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FLUORESCENCE POLARIZATION AND PIGMENT ORIENTATION IN PHOTOSYNTHETIC BACTERIA

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SUMMARY

Fluorescence polarization of photosynthetic bacteria with various types of chromatophores suggests an orientation of bacteriochlorophylls, but not of carotenoids. Comparison of the *in vitro* fluorescence polarization spectra of bacteriochlorophyll at high and low concentration and at 77 °K with those *in vivo* at various temperatures and in the presence of carbowax indicates that dichroism of shape effects the *in vivo* spectra. Both orientation and shape effects are highest for bacteria containing lamellar-type chromatophores, and lowest in those containing vesicle-type ones.

The polarization values of the bacteria studied are similar for the various red bands, indicating a nearly parallel orientation of the adjacent bacteriochlorophylls.

INTRODUCTION

The shape of chromatophore membrane structures in photosynthetic bacteria is rather specific; Oelze and Drews[1] describe eight types. For *Rhodospeudomonas palustris* a nearly parallel arrangement of the lamellae is found in rod shaped cells[2]. Morita and Miyazaki[3], therefore, used this species to investigate pigment orientation by measuring dichroism in whole cells, oriented in a flow system, and chromatophores, oriented in a dry film. A positive dichroism, defined as a higher absorption of light vibrating parallel to the plane of the lamellae than of light vibrating perpendicular to it, was measured in both far red bands (800 and 860 nm), and a weak negative dichroism observed in the yellow band (590 nm).

With chromatophores of *Rhodospirillum rubrum*, Sauer and Calvin[4] did not detect any "electric dichroism" (dichroism induced by an electric field), which might be due, *e.g.* to the use of sonicated chromatophore fractions.

A more general method for studying pigment orientation is the measurement of fluorescence polarization in a randomly oriented suspension of cells or chromatophores. If a number of conditions are fulfilled[5,6], a dilute solution of randomly oriented pigment molecules *in vitro* shows fluorescence polarization. At high pigment concentration, energy transfer from absorbing to emitting molecules will result in depolarization. Although the theory of this phenomenon is complicated (*cf.* ref. 8), an estimation of the number of transfers resulting in polarization, below the level of

detection under the conditions used, can be made. In the chromatophore a very high pigment concentration and energy transfer occurs between the different bacteriochlorophyll "forms"[7].

If these pigments were randomly oriented in the chromatophore, a strong depolarization would occur. However, at parallel orientation, energy transfer between them does not result in depolarization.

Polarization spectra of bacteriochlorophyll fluorescence in chromatophores of *Rhodospirillum rubrum* and *Rhodospseudomonas spheroides*, determined by Clayton and Ebrey[9] and Ebrey[10], showed the occurrence of an unexpectedly large, negative, polarization in the yellow band, while the positive polarization in the far red region was much less pronounced. From these results an unconventional mechanism of energy transfer was postulated[10]. The results, however, may also be explained if dichroism of shape, caused by the presence of layers of alternating different refractive indices[11–13], occurs in the chromatophore. Such an effect can best be studied with species which contain lamellar chromatophores, like *Rhodopseudomonas palustris*.

In the present study the fluorescence polarization and action spectra in this species and various others containing chromatophores of several structural types[1] are determined in order to study pigment orientation in photosynthetic bacteria. Some *in vitro* experiments were also performed to investigate intrinsic pigment polarization properties.

METHODS

Fluorescence is excited by light isolated with a Bausch and Lomb grating monochromator (focal length 500 mm, slit width 1–1.5 mm) from a tungsten halogen lamp operated at d.c. constant voltage. For polarization spectra, a slit width of 3 nm and an interference filter (Balzer $\lambda_{\frac{1}{2}}$ 8–10 nm), added to remove scattered light, were used. Checks are made at each wavelength for light transmitted outside the region of transmission of the filter. Linearly polarized light between 500 and 700 nm was obtained with a polaroid filter Type H and between 700 and 1000 nm with a filter Type HR. Fluorescence is isolated from exciting light with appropriate Kodak Wratten filters (87, 87B, 87C, 88A) in 1, 2 or 3 layers. To exclude scattered light, fluorescence for the 860–880 nm bands was measured at wavelengths longer than 920 nm. With the 800-nm band it was checked by using different filter combinations whether a difference in polarization is found between light emitted in the main fluorescence band (895 nm in *Athiorhodaceae* and 910 nm in *Thiorhodaceae* at 20 °C[11]) and light emitted beyond 920 nm. Though some decrease in polarization of long wave emission was found, it is too small to require corrections under our conditions. In the spectra measured at liquid nitrogen temperature, a shift of the long wave emission and absorption bands towards longer wavelengths occurs[14]. In this case the main fluorescence band is measured with all filter combinations.

Fluorescence is detected at right angles to the incident beam, with a Philips XP 1005 photomultiplier cooled with liquid nitrogen. The vertical position of the detector requires the use of a surface-silvered mirror, which induces a small polarization of the fluorescence beam. This could be compensated by a glass plate, the angle of which could be adjusted. Polarization is measured by turning the detection polaroid (HR) 90°, either by hand or with a motor at 30 rev./min. The degree of polarization, defined as $p=(I_{||}-I_{\perp})/(I_{||}+I_{\perp})$, is calculated with a Wang 720 table computer.

This is also used for correction of the action spectra, the correction factor for equal number of incident quanta being measured with a thermopile and a silicon cell.

Fluorescence polarization in dilute solution is measurable only if the average time between absorption and emission (mean life time τ) is short compared to the time of molecular rotation θ_r. The latter is related to viscosity η and molecular volume V[5]. With a fixed mean life-time (related to the quantum efficiency of fluorescence, Q)[15], polarization values may increase with either viscosity or volume. The latter can be visualized by attaching the pigment to a larger molecule, e.g. a detergent micelle or a protein molecule.

The reciprocal values of fluorescence polarization and viscosity show, in first approximation, a linear relationship. From these values the mean life-time can be calculated, provided this value is independent of viscosity in the solvents used.

In Ricinus oil the maximal value of polarization with bacteriochlorophyll and chlorophyll *a* is found to be $p=0.36$ at room temperature. A similar value is observed with bacteriochlorophyll in Triton-containing buffer. At -196 °C the maximal value is $p=0.38$. As relatively large opening angles had to be used, resulting in a slight depolarization, the theoretical maximal values are somewhat larger.

To investigate the influence of dichroism of shape, in some experiments a high molecular weight reagent (polyethyleneglycol 4000) is added to the buffer solution (0.02 M phosphate, pH 7.3) in which the cells are suspended. Photosynthetic bacteria are normally used 3–5 days after inoculation. Chromatophore suspensions are obtained after crushing the washed cells in a pressure gradient (French press, 0.6 ton/cm^2). Absorption spectra are recorded with a Cary 14 R spectrophotometer. Absorption, fluorescence and fluorescence polarization spectra at -196 °C are obtained with a Cary low temperature attachment with flat windows, which allow fluorescence measurements under a 90 ° angle. Samples are frozen in a buffer–glycerol (45:55; v/v) mixture to obtain a clear glass at low temperature.

For *in vitro* measurements the pigments are extracted from the cells with either methanol or acetone. In the latter case, the pigments are purified by thin-layer chromatography[16], in the former, they are used as a crude extract. Fluorescence emitted under 90 ° with incident non-polarized light can also be partly polarized. With the measurements of fluorescence action spectra a correction for this effect is applied.

RESULTS

In vitro measurements

An investigation of whether the shape of the fluorescence polarization spectrum depends upon pigment concentration, temperature or viscosity of the solvent was carried out. Therefore, in Fig. 1 the fluorescence polarization, fluorescence action and absorption spectra of a "solution" of bacteriochlorophyll (about $3 \cdot 10^{-6}$ M) in phosphate buffer containing 0.5% and 0.05% of the detergent Triton X-100 are given. The spectra at 0.5% and higher are similar to those found in viscous organic solvents, while the fluorescence intensity is also of a similar order of magnitude. If the Triton concentration is decreased, the fluorescence action spectra remain similar in shape, but fluorescence intensity and fluorescence polarization values drop. Energy transfer between pigment molecules attached to the same Triton micelle is

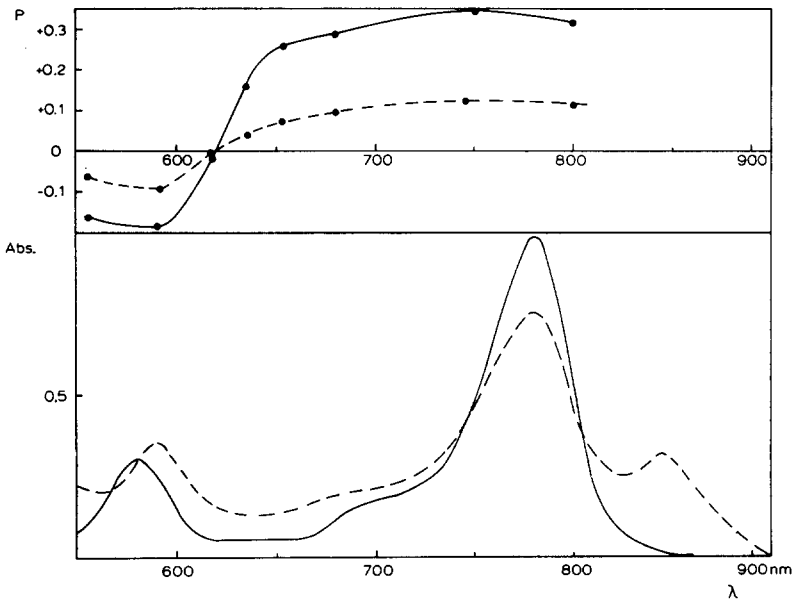

Fig. 1. Absorption and fluorescence polarization spectra of a "solution" of bacteriochlorophyll in phosphate buffer (pH 7.3, 0.02 M), containing 0.5% (—) and 0.05% (---) of the detergent Triton X-100. The fluorescence action spectra of both "solutions" resemble closely the absorption spectrum with 0.5% Triton, the fluorescence intensity is much lower.

TABLE I

FLUORESCENCE POLARIZATION OF BACTERIOCHLOROPHYLL "DISSOLVED" IN A MIXTURE OF 45% PHOSPHATE BUFFER (pH 7.3, 0.02 M) CONTAINING 0.5% OF THE DETERGENT TRITON X-100 AND 55% GLYCEROL, MEASURED AT 20 AND −196 °C (I), OF BACTERIOCHLOROPHYLL IN GLASSY ETHANOL MEASURED AT −196 °C (II) AND OF BACTERIOCHLOROPHYLL IN PHOSPHATE BUFFER CONTAINING 0.1% TRITON AND WITH INCREASING FINAL PIGMENT CONCENTRATION (III)

λ (nm)	p					
	I		II	III ($A_{770\ nm}$)		
	20 °C	−196 °C	−196 °C	0.5	1.0	2.0
750	0.32	0.38	0.26	0.29	0.17	0.11
680	0.23	0.27	0.10			
650	0.19	0.20	0.03			
635	0.11	0.32	−0.03			
620	0.00	−0.09	−0.07			
600	−0.11	−0.13	−0.06			
590	−0.12	−0.13				
555	−0.11	−0.08				
535	−0.08	−0.05				
510	0.04	0.10				
490	0.13	0.16				

assumed to produce the depolarization. The difference in absorption spectra and the decrease in fluorescence intensity is then brought about by formation of non-fluorescing colloids, with far red bands at 780 and 840 nm[14]; this also occurs in the absence of detergents. Similar changes in fluorescence polarization and absorption are found when, at a fixed Triton concentration (0.1%), the bacteriochlorophyll concentration is increased (Table I).

These experiments show that, under our conditions, concentration depolarization *in vitro* occurs and is independent of wavelength of excitation.

Table I also gives the polarization values of bacteriochlorphyll in 1% Triton buffer and in ethanol, both at −196 °C. The ratio of polarization in the yellow and far red band is unaffected by cooling. In ethanol the yellow band is shifted to 635 nm but the ratio mentioned is similar to that in Triton containing buffer or in viscous solvents at room temperature. These experiments indicate that the shape of the polarization spectrum *in vitro* shows little temperature dependence.

It was also investigated whether the fluorescence lifetime, and, consequently the fluorescence yield, depends *in vitro* on wavelength of excitation. Therefore, the fluorescence polarization values at 750 nm and at 580 nm are determined in solvents of different viscosity. As shown in Fig. 2, the fluorescence lifetime of bacteriochlorophyll in various alcoholic solvents does not differ appreciably for the wavelengths used. It is about 0.25 of that of chlorophyll *a* excited in the red band. If a value of $\tau = 6.3$ ns, measured with the phase fluorimetric method, is accepted for chlorophyll *a*[17],

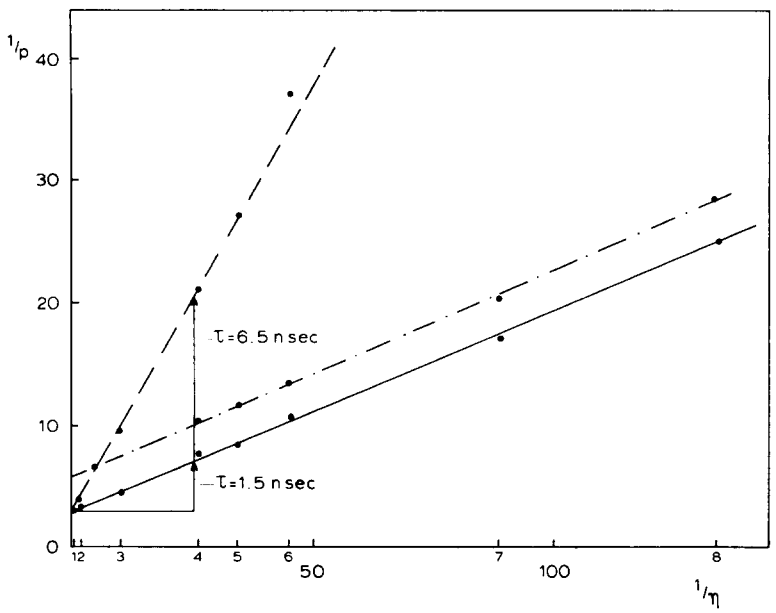

Fig. 2. Relation of reciprocal values of fluorescence polarization p with those of solvent viscosity η, measured with bacteriochlorophyll at 750 nm in the band ascribed to the first electron transition (—), at 590 nm in that ascribed to the second one (–.–) and with chlorophyll *a* at 660 nm (first transition, – –). 1 represents Ricinus oils as solvent, 2 cyclohexanol, 3 octanol, 4 heptanol, 5 butanol, 6 propanol, 7 ethanol, 8 methanol. The slope of the plot is a measure of the fluorescence lifetime. The value for bacteriochlorophyll is about 1/4 of that of chlorophyll *a*.

a $\tau = 1.7$ ns results for bacteriochlorophyll in alcoholic solvents. This value is similar for absorption in the far red and yellow band. Accordingly, the absorption spectrum matches the fluorescence action spectrum in the region concerned.

In vivo experiments

Fluorescence polarization, fluorescence action and absorption spectra with cells of *Rhodopseudomonas palustris* are given in Fig. 3. The spectra are measured at 20 °C (Fig. 3a) and at −196 °C (Fig. 3b). At room temperature the negative values around 590 nm are about equal to those in the far red, while in the various bands and shoulders (at 800, 860 and 880 nm at 20 °C), the polarization values do not differ markedly. Cooling to −196 °C results in the disappearance of the negative values in the polarization spectrum. The total span between maximum and minimum values of polarization decreases on cooling. The horizontal broken lines in the figure indicate the shape of the polarization spectrum, if the negative values at 590 nm and the positive ones in the far red are in a similar ratio to the *in vitro* experiments. As a result of cooling, this broken line is moved upwards.

The absorption spectrum of this bacterium is influenced by the presence of free porphyrins. Formation of free prophyrins is largely accelerated by crushing the cells when chromatophore preparations are made. As the fluorescence action spectrum is similar to that of *Rhodospirillum rubrum*, which does not contain such free porphyrins, little or no energy transfer from these highly fluorescing pigments to bacteriochlorophyll occurs.

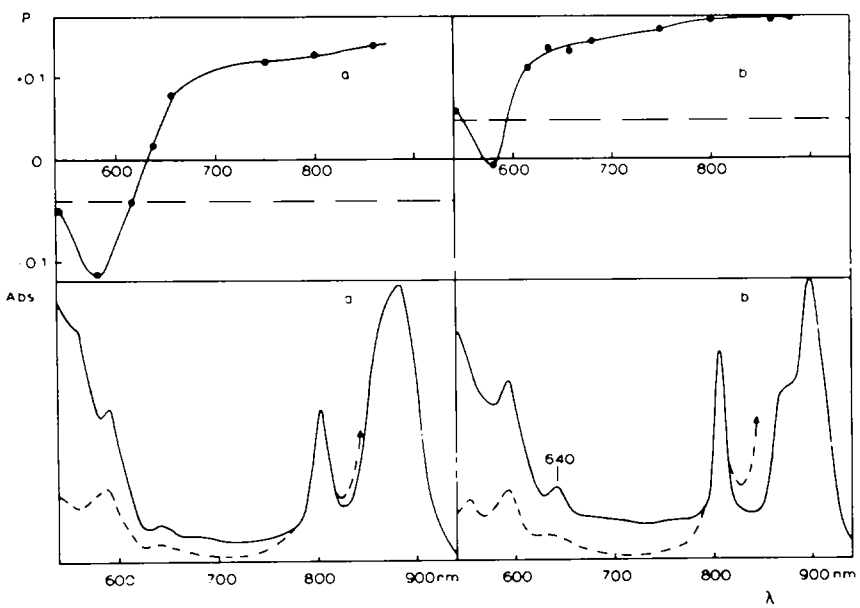

Fig. 3. Absorption (———), fluorescence action (– – –) and fluorescence polarization (●—●) spectra of intact cells of *Rhodopseudomonas palustris*, measured at 20 °C (a) and at −196 °C (b). Fluorescence and absorption spectra are made equal at 800 nm. The broken horizontal line in the polarization spectrum indicates the baseline if the shape of the spectra were similar to the *in vitro* ones. The difference between this line and the base line is assumed to be caused by shape effects

With *Rhodospirillum molischianum*, preparation of chromatophores does not result in a change in absorption spectrum. Fig. 4 gives fluorescence polarization and absorption spectra of chromatophores of this bacterium. The values of polarization

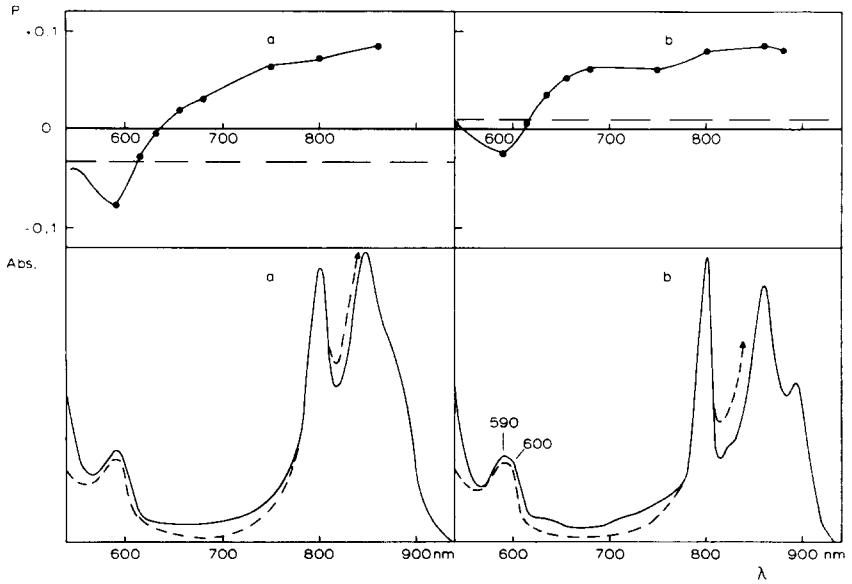

Fig. 4. Absorption (———), fluorescence action (– – –) and fluorescence polarization (●—●) spectra of chromatophores of *Rhodospirillum molischianum*, measured at 20 °C (a) and at −196 °C (b).

TABLE II

FLUORESCENCE POLARIZATION AT VARIOUS WAVELENGTHS OF INTACT CELLS OF THE SULFUR BACTERIUM *THIOCYSTIS VIOLACEA* AND THE NON-SULFUR ONES *RHODOPSEUDOMONAS GELATINOSA* AND *RHODOPSEUDOMONAS VIRIDIS* (BACTERIOCHLOROPHYLL B)

The polarization values are measured at room and liquid-nitrogen temperatures.

λ (nm)	p					
	Thiocystis violacea		*Rhodopseudomonas gelatinosa*		*Rhodopseudomonas viridis*	
	20 °C	*−196 °C*	*20 °C*	*−196 °C*	*20 °C*	*−196 °C*
880	0.053	0.080	0.062	0.100	0.090	0.105
860	0.047	0.066	0.059	0.093	0.090	0.095
800	0.032	0.060	0.051	0.080	0.074	0.080
750	0.028	0.051	0.037	0.074	0.074	0.070
680	0.024	0.048	0.035	0.044	0.068	0.070
625	0	0.019	−0.045	−0.020	−0.098	−0.040
600					−0.137	−0.046
590	−0.024	0	−0.063	−0.044		
535	−0.020	0.025	−0.027	0	−0.050	0.036

are somewhat lower than with the former species, although the shape of the polarization spectrum is similar to that in Fig. 3. The spectra of intact bacteria, in which the chromatophores consist of stacks of double membranes bound to the cytoplasmic membrane (Type 5, *cf.* ref. 1), are similar to those of chromatophore suspensions (Table II).

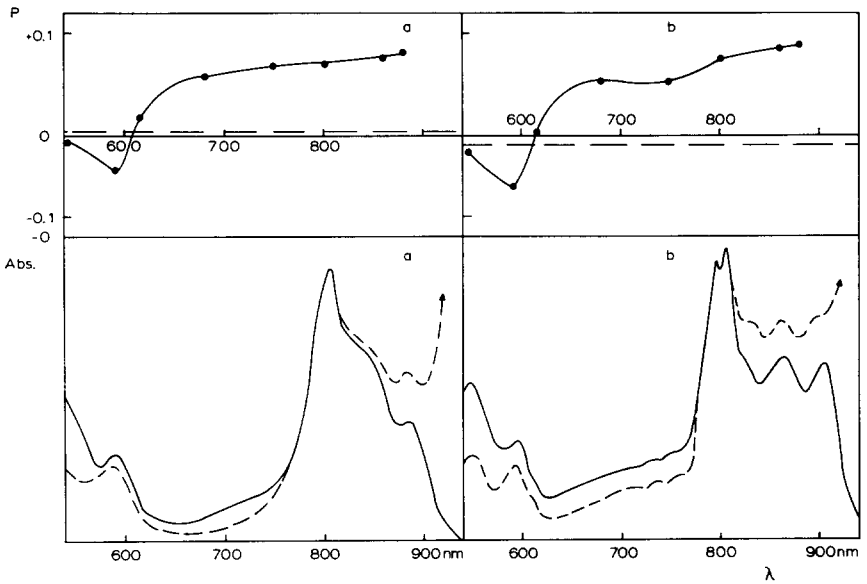

Fig. 5. Absorption (———), fluorescence action (– – –) and fluorescence polarization (●—●) spectra of chromatophores of *Chromatium* strain D.

Lower polarization values are found with cells and chromatophore suspensions of *Rhodopseudomonas spheroides* (Table II) and the *Thiorhodaceae Chromatium* strain D (Fig. 5) and *Thiocystis violacea* (Table II). Also the changes in shape induced by cooling to −196 °C are less pronounced. With the *Thiorhodaceae* the ratio of the negative polarisation at 590 nm to the positive one in the far red is similar to that *in vitro*. It decreases slightly upon cooling, with *Rhodopseudomonas spheroides* a slight increase in ratio occurs. All these bacteria contain vesicular membranes with a diameter of 30–50 nm (Type 2, *cf.* ref. 1). Such vesicular systems are also present in *Rhodospirillum rubrum* cells. The fluorescence action, fluorescence polarization and absorption spectra of this species are given in Fig. 6. The negative values around 590 nm are appreciably larger than the positive ones in the far red. Cooling results in an upward shift of the broken line, but not as far as in Figs 3 and 4. With chromatophore suspensions, the negative values are slightly lower and the positive ones higher than in whole cell preparations (Table II). As shown in Table III, addition of polyethylene glycol 4000 to cell suspensions of *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* results in a decrease of the positive values and an increase in the negative ones.

The polarization data of *Rhodopseudomonas gelatinosa* (Type 4 chromatophores, *cf.* ref. 1) and of *Rhodopseudomonas viridis* (Type 8) are also given in Table II. Although the main far red band of *Rhodopseudomonas viridis* is located at 1010 nm at

20 °C (and 1034 nm at −196 °C) and therefore cannot be measured with our filter combinations, the ratio of negative to positive polarization in the rest of the spectrum is similar to that in *Rhodospirillum rubrum*, but the absolute values of polarization are higher.

It is also checked whether the polarization depends upon quantum yield of fluorescence. The quantum efficiency of fluorescence of chromatophores is decreased to 1/6 of its original value by addition of $2 \cdot 10^{-4}$ M potassium ferricyanide. As shown in Table II with *Rhodospirillum rubrum* and *Rhodospirillum molischianum* chromatophores, such a decrease corresponds to an increase of 30–50% of the polarization values.

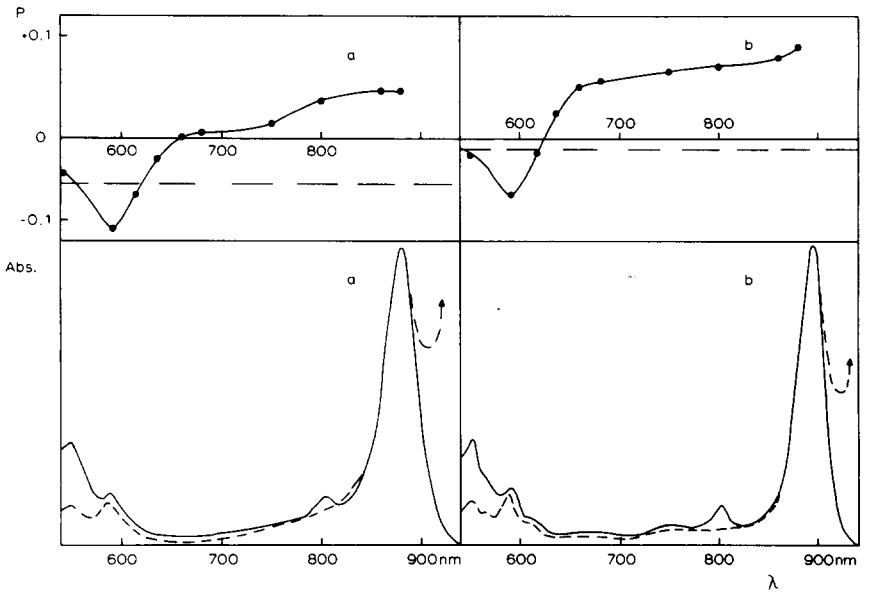

Fig. 6. Absorption (———), fluorescence action (– – –) and fluorescence polarization (●—●) spectra of cells of *Rhodospirillum rubrum* strain 4.

TABLE III

FLUORESCENCE POLARIZATION OF: (1) CHROMATOPHORES OF *RHODOSPIRILLUM RUBRUM* AND *RHODOSPIRILLUM MOLISCHIANUM* IN THE ABSENCE AND PRESENCE OF $1 \cdot 10^{-2}$ M POTASSIUM FERRICYANIDE, AND (2) CELLS OF THESE BACTERIA IN THE ABSENCE AND PRESENCE OF 25% CARBOWAX 4000

λ (nm)	*Fluorescence polarization*							
	Chromatophores with and without ferricyanide				*Cells with and without carbowax*			
	Rhodospirillum rubrum		*Rhodospirillum molischianum*		*Rhodospirillum rubrum*		*Rhodospirillum molischianum*	
	−	+	−	+	−	+	−	+
800	0.055	0.090	0.068	0.091	0.050	0.034	0.090	0.086
590	−0.078	−0.120	−0.078	−0.094	−0.083	−0.096	−0.100	−0.141

DISCUSSION

Although the measurement of fluorescence polarization is less specific for determination of pigment orientation than the measurement of dichroism, it can be used under many conditions in which the latter fails. Both methods are influenced by dichroism of shape, occurring in layered pigmented structures, such as lamellae.

The occurrence of a relatively high degree of fluorescence polarization with cells of *Rhodopseudomonas palustris*—Type 2 chromatophores; a system of parallel lamellae—is in line with the results from dichroism measurements[3].

Fluorescence polarization measurements cannot detect the direction of anisotropy. According to the dichoism measurements, however, maximal absorption occurs in the plane of the lamellae. Both fluorescence polarization and dichroism do not differ markedly for the various far red bands (B 800, B 860 and B 880 at 20 °C). This suggests that a nearly parallel orientation of the far red absorption vectors of these bacteriochlorophyll "forms" occurs. According to the action spectra, energy transfer from B 800 or B 860 to B 880 occurs with high efficiency. If the vectors were oriented at random, this should result in a marked depolarization. Negative dichroism and polarization occur at 590 nm, suggesting that the vector for absorption of the yellow band is perpendicular to the lamellar plane.

The dichroism measurements cannot discriminate between intrinsic dichroism and dichroism of shape. From the polarization spectra, however, an estimation of the latter effect can be made in the following way. *In vitro* the shape of the polarization spectrum is found independent of pigment concentration and temperature. The ratio between maximum negative and positive polarization is about 0.5. *In vivo* different and temperature-dependent ratios are measured in most species, while the ratio is also dependent on the presence of carbowax. Dichroism of shape will tend to decrease the apparent absorption parallel to the lamellae, if the refractive index of the pigmented layer is lower, and increase it when it is higher than in the non-pigmented one. Dichroism caused by parallel orientation of the pigments will then be decreased in the former and increased in the latter condition. In the fluorescence polarization spectrum this results in a shift of the base line.

The refractive index of protein aqueous layers can be influenced by imbibition of the cells in a solution of high osmotic pressure[12,13]. Also temperature changes influence the refractive indices *in vivo* and absorption properties[14]. In a lamellar structure this may affect the systems of layers in a different way. Both the temperature effect and the effect of imbibition were found to affect the polarization spectrum more in lamellar type chromatophores than in vesicular-type ones (Table III).

The polarization spectrum of *Rhodospirillum rubrum* shows a large negative polarization in the yellow bacteriochlorophyll band compared to the positive one in the far red, the effect being more pronounced in whole cells than in chromatophores. A similar room temperature polarization spectrum was measured by Ebrey[10]. His conclusion, that this high negative polarization is due to anomalous energy transfer from the second excited state (yellow band) to the first excited state (far red band) does not fit in with the present results. The yellow band in the action spectrum of fluorescence, when normalized at 800 nm, is of about equal height with the one in the absorption spectrum, provided the absence of the weak 800 nm absorption band (this band being intimately connected with the non-fluorescing energy acceptor

P 880[18,19]) and the spectral overlap with inactive carotenoids is taken into account. This indicates an efficiency of energy transfer of nearly 100%. Also at −196 °C the transfer efficiency approaches 100%, while the polarization spectrum differs from that at room temperature. No difference in shape of fluorescence action spectrum occurs between cell suspension and chromatophores, or between cell suspensions with and without carbowax.

Shape effects combined with pigment orientations are more likely the cause of polarisation anomalies than anomalous energy transfer.

With the *Athiorhodaceae* measured, the shape effect is weak. All five far red absorption bands, seen with these bacterial species at low temperature, are present in the fluorescence action spectrum.

The grana of the bacteriochlorophyll B-containing bacterium *Rhodopseudomonas viridis* consist of tacks of double membranes (Type 7, *cf.* ref. 1). The shape of the absorption spectrum resembles that of *Rhodospirillum rubrum*, although the far red bands are shifted towards the infrared. The fluorescence polarization spectrum is also similar to that of *Rhodospirillum rubrum*, although the absolute values are higher. Cooling results in an upward shift of the base line. As with *Rhodopseudomonas palustris* the total span of the polarization spectrum is decreased by cooling.

In general the results show that in bacteria with more or less flat lamellae the highest polarisation values are found and temperature effects are most pronounced, while those with small vesicles show lower values and a weak temperature effect. With all species, little depolarization occurs in the far red band system. This suggests that, at least with respect to the far red absorption vector, an orientation of adjoining bacteriochlorophyll molecules occurs.

In the carotenoid region, the polarization is negligible after correction for the shape effect. This suggests that the carotenoid molecules are not oriented *in vivo*.

As indicated before, fluorescence polarization may be observed also with a random orientation, but with a small average number of transfers. This occurs either at low pigment concentration or at low fluorescence lifetime[15]. If such was the cause of polarization, a decrease in fluorescence lifetime would result in a marked increase in polarization. Fluorescence yield is usually a measure of fluorescence lifetime. A strong decrease in yield with *Rhodospirillum rubrum* and *Rhodospirillum molischianum*, both containing vesicular chromatophores, corresponds to only a weak increase in polarization. Such a weak increase, corresponding to a decrease in radius of energy transfer, does not seem unlikely with this type of chromatophore.

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