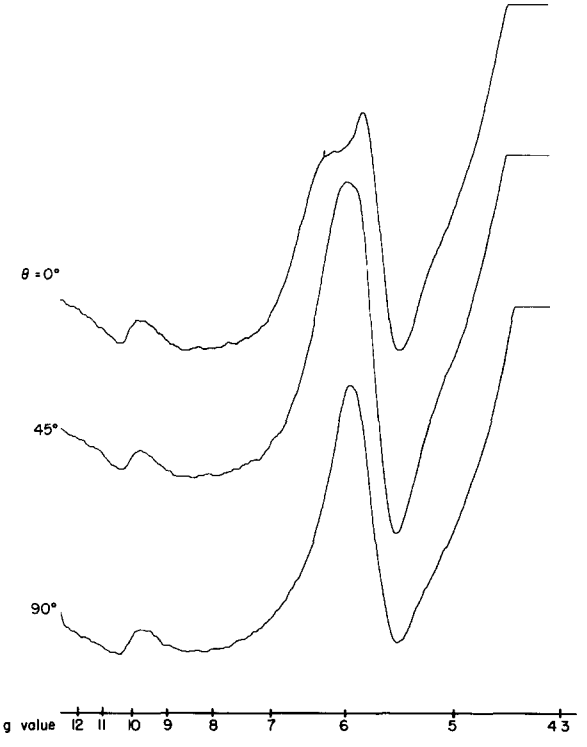

Fig 7 EPR spectra of the $g = 4\,3$–12 region of dark-adapted, oriented *D parva* membrane fragments Sample conditions and EPR spectroscopic conditions identical to those of Fig 5

from dark-adapted preparations in the presence of the oxidant DDQ at a concentration of 50 μM. The interpretation of these spectra is more difficult due to their complexity; therefore, detailed polar-angle plots were attempted only for the $g = 3\,1$ signal illustrated in Fig. 6. However, some qualitative inferences can be drawn about the angular dependencies of the signals. There are at least two signals with g values at approx. 3.0 (3 1 and 2 95) with additional minor components at g values of 2.8 and 2.23 The signal at $g = 2.8$ appears to show a significant shift in g value, most noticeably in the spectra recorded from 60 to 90° This behavior suggests significant orientation for this component. EPR spectra taken at low magnetic field in the presence of DDQ show at least two components centered around $g = 6$. The signal at $g = 5.8$ is oriented at 45° from the membrane plane, the signal at $g = 6.1$ is oriented parallel to the membrane plane.

Figs 8 and 9 present the EPR spectra and associated polar-angle plots of signals produced after the preparations were exposed to broad-band red light (640–800 nm) at an intensity of 5 mW · cm^{-2} for 1 min with the sample kept at 10 K. These figures are presented as (light minus dark) difference spectra in order to display only the photoinduced components. As previously reported [9], photoreduction of unoriented *D parva* membrane fragments results in the observation of six distinct g values within the '$g = 1.94$' manifold associated with the photoreduction at 10 K of both iron-sulfur clusters A and B This is in contrast to the situation in spinach were only cluster A is photoreduced at this temperature [11]. In the oriented preparations of *D parva* seven distinct g values are present at 10 K. The additional signal at $g = 2.12$, generally not observed in unoriented preparations, is broad at 10 K, sharpens at 5 K, and shows an orientation parallel to the membrane plane The signal at $g = 2.072$ is oriented at 55° with respect to the membrane plane. The signal at $g = 2.053$ exhibits two components, one oriented at 25° to the membrane plane and a second component oriented perpendicular to the membrane

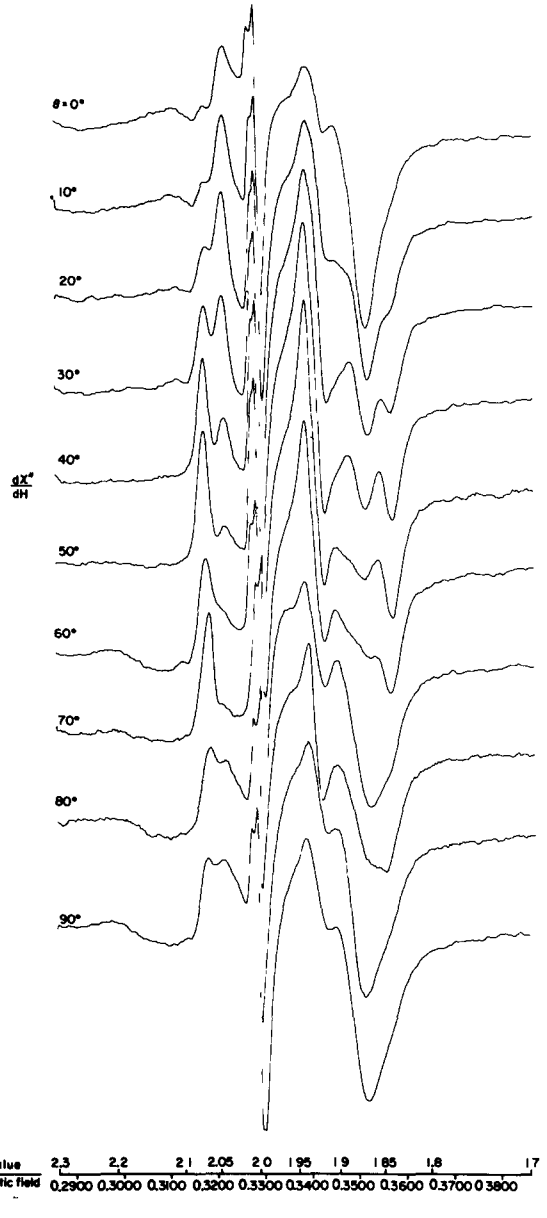

Fig 8 EPR spectra of the $g = 2$ region of oriented *D parva* membrane fragments Each spectrum is a light minus dark difference spectrum taken at the same angle The observed signals are, therefore, light-induced components only Ascorbate was included in the sample preparation before the dehydration-orientation process EPR spectroscopic conditions are the same as for Fig 1

plane. The signals at $g = 1.94$ and 1.925 were plotted as a sum because an inflection point between these two intense signals is difficult to resolve. Both signals must, however, have the same orientation (35° with respect to the membrane plane), since only this single orientation was observed for the two signals taken collectively. The signal at $g = 1.876$ also exhibits two components, one oriented parallel and the other oriented perpendicular to the membrane plane. The signal at $g = 1.844$ has its magnetic axis oriented at 45° with respect to the membrane plane

Figs. 10 and 11 illustrate the EPR spectra and associated polar-angle plots of samples prepared and exposed to red light under similar conditions as the data displayed in Figs. 8 and 9, but measured at 5 K. Qualitatively, the EPR spectra at 5 K are similar to those taken at 10 K, but with some evidence of microwave power saturation The signal with a g value of 2 12 becomes more apparent and a new signal is observed at $g = 1$ 970. This new signal displays two orientation components, one at 70° from and one parallel to the membrane plane. It should be noted that the component oriented parallel to the membrane plane could be an artifact due to an oriented component at a slightly higher field going to a minimum. Including the additional signal observed at 5 K, there are

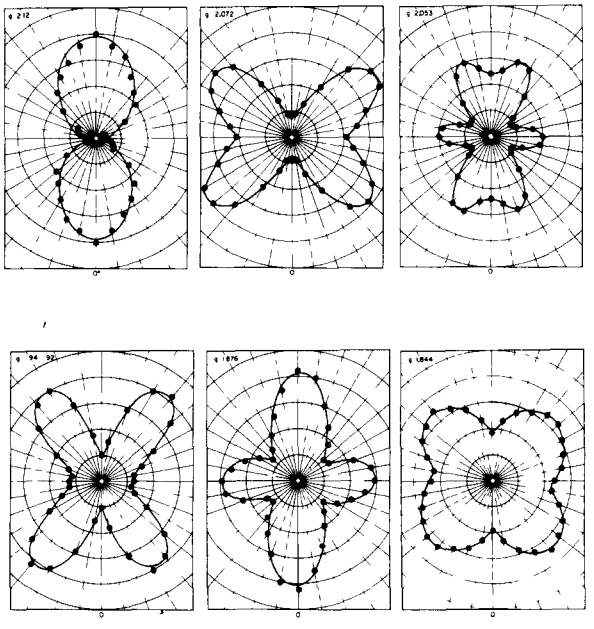

Fig 9 Polar-angle plots for the observed g values presented in Fig 8 Plots generated as in Fig 2

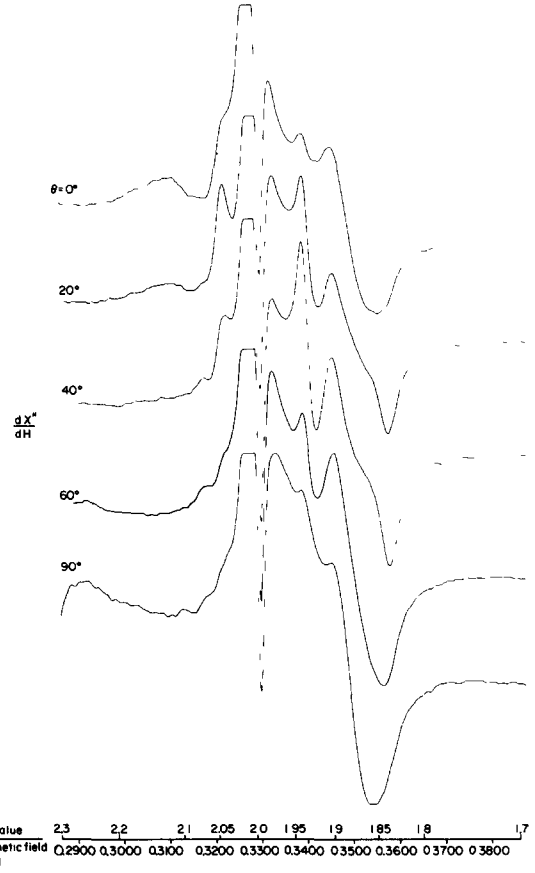

Fig 10 Sample and spectroscopic conditions similar to those of Fig 8 except that the EPR spectra were taken at 5 K

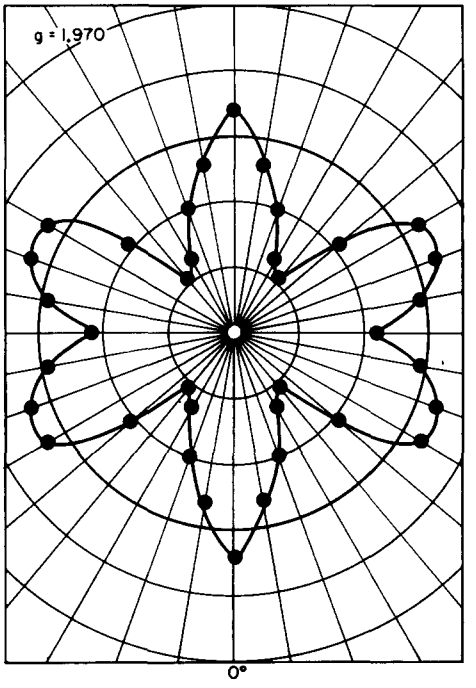

Fig 11 Polar-angle plots of the $g = 1\,970$ signal shown in Fig 10 Plot generated as in Fig 2

a total of eight g values observed at low temperatures in *D. parva* with eleven oriented components; three g values exhibit two orientations each.

Fig. 12 illustrates EPR spectra from an oriented membrane sample that was treated with a strong reductant (sodium dithionite) anaerobically after the preparation process and then frozen while under intense while light illumination. The signals are intense, being considerably larger than in the case of red light illumination without the addition of reductant. Qualitatively, the spectra appear sim-

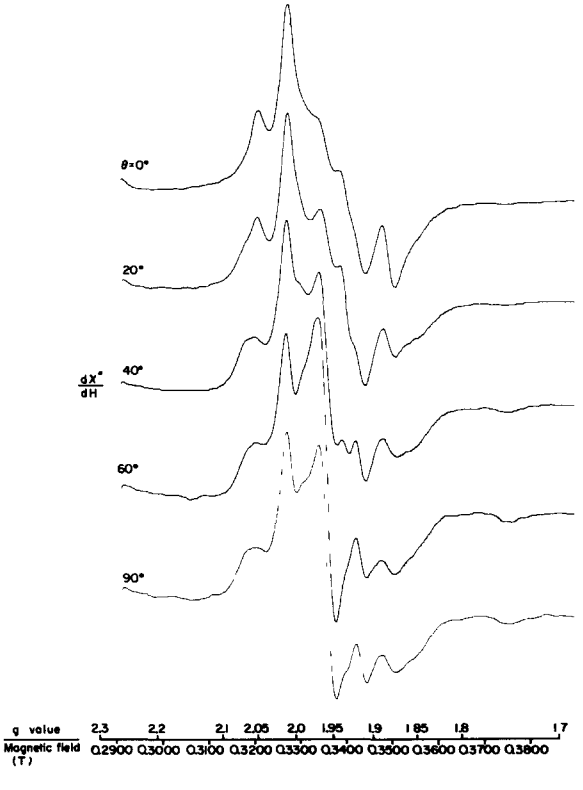

Fig 12 EPR spectra of the $g = 2$ region of oriented *D parva* membrane fragments that were exposed to intense while light illumination while freezing from 300 to 77 K in the presence of sodium dithionite EPR spectroscopic conditions were the same as in Fig 1

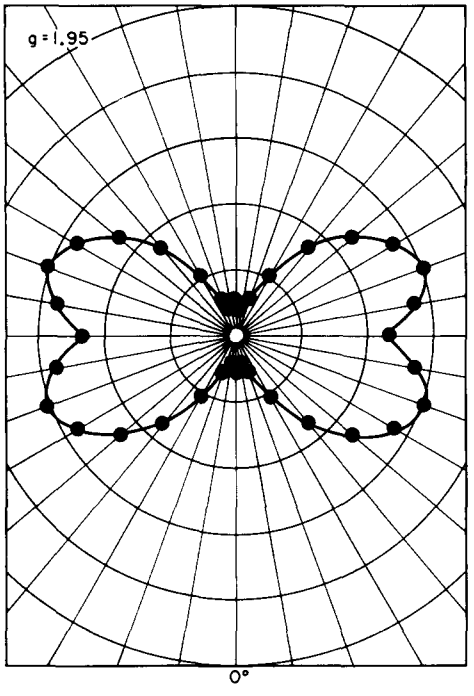

Fig 13 Polar-angle plot of the $g = 1\ 95$ signal observed in the EPR spectra of Fig 12 Plot was generated as in Fig 2

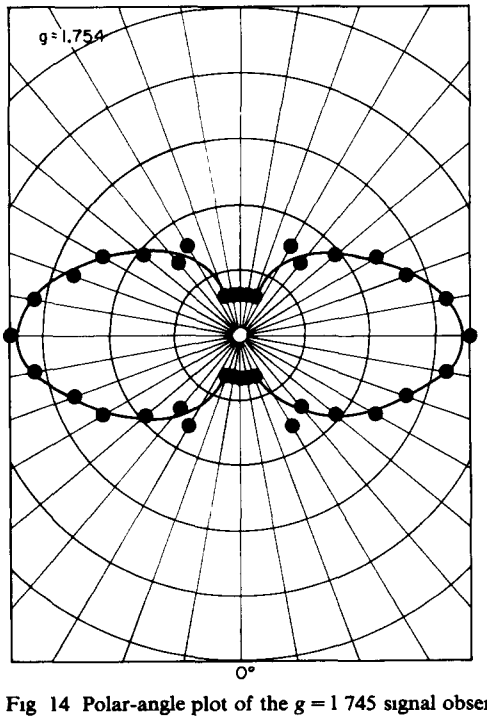

Fig 14 Polar-angle plot of the $g = 1\ 745$ signal observed in the EPR spectra of Fig 12 Plot was generated as in Fig 2

ilar to those in Fig. 8 with three exceptions. The signal at $g = 2.0$ now appears to be power saturated at 10 mW and reduction of the power level to about 50 μW restores the more usual isotropic nonsaturated signal frequently observed at $g = 2.0$ In addition, there is another signal with $g = 1.95$ which is oriented at 70° with respect to the membrane plane. This signal does not display power saturation even with microwave powers in excess of 10 mW The signal with a g value of 1.754 (oriented perpendicular to the membrane) also becomes quite evident at 5 K with higher microwave power levels. Polar-angle plots of these signals are presented as Figs 13 and 14.

Discussion

Recently, much attention has been given to EPR studies of photosynthetic systems where partial orientation of the membranes has been achieved by a variety of methods, including application of a large magnetic field [6,8,12,13], partial dehydration, [1–5,7,10,14–17] and viscous flow [12]. The most effective method has been allowing suspensions of membrane fragments to dehydrate partially [18] while in contact with a hydrophilic layer (collodion) coated on a Mylar film. This orientation along a single axis is imperfect, being subject to several types of disorder as discussed by Blum et al. [1,19]. There may be disorder in the orientation between the membranes and the planar Mylar surface on which the membranes are deposited, disorder in the orientation of the protein with respect to the membranes in, or on, which they are bound ('chromophore disorder'), and nonrigid orientation of the chromophore in the protein itself ('stacking disorder', or in an EPR context, 'g-strain'). Collectively, these disordering influences have been labeled 'mosaic spread'. The spectroscopic consequences of these disordering parameters are two-fold. EPR g values remain independent of the angle between the applied magnetic field (H_0) and the membrane normal, while the amplitudes of the observed signals exhibit an orientation-dependent angular variation as the membrane normal is rotated with respect to the direction of the H_0 field. The maximum amplitude of the first-derivative EPR signal intensity occurs when H_0 is at the same angle to the normal

as the particular magnetic axis being investigated [1,10].

Dark spectra the Rieske center

The Rieske iron-sulfur center, observed in experiments with spinach chloroplasts, has g values of 2 037 and 1.90 and an oxidation-reduction potential, as measured with exogenous electron donors and acceptors, of approx +290 mV at pH 7 0 [20]. This component is believed to transfer electrons between the plastoquinone pool and cytochrome f [21]. Iron-sulfur clusters of this type have also been observed in mitochondrial systems and in photosynthetic bacterial membranes [22], the EPR signals have rhombic symmetry with the g_x component at $g = 1.8$ [23,24]. In photosynthetic membranes isolated from spinach, the signal at $g = 2.03$ is oriented parallel to the membrane plane and the signal at $g = 1.90$ is oriented perpendicular to the membrane [6,7]. It is still unsettled as to whether this signal is axially symmetric or rhombic with the g_x component unresolved perhaps due to a broadened EPR line width

Table I lists the angular dependence of each EPR signal observed in dark-adapted *D parva* samples Although the signal at $g = 2.023$ is oriented parallel to the membrane plane, the signal at $g = 1\ 895$ appears not to be oriented in the *D parva* material This is in contrast to the results in spinach chloroplasts [7] Two explanations for this difference are possible. (1) the signal could be randomly oriented in the membrane as isolated, which seems unlikely since g_z is well oriented, or, (2) the Rieske center may be axially symmetric In this case, the $g = 1.895$ signal would represent the $g_{perpendicular}$ and exhibit minimal orientation dependence since the $g_{perpendicular}$ has components both parallel and perpendicular to the membrane. The $g = 1.735$ signal can be eliminated as a candidate for the g_x component of the Rieske center since this component is always oriented. (If the g_z and g_x components of a rhombic EPR center are oriented, then the g_y component must also be oriented) It is important to note that 'dichroic ratios' of at least 3 : 1 were observed for most of the signals in this preparation If there were a unique orientation for this component, it would have been resolved. Therefore, the present study tends to support the assignment of axial symmetry in this alga

TABLE I

ORIENTATION DEPENDENCE OF THE EPR SIGNAL INTENSITY OF *D PARVA* DARK-ADAPTED PREPARATIONS

g value	Orientation (θ) (°)	Orientation (ϕ) (°)	$\cos^2\phi$
(a) Ascorbate-reduced preparation			
2 23	90	0	1 0
2 023	0	90	0
2 0	–	–	–
1 895	–	–	–
1 735	90	0	1 0
(b) DBMIB preparation			
2 0	0	90	0
1 945	0	90	0
1 895	90	0	1 0

The addition of DBMIB to the samples before partial dehydration, dark adaptation, and subsequent EPR examination results in several alterations in the EPR spectra. First, the previously unoriented signal at $g = 1\ 895$ now exhibits an orientation perpendicular to the membrane plane, this orientation is the same as observed for this component in spinach preparations in the absence of DBMIB Concurrently, a new signal is observed at $g = 1\ 945$ which we believe results from an interaction between DBMIB and the Rieske center It is a possibility, if the Rieske center is axially symmetric, that the $g_{perpendicular}$ at $g = 1\ 895$ breaks into a $g_x = 1.895$ and $g_y = 1.945$ due to an interaction with DBMIB This idea is given additional support in that the sum of the squared direction cosines of the components at g values of 2 023, 1 945 and 1 895 equals unity It should be considered, however, that the 50 μM DBMIB concentration used suggests that this plastoquinone analog may be interacting with only a fraction of the Rieske centers in the preparation shifting those centers' EPR signals to lower fields. In support of this possibility is the fact that, at least qualitatively, the spectral intensity of both the $g = 1.895$ and 1.945 signals taken together approximately equals the original intensity of the signal at $g = 1\ 895$ in untreated material. Malkin [25] has studied the effect of DBMIB on the signals in spinach

chloroplasts at $g = 1.895$ and 1.945 and has observed a correlation between the appearance of the signal at $g = 1\ 945$ and the disappearance of the signal at $g = 1\ 895$

The decrease in signal intensity of the $g = 2\ 023$ signal along with the presence of a new signal, with the same orientation, at $g = 2.00$ after the addition of DBMIB suggests that the g_z component of the reduced Rieske center also shifts in g value. Indeed, Malkin [26] has observed a similar shifts of g_z in a purified cytochrome b_6-f complex which contains the Rieske center. It is also important to note that the signal observed at $g = 1\ 735$ before the addition of DBMIB is now unobservable. This effect may be related to the diminished intensity of the $g = 2\ 23$ component, and may result simply from the alteration of the ambient potential of the membrane preparation by the addition of DBMIB

Dark spectra. cytochrome b-559

Cytochromes have been investigated extensively by EPR spectroscopy. Since Taylor [27] determined that the g_z component of the EPR signal of oxidized cytochrome *c* is oriented perpendicular to the heme plane, orientation studies of this component then allow the determination of the orientation of the heme plane relative to the biological membrane Previous studies of cytochrome orientation in cytochrome *c* oxidase and other *c*-type cytochromes in both bacterial photosynthetic and mitochondrial systems show that generally the heme plane is always either parallel or perpendicular to the membrane plane [1–3,28,29].

Theoretical studies on the EPR of low-spin [27,30] and high-spin [31,32] heme complexes in addition to experimental studies [33] on a number of low-spin eukaryotic and prokaryotic cytochrome *c* complexes have been made, this work allows us to make spectral identification of cytochromes in photosynthetic materials. EPR spectroscopy of the plant cytochromes has been less well characterized but has been investigated in a variety of membrane materials including chloroplasts [8,34], subchloroplast particles [35], and purified cytochrome *b-f* complex preparations [36]

Figs 5 and 7 show the EPR spectra obtained from dark-adapted materials at a temperature of 10 K in the presence of 50 μM concentrations of the oxidant DDQ. Due to less well defined spectra than in the case of the iron-sulfur EPR signals only qualitative features of these spectra will be discussed. There are at least two signals with g values near 3, a signal at $g = 3.1$ (Fig. 6) and another at $g = 2\ 95$. These signals appear to be oriented parallel to the membrane plane. Following Taylor's analysis, the heme plane is found to be oriented perpendicular to the membrane plane, in agreement with the findings on spinach chloroplasts reported by Bergstrom and Vanngård [8] Those studies employed magnetic field orientation for studies of membrame-bound cytochrome *b*-559 The values $g = 3\ 1$, 2 95, 2 23 and 1 735 are close to the values reported for the high- and low-potential forms of cytochrome *b*-559 [8,34–36]. Accurate assignments of the high-spin signals centered around $g = 6$ are difficult The preparations with added sodium dithionite showed no signals in this region and preparation with ascorbate showed only a small EPR signal at $g = 6$. On the basis of oxidation-reduction titrations [36], these signals have been tentatively identified as arising from cytochrome *b*-563.

Light-induced signals iron-sulfur clusters A and B

The Photosystem I electron acceptor complex in eukaryotic organisms has been shown to contain at least two membrane-bound iron-sulfur clusters on the basis of low-temperature EPR spectroscopy [11,37–39]. In *D parva*, these two clusters, A and B, can both act as phototraps for electrons from the reaction-center chlorophyll, P-700, at low temperatures [9] Photoreduction of the iron-sulfur clusters observed by EPR spectroscopy at 10 K produces a total of eight g values as illustrated in Fig. 8 and tabulated in Table II. These orientation data, when combined with the chemical reduction, photoreduction and EPR power saturation data, make the assignments of the g values and the orientations of the magnetic axes unambiguous A comparison between the orientation values in *D parva* and those previously reported for spinach [7] is included in Table III. Within experimental error, the orientation data for cluster A in *D. parva* agree with the orientation proposed from the spinach data. In the case of iron-sulfur cluster B, two possible orientations have been proposed based on the data from spinach membrane frag-

TABLE II

ORIENTATION DEPENDENCE OF THE EPR SIGNAL INTENSITY OF *P PARVA* ILLUMINATION AT 10 K, DIFFERENCE SPECTRA

g value	Orientation (θ) (°)	Orientation (ϕ) (°)	$\cos^2\phi$
2 12	0	90	0 0
2 072	55	35	0 67
2 053	25	65	0 18
	90	0	1 0
1 97	0	90	0 0
	70	20	0 88
1 94	35	55	0 33
1 92	35	55	0 88
1 876	0	90	0 0
	90	0	1 0
1 844	45	45	0 50

ments [7], i e , the orientation in which the components g_y and g_z are either oriented at 0 and 90° or at 50 and 40°, respectively. The *D. parva* data as presented in Table III clearly agree only with the latter possibility. The similarity in orientations for iron-sulfur clusters A and B between *D parva* and spinach provides further support for the similarity of mechanism in electron transport in both a green alga and a higher plant.

Light-induced signals the possibility of a third iron-sulfur cluster?

There are still several unassigned components observed in light minus dark difference EPR spectra as shown in Figs. 8 and 10. Three of the components have increased EPR absorption when the temperature of measurement is reduced from 10 to 5 K and the microwave power is increased from 10 to 70 mW Based on a study of microwave power saturation behavior, it is tempting to assign the signals with *g* values of 2.12, 1.97 ($\theta = 0°$), and 1 876 ($\theta = 90°$) as belonging to the same cluster. Added support for this assignment is provided by the fact that the sum of the squared direction cosines for these components equals unity It is important to note that these signals also appear when longer, narrow-band far-red light (730 nm) is

TABLE III

ORIENTATION DEPENDENCE AND *g* VALUE ASSIGNMENT OF THE IRON-SULFUR CLUSTERS OF *D PARVA* AND OF SPINACH (IN BRACKETS)

Fe-S cluster	*g* value	Orientation (ϕ) (°)	$\cos^2\phi$	$\Sigma\cos^2\phi$
Cluster A	2 053 g_z (2 05)	65 (65)	(0 18) 0 18	
	1 94 g_y (1 94)	55 (50)	(0 41) 0 33	1 01
	1 844 g_x (1 86)	45 (50)	(0 41) 0 50	
Cluster B	2 072 g_z (2 07)	35 (40)	(0 59) 0 67	
	1 92 g_y (1 94)	55 (50)	(0 41) 0 33	1 00
	1 876 g_x (1 89)	90 (90)	(0 0) 0 0	
Cluster C	2 12 g_z	90	0 0	
	1 97 g_y	90	0 01	1 00
	1 876 g_x	0	1 00	
Rieske center	2 023 $g_{\parallel}$	90	0 01	
	1 895 $g_{\perp}$	–		
+ DBMIB	2 00 g_z	90	0 0	
	1 945 g_y	90	0 01	1 00
	1 895 g_x	0	1 00	
X^-	? g_z	90 (deduced)	0 0	
	? g_y	90 (deduced)	0 0	1 00
	1 745 g_x	0	1 00	

TABLE IV

ORIENTATION DEPENDENCE AND g VALUE ASSIGNMENT OF THE IRON-SULFUR CLUSTERS OF *D PARVA*

Fe-S cluster	g value	Orientation (ϕ) (°)	$\cos^2\phi$	$\Delta\cos^2\phi$
Cluster A	2 053 g_z	65	0 18	
	1 94 g_y	55	0 33	1 01
	1 844 g_x	45	0 50	
Cluster B	2 072 g_z	35	0 67	
	1 92 g_y	55	0 33	1 00
	1 976 g_x	90	0 01	
Cluster C	2 12 g_z	90	0 0	
	1 97 g_y	90	0 0	1 00
	1 876 g_x	0	1 00	
Rieske center	2 023 $g_{\parallel}$	90	0 0	
	1 895 $g_{\perp}$	-		
+ DBMIB	2 00 g_z	90	0 0	
	1 945 g_y	90	0 0	1 00
	1 895 g_x	0	1 00	
X^-	? g_z	90 (deduced)	0 0	
	? g_y	90 (deduced)	0 0	1 00
	1 745 g_x	0	1 00	

used to illuminate the samples, an indication of the association of all these signals with Photosystem I. The g values, low-temperature dependence of the EPR signal intensity and EPR symmetry associated with this cluster make it likely that these signals are due to the reduced form of a previously unidentified iron-sulfur cluster active in Photosystem I.

Light-induced signals an intermediate electron acceptor, X^-

Studies on the reversibility of the P-700$^+$ EPR signal ('Signal I') and the irreversibility of the EPR signals associated with the reduced iron-sulfur clusters A and B at cryogenic temperatures suggested that an intermediate acceptor must exist Studies by several groups [40–44] have shown the presence of a signal which they associate with an intermediate electron acceptor in Photosystem I that precedes iron-sulfur clusters A and B. The EPR spectrum obtained under strong reducing conditions shows g values at 2.08, 1.88 and 1.78 in spinach chloroplasts; this signal is alternately specified as X^- or by A_2. This signal does not show microwave saturation even at 100 mW

Illumination of oriented layers of *D parva* by strong white light while freezing after the addition of sodium dithionite results in a complicated orientation spectrum (Fig. 12). Although qualitatively similar in many respects to the spectra obtained at low temperature with red light illumination, the signals are more intense and more closely spaced making spectral assignments difficult. There are two signals observed at $g = 1.95$ and 1.756 which do not show microwave power saturation at high powers, it is tempting to assign these signals to the same center on this basis, but our orientation data show this to be a mistake. The signal at $g = 1.95$ shows a single orientation that is identical to the signal at $g = 1.97$ ($\theta = 70°$) shown in Fig. 11 under less reducing conditions (see Fig. 13) However, the signal at $g = 1.756$ can be assigned as the g_x component of X^- on the basis of its appearance only under strong reducing conditions and the similarities of its g values to the case in spinach The orientation of this component perpendicular

to the membrane is in agreement with the orientation of the g_x component in spinach in a study where the orientation was produced by an applied magnetic field [6]. Fig. 14 shows a polar-angle plot of the $g = 1.756$ signal ascribable to the g_x component of X^-. Since signals in the intermediate field region are intense and closely spaced, assignment of the g_y component is impossible. Interpretation of spectra such as these for polar plotting is difficult, since the orientation of one component at a particular g value can result in the assignment of a false orientation for a second component with a slightly different g value. It is unclear why we do not observe the g_z component of X^- Since the g_x component of X^- is observed to be oriented perpendicular to the membrane, the g_y and g_z components must, therefore, be oriented parallel to the membrane plane.

What is clear is that the X^- signal can be generated in *D parva* and that it has the same g tensor orientation as in spinach chloroplasts.

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