

## Properties of photosynthetic bacteria in anisotropic rigid matrix and in suspension

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### Abstract

The photosynthetic bacteria (*Rhodospirillum rubrum*, *Synechococcus* and *Anabaena variabilis*) as well as their fragments embedded in isotropic and anisotropic polymer film were investigated. The orientation of photosynthetic pigments inside these organisms was compared, on the basis of the polarised absorption and fluorescence spectra, with the macroscopic orientation of investigated objects seen under microscope. The anisotropy of fluorescence was much higher than anisotropy of absorption. It showed strong influence of the photoselection by polarised radiation on the various bacterial chromophores exhibiting different orientations in the cells and various yields of fluorescence. The dimensions of cells were investigated on the basis of their photographs and by the scattering of the monochromatic radiation.

**Additional key words:** *Anabaena variabilis*; cyanobacteria; fluorescence microscope photographs; orientation; polarized spectra; polyvinyl alcohol film; purple bacteria; radiation scattering; *Rhodospirillum rubrum*; *Synechococcus*.

### Introduction

Spectra and photographs of biological objects taken in polarised radiation deliver several information about the structure and function of the investigated samples (Breton and Vermeglio 1982, Frąckowiak *et al.* 1986, Michl and Thulstrup 1986, Martyński *et al.* 1998). The anisotropy obtained on the ground of polarized absorption and fluorescence spectra is a function not only of the orientation of transition moments (TM) of absorption and emission of absorbing and emitting molecules located in such objects, but it also depends on the angular distribution of whole objects around the orientation axis (Frąckowiak *et al.* 1986, Martyński *et al.* 1998). In order to separate the contributions related to macroscopic objects orientation from the effects due to ordering of chromophores in the cell frame besides the polarised spectra, the photographs of samples with fluorescence and transmission microscope equipped with polarisers should

be taken (Martyński *et al.* 1998).

In this paper photographs were taken of the following organisms: *Rhodospirillum rubrum* (purple bacterium), *Synechococcus* and *Anabaena variabilis* (cyanobacteria) embedded in isotropic and stretched polyvinyl alcohol polymer film and located on a glass plate. The orientation of the investigated bacteria in polymer film was established and compared with anisotropies of absorption and fluorescence as calculated from polarised spectra. The fluorescence spectra were measured in the region 400-900 nm. The results received from the photographs are discussed together with polarised absorption and fluorescence spectra as measured previously for similar samples (Erokhina *et al.* 1980, Frąckowiak *et al.* 1990, Cegielski *et al.* 1992, Planner *et al.* 2000).

Martyński *et al.* (1998) showed for green bacteria that in a polymer matrix the clusters of bacteria and

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**Abbreviations:** BChl *a* = bacteriochlorophyll *a*; BPhe *a* = bacteriopheophytin *a*; DHP = dihydroporphyrin; PVA = polyvinyl alcohol; TM = transition moment.

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bacteria fragments occur. Therefore, now the dimensions of the investigated objects were established not only from photographs of polymer samples and samples adsorbed on glass plates but also from scattering of radiation on bacteria and bacteria fragments suspended in culture medium. It was done in order to proof if clusters were

## Materials and methods

The cyanobacteria (*Anabaena variabilis* and *Synechococcus*) and purple bacteria (*Rhodospirillum rubrum*) were from the collection of the Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Puschchino (Russia) (Frąckowiak *et al.* 1990, 1995). Methods of culturing bacteria were those used previously (Erokhina *et al.* 1980, Erokhina 1991, Frąckowiak *et al.* 1995). For the exact description of growing medium for cyanobacteria see Watanabe (1960). Purple bacteria were cultured using the Hunter medium and irradiation by incandescent lamp.

Whole bacteria and their fragments (obtained by sonication for 15 min at 4 °C and centrifugation at 36 000×g) were introduced into an aqueous solution of polyvinyl alcohol (PVA). Films were prepared from PVA solution mixed with resin in order to obtain non-acidic solvent (pH from 7.0 to 7.4). The PVA films were prepared and stretched as previously described (Fiksiński and Frąckowiak 1980). Fragments of bacteria for scattering experiments were obtained by sonication at room temperature at frequency 40 kHz and power of ultrasounds 50 W. The observation of the sonicated suspension shows that only a part of bacteria was disrupted.

The sample photographs were taken by means of a fluorescence microscope (*Olympus, AX80TR*, Japan) equipped with a 4CCD Colour Camera (*Olympus*) with image improve and HCl amplifier (*Olympus Flower*). Most pictures were taken at 100×, 200×, or 500× magnifications. The fluorescence was excited by polarised radiation using the 420–480 nm band for excitation. Additionally, polariser was also located in the fluorescence beam. The average lengths of objects measured from 15 objects in the same picture were established (Table 1). The averaged angles between the long axis of cells or cell fragments and film axis were also established (Table 2). The pictures of the samples in transmitted

radiation were also taken and used for measurements of lengths and angles (Tables 1 and 2).

The absorption spectra were collected using a *Shimadzu UV-V-1601* spectrophotometer, whilst the fluorescence spectra were obtained with a *Hitachi F4500*. From the polarised absorption the orientation factor  $S_{abs} = (A_{||} - A_{\perp}) / (A_{||} + 2A_{\perp})$  was calculated (Table 2). For stretched samples the four following polarised components of fluorescence were measured: VVV, VVH, VHV, and VHH (V - vertical, H - horizontal). The first and last letters refer to the direction of electric vector of exciting and emitted radiation, respectively, and the middle to the orientation of the axis of PVA film. In a case of non-stretched samples two components (VOV and VOH) were measured (middle O means non-stretched sample). For stretched samples, anisotropy of emission coefficients  $r_c = (VVV - VHV) / (VVV + 2VHV)$  for various bands were calculated.

The radiation scattering measurements were performed by means of a Master Sizer S instrument (*Malvern Instruments*, Malvern, U.K.). As a parameter describing the object dimensions the so-called equivalent sphere diameter (Rawle 1994) was determined. Of course, it is a crude approximation because most of the investigated bacteria are elongated, but from monochromatic radiation scattering some information for the object dimensions can be reached.

The scattering method was applied only in order to evaluate the dimensions of investigated objects, therefore for scattering experiments the wavelength of radiation (632.8 nm) only slightly absorbed by photosynthetic pigments was applied. In such condition, it is predominantly observed the scattering on whole cells or their fragments treated as non-pigmented objects having different refractive index than the culture medium.

## Results

**Photographs:** Fig. 1 shows the photographs of investigated cells and cell fragments taken with the transmission and fluorescence microscope. Fig. 1A–C presents *Synechococcus* cells under fluorescence microscope: A on glass plate, B and C in stretched film. In green radiation observation at 500× magnification (Fig.

1B) dark parts due to glycogen granules are seen, the fluorescent chromophores are distributed rather uniformly in cell volume. The separated chromophores are not visible. Similar pictures for *A. variabilis* whole bacteria are shown in Fig. 1D–F: in D on glass plate in transmitted radiation, magnification M = 500×; in E in green

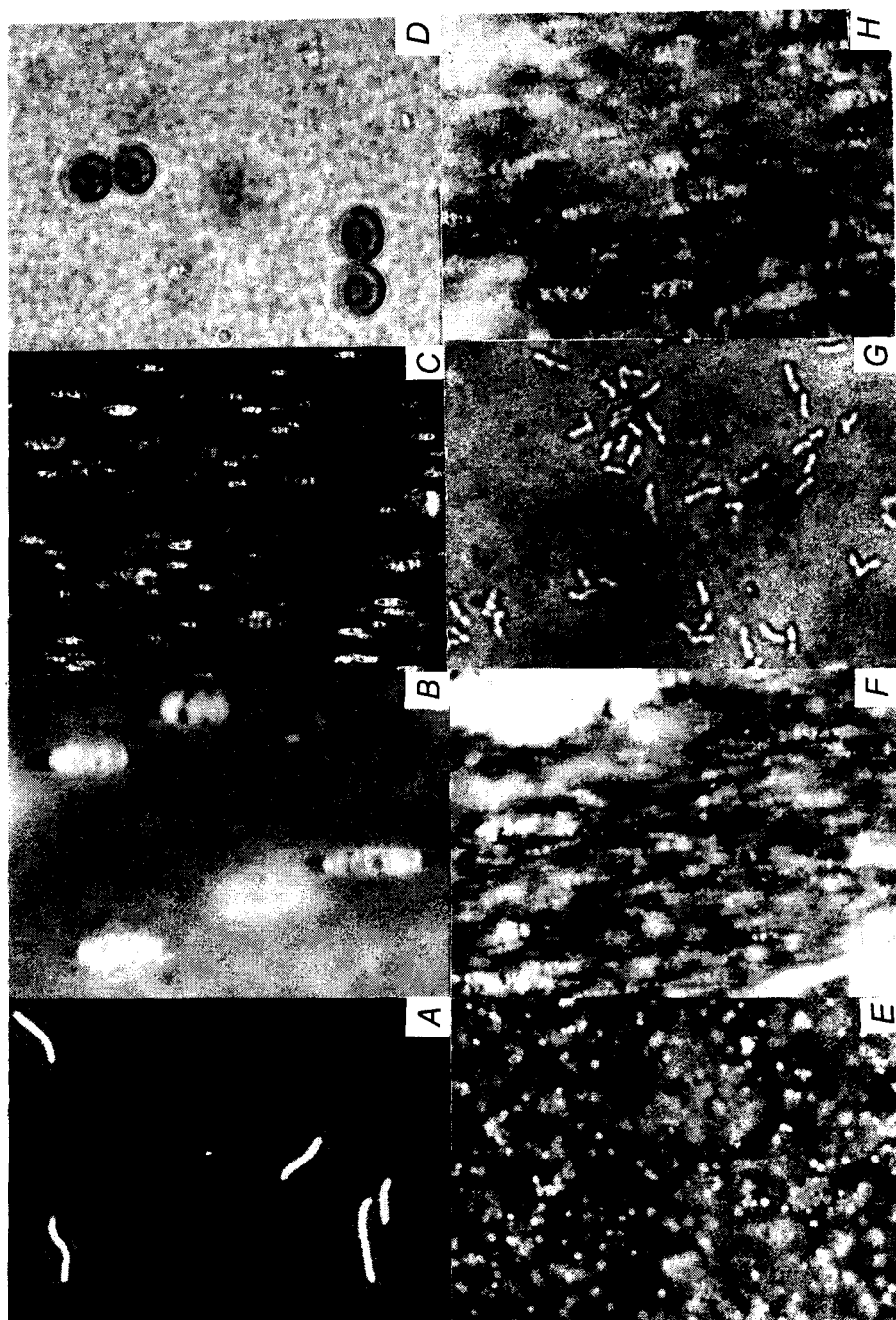


Fig. 1. Photographs of the investigated bacteria taken in the fluorescent microscope (A, B, C, E, F, H) and in transmitted radiation (D, G). The wavelengths of fluorescence excitation were from 330 to 385 nm. A, B, and C show whole cells of *Synechococcus*: A on the glass plate, magnification  $M = 500\times$ , observation at wavelengths longer than 590 nm; B in stretched PVA film,  $M = 500\times$ , observation in green radiation; C the same sample as in B,  $M = 100\times$ , observation in red radiation. D, E, and F whole cells of *Anabaena variabilis*: D on the glass plate,  $M = 500\times$ , E in isotropic PVA film,  $M = 100\times$ , observation in red radiation; F in stretched PVA film,  $M = 100\times$ . G and H whole cells of *Rhodospirillum rubrum*: G in isotropic PVA film,  $M = 200\times$ ; H in stretched PVA film, observation in green radiation,  $M = 200\times$ .

radiation, isotropic PVA film,  $M = 100\times$ ; in *F* stretched PVA film,  $M = 100\times$ . Fig. 1*G-H* shows whole bacteria of *R. rubrum*: *G* in transmitted radiation, in isotropic PVA film; *H* in fluorescence radiation in stretched film,  $M = 200\times$ . All three organisms are in stretched films oriented in high degree. The photographs were taken using different combinations of filters in exciting and fluorescence beams, with and without polariser in fluorescence beam. In most cases the pictures observed in radiation polarised parallel to the stretched axis of the film need shorter time of exposition than the picture taken perpendicular to this axis direction of the electric vector of fluorescence radiation to reach optimal exposition established automatically by microscope arrangement. It suggests that more fluorescence TM of BChl *a* is located along long axis of bacteria than under large angle with this direction.

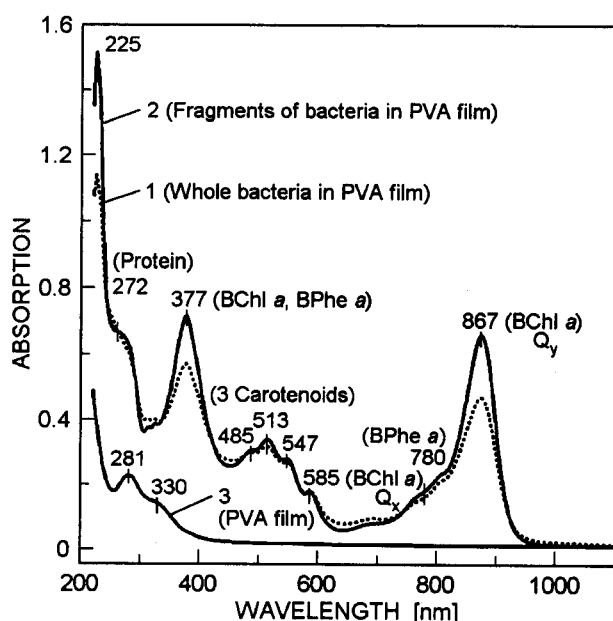


Fig. 2. Absorption spectra of the purple bacteria *Rhodospirillum rubrum* in isotropic PVA film. Curves: 1 - whole bacteria, 2 - fragments of bacteria, 3 - pure PVA film.

The lengths of bacteria cells and cell fragments obtained by different methods were comparable (Table 1). The photographs show that fragments are formed predominantly by the division of cells along their long axis. The orientation factor for bacteria cells calculated from photographs of oriented sample was very high (Table 2). The average angle of cell longer axis with film axis was low (Table 2).

**Absorption and fluorescence spectra:** Both stretched and unstretched samples exhibited some anisotropies of absorption (Fig. 2) and emission (Fig. 3 and Table 2). For

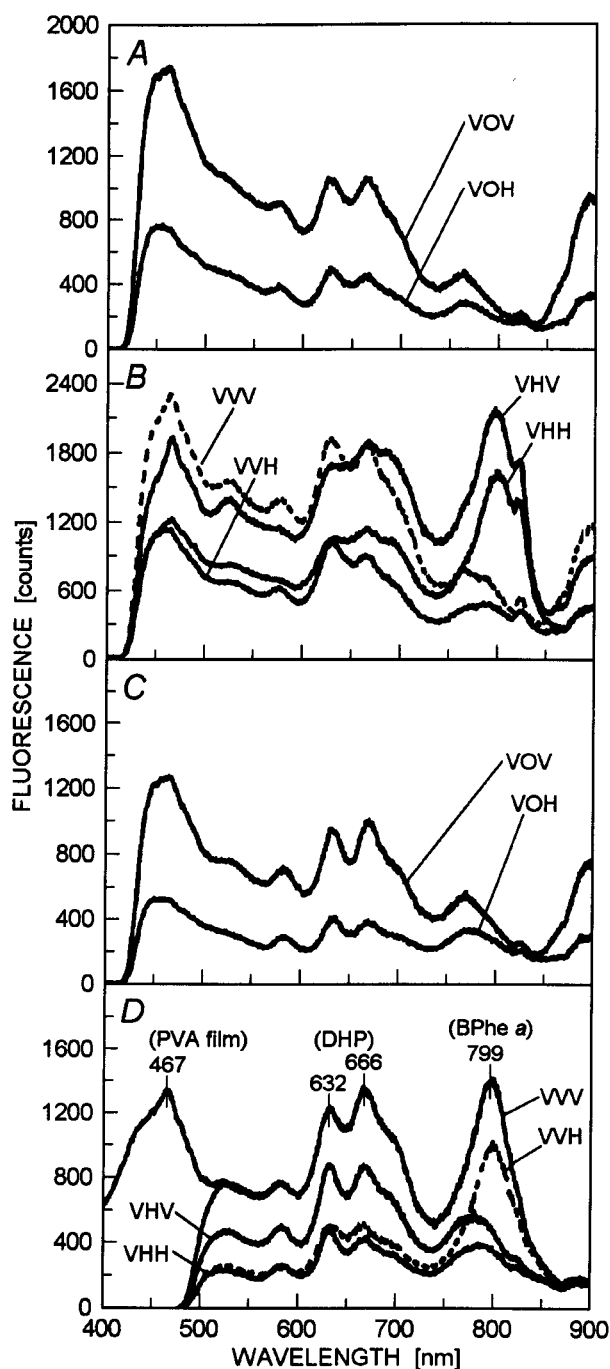


Fig. 3. Examples of polarised fluorescence spectra of *Rhodospirillum rubrum* cells (*A*, *B*) and fragments (*C*, *D*) in unstretched (*A*, *C*) and stretched (*B*, *D*) films. Polarised components are marked. The four following polarised components of fluorescence were measured: VVV, VVH, VHV, and VHH (V - vertical, H - horizontal). The first and last letters refer to the direction of electric vector of exciting and emitted radiation, respectively, and the middle to the orientation of the axis of PVA film. In non-stretched samples, two components (VOV and VOH) were measured (middle O means non-stretched sample).

purple bacteria some polarised spectra are presented in this paper and some values were taken from literature (Cegielski *et al.* 1991, 1992). For cyanobacteria the polarised absorption and fluorescence spectra, measured for the same sets of sample, and reported previously (Planner *et al.* 2000) were used for discussion of the photographs. Anisotropy of absorption of *R. rubrum* in PVA film and the values  $S_{abs}$  of others samples were very low (Fig. 2 and Table 2). Anisotropy of fluorescence coefficient  $r_c$  (Table 2) was calculated for BPhe *a* maximum at 799 nm and for dihydroporphyrin (DHP) at 632 and 666 nm.

Similar results as from photographs followed from polarised fluorescence spectra (Fig. 3 and Table 2). Polarised fluorescence spectra were measured only in the region from 400 to 900 nm, *i.e.*, for the emission of degraded BChl *a* only. It was because of the properties of used spectral arrangement. As follows from fluorescence spectra presented in Fig. 3, part of BChl *a* was in the investigated sample in a form of BPhe *a* or DHP but in native *R. rubrum* cells (Cegielski *et al.* 1991) BPhe *a* absorption is located in a similar region (about 800 nm).

Strongest contributions to emission were seen for BPhe *a* absorbing at 780 nm and fluorescing (Fig. 3) at 799 nm. The film stretching causes the increase of this emission what can be due to better orientation of DHP transition moment in film plain or/and to higher degree of degradation of BChl *a* in stretched than in non-stretched film. Primary donor emission was not observed because of low sensitivity of the used apparatus in long wavelength region and may be also because of photochemical reaction competing with fluorescence emission. Maximum at about 467 nm was due to fluorescence of the PVA film.

**Radiation scattering experiments:** The scattering of radiation on a sample with *Synechococcus* (*Anacystis nidulans*) located in culture medium, measured after various time of sonication is shown in Fig. 4. The disintegration of some clusters was followed by the increase of maximum of diameter distribution belonging to whole cells and probably also to cell fragments. For *Anabaena* cells, the used sonication did not change the dimension distribution (Table 2), and it is known from photographs and literature (Martyński *et al.* 1998) that some fragments are formed.

The supposition that cells and cell fragments are spherical, taken for results presented in Fig. 4, is a crude approximation, but it can be used for comparison of dimensions of investigated objects. The diameters of such sphere are comparable with dimensions of cells taken from literature (Trüper and Pfennig 1978) and obtained from photographs (Fig. 1 and Table 1). The diameter of equivalent sphere (Rawle 1994) is located usually between long and short axes of real cells and it is not

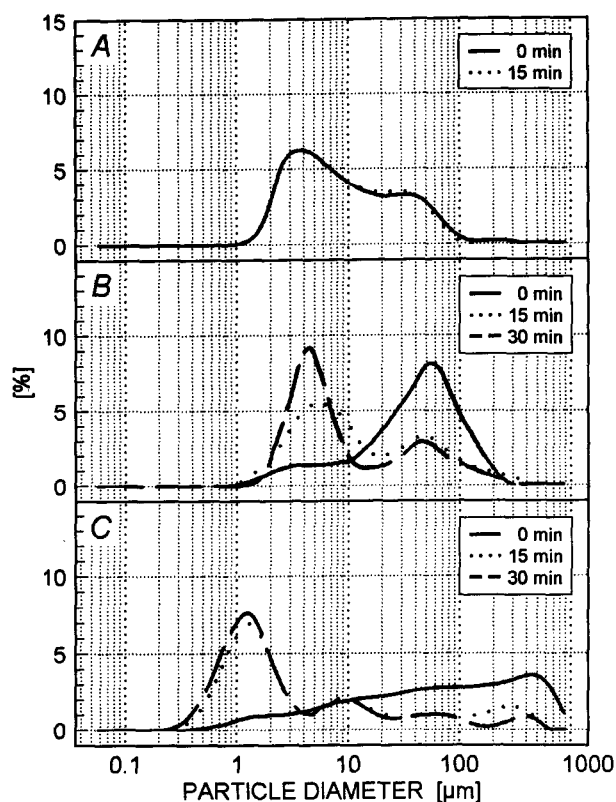


Fig. 4. Bacteria volume distribution obtained by radiation scattering (Master Sizer S) for bacteria in culture medium: A - *Anabaena variabilis*; B - *Synechococcus*; C - *Rhodospirillum rubrum*. The samples were sonicated in Malvern Automated Sample Dispersion Unit in given times.

changed dramatically with cell fragmentation. According to Frackowiak and Januszczuk (1974, 1975) and Januszczuk *et al.* (1969), not the prolonged sonication, using the power and frequency of ultrasound similar to used in this paper, causes the fragmentation only of a part of the cells. The same conclusion follows from Fig. 4 showing the distribution of cell dimensions before sonication and after it. The ultrasound caused predominantly the disruption of cell clusters (for *R. rubrum* with diameter from about 1.3 till 400  $\mu\text{m}$ ). After sonication, the maximum connected with separated cells (which is about 1.3  $\mu\text{m}$ ) increased. Last maximum was shifted towards smaller diameters as a result of sonication because of partial fragmentation of whole cells. Prolonged sonication caused the creation of fragments forming big clusters (Fig. 4). The small clusters of cells were also formed. The big clusters could be easily recognised and were not used for the calculation of object length and orientation from the photographs (Table 1).

The present results show that the used scattering measurements are not an effective method for observation of the fragmentation of cells. The diameter of equivalent

Table 1. Average lengths [ $\mu\text{m}$ ] of bacteria obtained from fluorescence (F) and transmission (T) pictures in microscopic photographs, from radiation scattering experiments (S) and from the  $L$  = width/length given in the literature.  $S_A$  and  $S_B$  mean diameter obtained from bacteria volume distribution before and after the 15 min sonication of the bacteria in culture medium. The samples were sonicated in Malvern Automated Sample Dispersion Unit (ultrasonic power = 50 W, frequency = 40 kHz, stirrer speed = 16.67 rps). The standard deviation of F and T is 0.4  $\mu\text{m}$ .

Sample	Dimension		$S_A$	$S_B$	$L$
	F	T			
<i>Anabaena variabilis</i>	3.1	3.3	6.8	3.7	0.5-0.6 (Wolk <i>et al.</i> 1994)
<i>Synechococcus</i>	7.0	7.5	15.1	5.5	0.6-0.8/2-4 (Simon 1987)
<i>Rhodospirillum rubrum</i>	7.0	8.0	63.0	3.4	0.8-1.0/7-10 (Pfennig 1978)

Table 2. Orientation degree of the cells and cell fragments in PVA stretched film.  $\Theta$  [degree] - average angle of inclination of the long axis of cell from the film axis,  $S_{\text{abs}}$  - pigment orientation coefficient calculated from polarised absorption spectra [ $S_{\text{abs}} = (A_{\parallel} - A_{\perp}) / (A_{\parallel} + 2 A_{\perp})$ ], where  $A_{\parallel}$ ,  $A_{\perp}$  are the absorption value measured for horizontally and vertically polarised radiation, respectively],  $r_c$  - emission anisotropy coefficient [ $r_c = (V_{VV} - V_{HV}) / (V_{VV} + 2 V_{HV})$ , notation of compound in text],  $S_{\text{phot}}$  - the orientation coefficient obtained from photographs in fluorescence radiation [ $S_{\text{phot}} = (L_{\parallel} - L_{\perp}) / (L_{\parallel} + 2 L_{\perp})$ ], where  $L_{\parallel}$ ,  $L_{\perp}$  are the projections of long axis of bacteria on film axis and on perpendicular direction to film axis, respectively],  $\lambda_{\text{abs}}$  [nm] - wavelengths of absorption maxima,  $\lambda_F$  [nm] - wavelengths of fluorescence maxima. \*From Cegielski *et al.* (1992).

Sample	Stretching	$\lambda_{\text{abs}}$	$S_{\text{abs}}$	$\lambda_F$	$r_c$	$S_{\text{phot}}$
<i>Anabaena variabilis</i> in PVA film	0 %	436	-0.020	684	-	
		671	0.130	738	-	
	200 %	436	-0.012	684	-0.25	$0.69 \pm 0.01$
		671	0.160	738	-0.26	
<i>Synechococcus</i> in PVA film	0 %	436	0.009	684	-	
		671	0.140	738	-	
	200 %	436	0.011	684	-0.24	$0.62 \pm 0.01$
		671	0.290	738	-0.24	
<i>Rhodospirillum rubrum</i> in PVA film	0 %	377	0.000	632	-	
		867	0.000	666	-	
				799	-	
				880	-	
	200 %	377	-0.008	632	0.05	$0.45 \pm 0.01$
		867	0.002	666	0.00	
				799	0.45	
				880	-0.08*	
Fragments of <i>Rhodospirillum rubrum</i> in PVA film	0 %	377	0.000	632	-	
		867	0.000	666	-	
				799	-	
				880	-	
	200 %	377	0.002	632	0.12	
		867	0.006	666	0.15	
				799	0.38	
				880	0.10*	

sphere for cells of *A. variabilis* was larger than that obtained from photographs in transmitted radiation what is an unexpected result (Table 2). Even worse is the result for *Synechococcus* cells because in this case the diameter of sphere was twice as large than found from photographs. Thus the cells, which are only partially transparent because of absorption of radiation by pigments, can not be investigated by the used apparatus at

approximation applied for the evaluation of their dimensions.

As shown by Frąckowiak and Januszczyk (1974, 1975), from radiation scattering using wavelengths absorbed by pigments of organisms and under compensation of the scattering on other membrane (by proper choice of medium) it is possible to observe the dimensions of pigmented elements located inside of

organisms. On such a way it was found that sonication changed the inner structure of photosynthetic apparatus (Frąckowiak and Januszczyk 1975) and caused the desegregation of pigments (Januszczyk *et al.* 1969). The

## Discussion

The dimensions obtained from fluorescence pictures were in most cases smaller than these calculated from photographs taken in transmitted radiation. It is logical because intensively fluorescent are only antennae located inside cells (Fig. 1A). These differences depend on the distribution of fluorescent elements in cells. In all cases the anisotropy of fluorescence was higher than that of absorption and it increased as a result of stretching (Table 2). The observed effects agree with other results obtained for oriented cyanobacteria (Frąckowiak *et al.* 1996).

The coefficient  $r_c$  (Table 2) shows if there is an orientation of pigment emission transition moments in the cells. In case of only photoselection effect, this coefficient is zero. As shown in Table 2, the DHP exhibited very low degree of orientations whereas BPhe *a* (at 799 nm) showed much higher  $r_c$  value. Hence DHP molecules were almost randomly distributed, whereas BPhe molecules were still located in oriented matrix. For fragments, the  $r_c$  values were higher for both spectral regions, which shows that also the DHP at higher degree of macroscopic orientation exhibits the anisotropy  $r_c$ . Previous results (Frąckowiak *et al.* 1990, 1992, 1995, 1996, Cegielski *et al.* 1991), obtained for various purple bacteria or their parts located in anisotropic media, show that also the DHP at higher degree of macroscopic orientation exhibits the anisotropy of  $r_c$  and that the anisotropy of emission is much higher than that of absorption. It is due to higher degree of orientation of pigment molecules responsible for fluorescence than average degree of orientation of all

last effect can be responsible for the large difference between results obtained by radiation scattering of sonicated cells and photographed cells embedded in PVA film.

absorbing molecules. In fluorescence spectrum, the largest peak in a region 600-700 nm is due to DHP, in region of about 800 nm to BPhe *a* (Frąckowiak *et al.* 1995). The shorter wavelength emission maxima in a region of 500-600 nm should be due to other BChl or BPhe *a* degraded forms. Such forms are usually created during embedding of organisms in stretched films. These forms exhibit much higher emission intensity in a case of stretched film. The maximum at about 820 nm can be due to BChl monomeric form (Frąckowiak *et al.* 1995).

It may be concluded that:

- (1) In all investigated organisms the anisotropy of emission radiation was higher than that of absorption. This shows that in bacteria there occur several pools of chromophores exhibiting different orientations and various yields of fluorescence.
- (2) Bacteria and bacteria fragments were in a high degree ordered in stretched polymer, but anisotropy of emission was observed even in isotropic films.
- (3) The dimensions of bacteria and bacteria fragments obtained from radiation scattering and from photographs were different. It shows that approximation applied in scattering experiments is not exactly applicable for investigated samples.
- (4) In investigated bacteria the fragments form clusters, therefore the fragmentation can not improve strongly the orientation. The anisotropy of fragments and whole cells is similar.

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