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CIRCULAR DICHROISM SPECTRA OF AXIALLY ORIENTED *RHODOSPIRILLUM RUBRUM* CELLS AND THEIR FRAGMENTS IMMOBILIZED IN POLYMER FILM*

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Circular Dichroism (CD) spectra of *Rhodospirillum rubrum* cells, and their fragments embedded in isotropic and uniaxially oriented by stretching polyvinyl alcohol films were measured. Effects responsible for CD signal generation such as assymetry of chromophores itself, the helical organisation of lamellar system, the helical texture of stretched PVA matrix, the contribution from circular and linear birefringence, as well as from exciton splitting resulting from pigments mutual interactions are discussed.

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1. Introduction

A lamellar system of photosynthetic organisms is anisotropic and therefore can exhibit several optical effects: linear dichroism (LD), linear birefringence (LB), circular dichroism (CD) and circular birefringence (CB) [1, 2]. The CD signal obtained by the photosynthetic apparatus of organisms , can be related to the following effects: the asymmetry of chromophores, the helical organization of the lamellar system resulting in similar organization of the pigment transition moments, the combination of the influence of LD, LB and CB on the measured CD

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signal, the influence of light scattering, and the exciton splitting caused by mutual interaction between pigment molecules [1, 3–8]. Circular dichroism spectroscopy has been developed predominantly for studying biopolymers . There seems to have been little, if any, application to the field of synthetic polymers [9]. Therefore, the CD spectra of PVA matrices, both isotropic and stretched, have also to be investigated. The PVA matrix exhibits a helical organization, especially after stretching. This can be mimicked by the small fragments of organisms embedded in the film. Previously [1, 10], the CD and MCD spectra of the model system which consists of a chlorophyll solution in a nematic liquid crystal were measured. This model is organized in a helical texture, diminished by a strong magnetic field. It was shown that a big CD signal can be caused predominantly by a helical structure of the matrix orienting the pigment molecules attached to it.

Pearlstein and Hemenger [4] have shown that bacteriochlorophyll (Bchl) complexes possess a CD structure which cannot be explained simply by exciton splitting. Also the CD signal of chloroplasts [5] is considerably larger than that of isolated complexes and much larger than an intrinsic CD signal of pigment. This "giant" signal can be, at least partially, related to the scattering effects occurring in the helical structures of chloroplasts. For some natural biological samples (such as nucleic acids), the relation between their secondary structure and the shape of the CD spectra is known [11]. Even in this case however, the interpretation of the changes in shape and amplitude of the CD signal as the effect of perturbation of natural sample structure is presently still controversial [11]. The light scattering changed by the perturbation of sample structure influences the CD spectra, predominantly in the short wavelength region [12].

The helical structures of photosynthetic apparatus have been lately investigated by computer-controlled confocal scanning differential polarization microscopy [7, 8]. In the present paper we are showing that information about such structures can be delivered by CD spectroscopy applied to uniaxially oriented biological samples. Therefore, an analysis of contributions of the various effects to the CD signal for oriented biological sample was performed. The CD signal related to the structure of PVA matrix is not only the "background" effect (which has to be eliminated before the interpretation of the CD signal of bacteria or bacterial fragments), but also provides information about the organization of the matrix in which bacterial samples are embedded and oriented.

2. Material and methods

The methods of culturing bacteria, introducing them in to PVA films, and film stretching were given previously [13]. The isotropic films and films elongated to four-times l_0 (their initial length), e.g. $((l - l_0)/l_0) \times 100\% = 300\%$ were investigated. The CD spectra were recorded using either a Jasco J20 or Jasco J200B CD spectrometer. Since linear and circular effects for partially oriented samples are interwined [2], we have to consider them jointly. In a region of strong absorption of oriented solute, the effects related to LD dominate. The effects related to birefringence are caused predominantly by the anisotropic matrix and occur even in a region of very low absorption of polymer.

In order to evaluate on the basis of Muller matrix [14] the contributions of LB and CB to the CD signal obtained directly from measurments, the uncolored PVA films at various elongations were also investigated. The CD and LD of stretched (reference and with bacteria) PVA films were measured under the following angles between film axis and the horizontal direction : 0°, 90°, 45°, 135°. In the comparison of the CD shapes of various samples the same (horizontal) position of anisotropic film axis was always used.

3. Results and discussion

Figure 1 shows the CD spectra of pure (uncolored) PVA films stretched into various degrees. Absorption of PVA in this spectral region is very low, therefore the presented CD signal is predominantly related to the film birefringence. The signal for unstretched film is low (but measurable). The film stretching causes at first the increase of CD signal, but for higher degrees of stretching the amplitude of the CD signal is diminished. The spectra measured at 0° and 90° between horizontal direction and film axis have similar shapes but are of opposite sign, whereas for 45° they strongly differ in shape. All spectra taken at 135° have similar shapes like those measured at 45° but are of opposite sign. This effect is shown in the figures only for one degree of elongation, but it was observed for all reference and pigmented samples. The absorption and LD spectra for uncolored PVA films are unmeasurable, therefore the observed CD signal can be related only to birefringence. Since the signal at 45° and 135° does not vanish, it seems that optical symmetry of birefringence of the film is not uniaxial and that this symmetry changes with film stretching [2]. The CD signal for unstretched film is low and it is flat in the spectral region used. The CD signals of the film stretched to 300% are higher than these of 0% stretching, but in the spectral region between 350 nm and 650 nm they are rather flat (Fig. 1(d). Figures 2(a) and 2(b) show absorption spectra of bacteria and their fragments in both, isotropic and stretched, PVA. The spectra of whole bacteria (Fig. 2(a)) are much stronger perturbed by light scattering than the absorption spectra of fragments (Fig. 2(b)). Figure 3 presents the CD spectra of isotropic (unstretched) films with whole bacteria and bacterial fragments.

The investigated films containing fragments of bacteria have a higher concentration (more than twice) than those with whole bacteria, but they exhibit a lower amplitude of the CD signal, (especially in Soret and carotenoids regions), compared to that of the whole bacteria. This result is in agreement with the observation of other authors [3, 5]. The spectra in Fig. 3 are not corrected for light scattering effects, therefore the signal is also high in the low absorption region (600–750 nm). Similary to the absorption spectra (Fig. 2), the scattering effect is lower for films with the bacterial fragments than for the samples with whole bacteria. The difference between the two types of samples is smaller in the infrared absorption band of Bchl (800–900 nm) (which suggests strongly that it is related to light scattering), but whole bacteria still have a stronger (recalculated on absorption unit) CD signal than that of bacterial fragments. Comparing the spectra

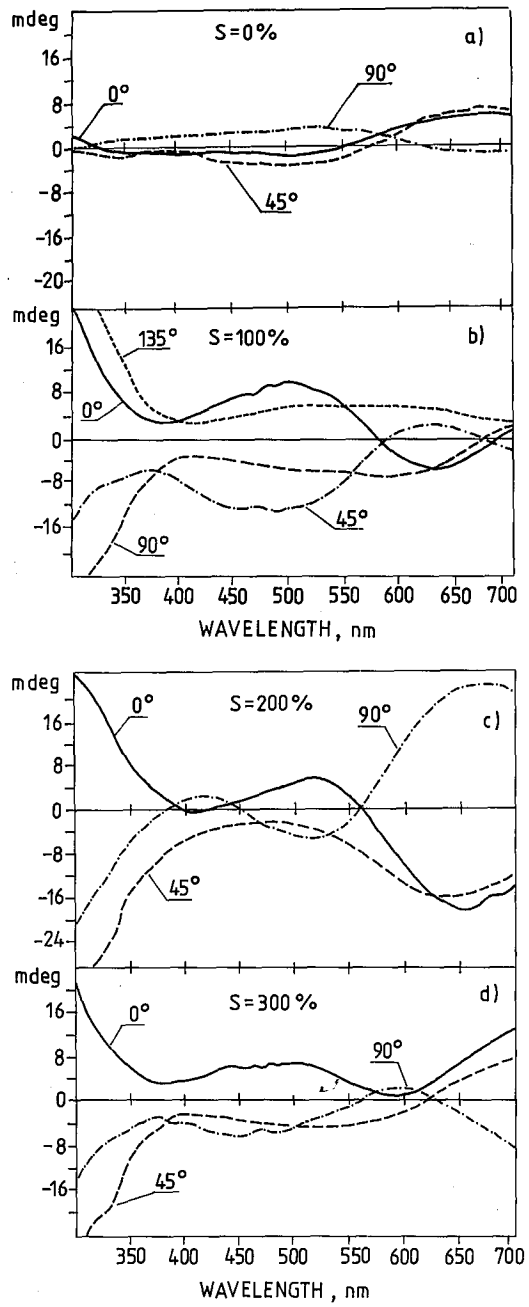

Fig. 1. CD spectra of uncolored PVA films. The degree of the film stretching ($S = \Delta l/l_0 \cdot 100\%$, where l_0 is the initial lenght of the film) and angles between the horizontal direction and the film axis are marked in the figure (for unstretched film an arbitrary direction is taken as the axis)·1 mdeg= $0.001^0 = 17.5 \times 10^{-6}$ rad.

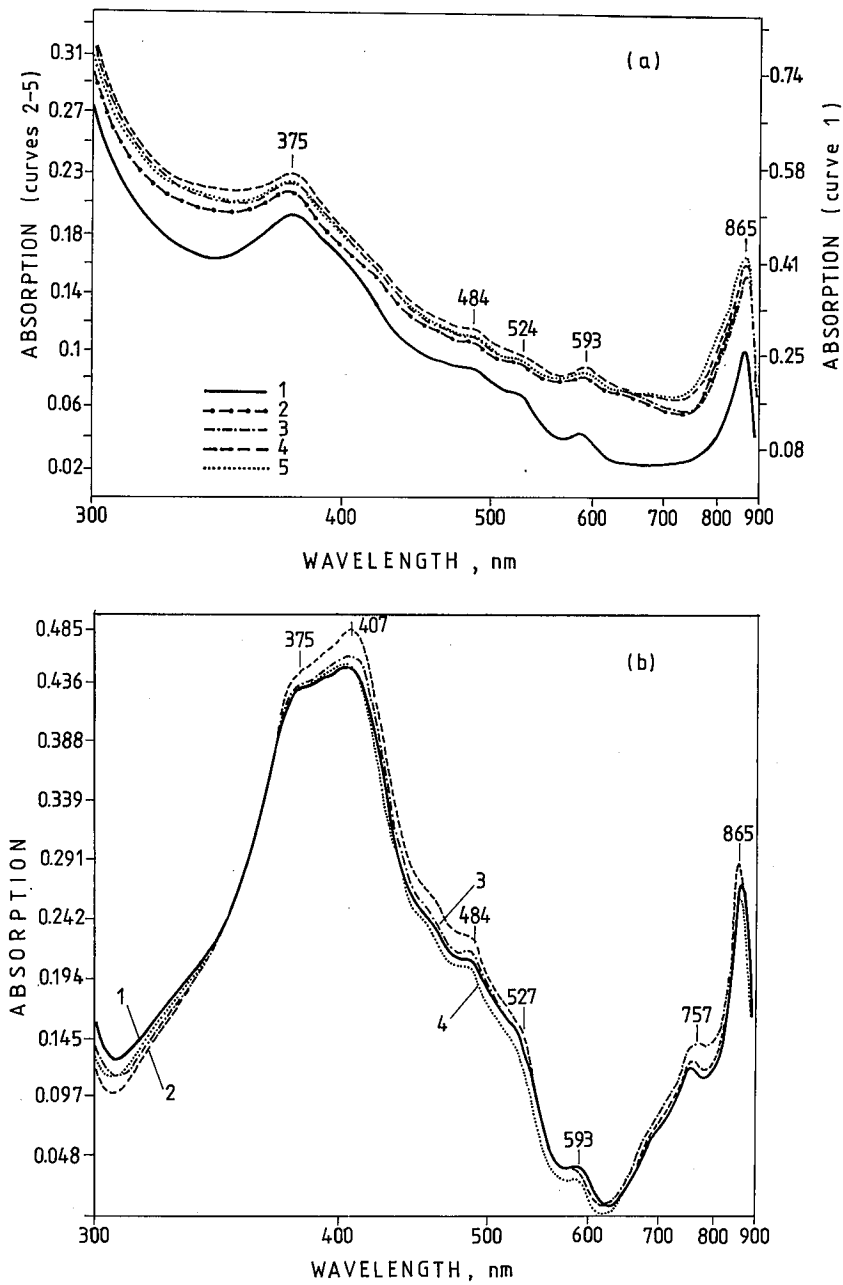

Fig. 2. (a) Absorption spectra of PVA film with whole bacteria: (1) unstretched film, natural light, (2)–(5) — film stretching 300%, angles between the polarizer and the film axis: (2) 0°, (3) 90°, (4) 45°, (5) 135°. Main maxima are marked in nm.
(b) Absorption spectra of stretched PVA with bacterial fragments: (1) 0°, (2) 90°, (3) 45°, (4) 135°.

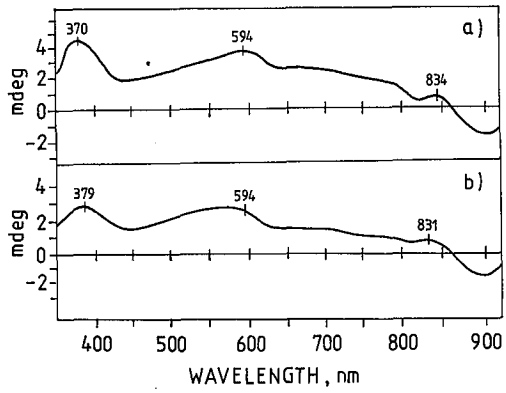

Fig. 3. CD spectra of whole bacteria (a) and bacterial fragments (b) in unstretched PVA film.

presented in Fig. 3 with those known from the literature [6], one can see some similarities (positive maximum at 831 nm, negative on the longer wavelength side of it etc), but the spectra are deformed by scattering. Figure 4(a) shows the CD spectra of chromatophores. These spectra are in a lesser degree disturbed by light scattering than those of whole bacteria and bacterial fragments. Therefore, the CD spectrum is very similar to that of Bchl [15] even in the Soret band. Figures 4(b) and 4(c) show the unstretched samples with bacterial fragments and whole bacteria measured at various angles (of rotation of sample). Because the axis created by film elongation is absent for unstretched samples, the angles given in these figures are measured from an arbitrary position of the sample. The results show that the samples have some anisotropic properties and that it is possible to establish a sample position in which the spectrum is located near the base line. For the sample with whole bacteria, the changes in the CD signal, obtained as a result of film rotation, are higher than those observed for bacterial fragments. It seems that these effects can be explained by circular differential scattering (CDS) observed also by Garab [12] in the local CD signals. The CDS signal of helical elements is formed because of various scattering of light with different circular polarization. This effect depends on the angle of observation and is a very sensitive function of the parameters of the helix. Liquid crystalline macroaggregates occurring in the photosynthetic apparatus display a strong CDS signal related to themacrohelical array of chromophores [5]. In the case of whole bacteria, statistical fluctuations in contributions to CD from various microdomains located in spectrometer light beam could be larger than for fragments because elements embedded in film are large and their number in observed part of film is lower. Also, other scattering effects in both cases have to be different because of the various dimensions of the scatterers. Generally, the shapes of the spectra presented in Figs. 3 and 4 are similar to the spectra of photosynthetizing organisms [16, 17] and their fragments [18]. In a Soret band, the CD signal is negative (or low) at shorter wavelength and

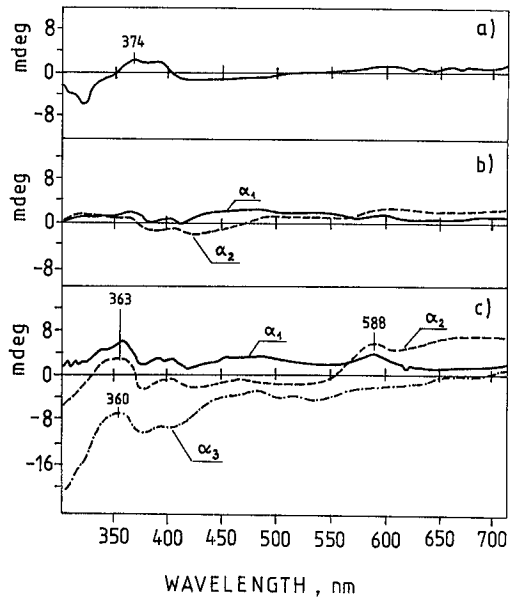

Fig. 4. CD spectra of: chromatophores (a), bacterial fragments (b), whole bacteria in unstretched films (c). α — angles between the horizontal direction and arbitrary direction in the film; $\alpha_1 - \alpha_2 = 45°$, $\alpha_3 - \alpha_2 = 45°$.

positive (or high) at longer wavelength ranges. The carotenoids region has a positive maximum at about 594 nm showing a highly organized fraction of molecules. In the near IR band a positive signal appears from the shorter wavelength side and negative from the longer. The CD signal is higher than can be expected from intrinsic Bchl chirality at the same concentration of the pigments in measured samples. Therefore overlapping of the several effects in the measured CD signal is suggested.

Figure 5 shows the CD spectra of pigmented stretched samples measured at various angles with respect to the stretching axis. As in the case of unpigmented films a mirror symmetry with respect to the base line for spectra measured at 0° and 90° as well as for 45° and 135° angles is always observed. Only the part of such symmetrical spectra is shown. The CD spectra of oriented samples are closly related to pigment absorption regions. Amplitudes of the CD signals are much higher than those of unoriented samples with the same concentration of pigments. The differences between oriented and isotropic samples are much higher than observed by Gingras [17] between randomly distributed and magnetically oriented samples. The shapes of spectra measured at 0° and 45° are different, which means that the contributions from various pools of pigments have different magnitudes. The shapes are also strongly different from those of unpigmented films and have much higher amplitudes. All spectra presented in Fig. 5 are done for 300%

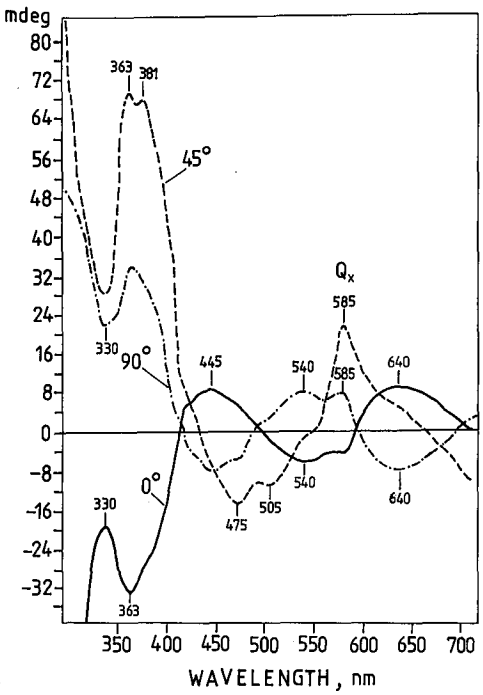

Fig. 5. CD spectra of whole bacteria in stretched 300% PVA film (main maxima in nm and angles between the horizontal direction and the film axis are marked on graphs).

of film elongation. The values of the CD signal for pure PVA film were lower than 8 mdeg at the same film thickness and elongation degree. The contribution of PVA to the CD signal of pigmented samples is lower since absorption cross section of embedded in film biological membranes is large. Therefore big part of the light is absorbed or transmitted by the biological membranes instead being transmitted by PVA film. All the features of the spectra shown in Fig. 5 suggest strongly the influence of textural (linear and helical) effects on the CD spectra.

Previously [1, 10], we have investigated the chlorophyll in nematic liquid crystal as a model of chlorophyll in organisms. Bchl in nematic liquid crystal (LC) [1] exhibits in the red band region, positive or negative sign of the CD signal depending on the type of LC (positive or negative dielectric anisotropy of LC). This is a result of various orientations of the pigment molecules in different LC, which suggests a strong influence of sample "texture" on the character and amplitude of the CD spectra.

In vivo the CD signal of chlorophylls usually has the "sigmoidal" shape in the red band, a part of it is positive, another part negative [5].

As it was shown [12], the big CD signal is correlated with light harvesting complexes (LHC) content in thylakoid membranes. The various macrodomains are

characterized by a different sign of the CD signal. The macroscopic CD features are obtained by averaging the contributions from the regions of different elipticity. The positive and negative CD bands in the red region could be separated spatially by recording the local CD in a confocal scanning CD microscope [7, 8, 12].Theoretical study has shown, that the sign and the amplitude of circularly polarized light scattering (CDS) occurring on the helical elements depends on the orientation of scattering particles [15].

The shape of the infrared CD spectrum of bacterial reaction centers (RC) depends on reduction or oxidation of the sample [19]. This was explained as a change in pigment interactions. This is not in disagreement with the microdomains hypothesis [7, 8, 12] since firstly, the contributions to the CD signal are also dependent on mutual pigment "aggregation" and secondly, the "giant" CD signal of the photosynthetic apparatus is related rather to LHC than to RC. In the case of RC the exciton splitting effect is very important [17], whereas in LHC the textural effects are rather predominant as the helical domains contribute to both, CDS and CD.

Figure 5(a) presents the CD spectra of whole bacteria. Such a sample has a low LD as well as LD' (linear dichroism of absorption measured at 0° and 90° as well as at 45° and 135°, respectively) (Fig. 2(a)). Both LD and LD' are measurable. The parallel component of absorption is higher than the perpendicular in the red band (865 nm), showing that a lot of Bchl Q_y transition moments are aligned parallel to the film stretching axis. The perpendicularly polarized absorption is higher than parallel one in the region of Q_x band (593 nm) indicating that for this pool of aligned molecules Q_x and Q_y transition moments are as usually almost mutually perpendicular. The spectra taken at 45° and 135° are different than those taken at 0° and 90°, especially in the Soret band and in the carotenoids region. It is known [20, 21] that different pools of chlorophyll molecules are contributing to the red and Soret bands of oriented bacterial and chloroplast samples. Predominantly TM with large projections on a plane perpendicular to spectrophotometer light beam are measured in absorption.

The presented spectra suggest that at least a part of the transition moments (TM) of Bchl and car molecules are forming large angles with PVA stretching axis. It is known [20] that the TM of 397 nm and 378 nm bands of Bchl form an angle of about 30° between themselves and that they always have different chirality [23]. As presented in Fig. 5, for a spectrum taken at 0° the maximum at 363 nm is negative and has a shoulder at about 380 nm. After the sample was rotated to the 90° positions of both the maximum and the shoulder became positive. At the 45° position, the CD signal increased almost twice compared to that at 90°. It exhibits two well resolved maxima at 363 nm and 381 nm and a strong positive maximum in a region of the Q_x band of Bchl. This last band is also better pronounced in this spectrum in comparison to spectra measured at 0° or 90°. It is clear that contributions from arranged pigments are strong in this particular position of the film. Unfortunately, these spectra were taken only with a Jasco 20, therefore, the IR region could not be measured. In the carotenoids absorption region, maxima were observed at 475 nm, 505 nm and 540 nm. These maxima are located close to the positions of the components of photoacoustic spectra previously measured for

similar samples [21]. The rotation of the film changes not only the sign of the CD signal, but also the shape of the spectrum. Effects related to the PVA matrix are much stronger at 0° and 90° than at 45° and 135°. Because the PVA chains are oriented parallel to the stretching direction, the LB can be formed as a result of stretching. The diminishing of the effects related to polymer at 45° suggests that it is rather LB than CB. The same conclusion can be drawn from the change of the sign of PVA effects following the rotation of film from 0° to 90°. An especially strong influence of PVA is observed in wavelengths shorter than 350 nm and longer than 600 nm.

From the CD spectra of whole bacteria, it appears that they are highly related to the pigment anisotropic absorption (linear and/or circular). A similar conclusion can be drawn from the linear and circular dichroism of bacterial fragments (Fig. 2 and Fig. 6). Fragments are in a higher degree oriented than whole bacteria and the

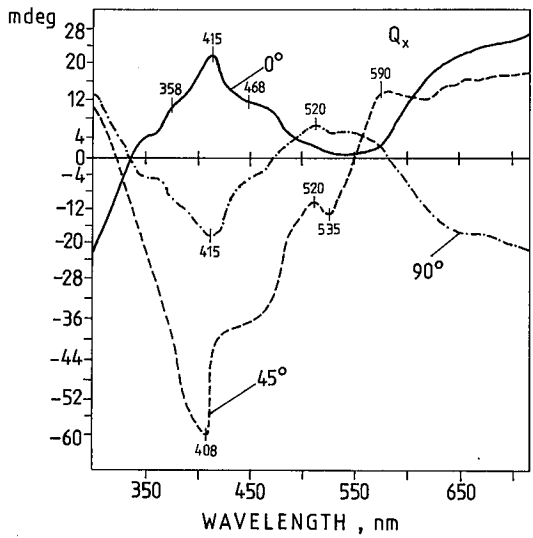

Fig. 6. CD spectra of bacterial fragments in stretched PVA (notation as in Fig. 5).

CD signal recalculated on the unit of absorption is also much higher. The shapes and signs of the CD spectra of fragments differ from that of whole bacteria, which suggests a different orientation of the lamellar system in respect to the matrix axis. This is also possible in a case of various shapes of biological elements embedded in PVA. As was already mentioned, several effects contribute to the CD spectra. If we neglect for a moment the light scattering and even CDS effects, the other contributions are interwined as is described by Muller matrix [2, 14]. The measured CD signal is a ratio of ac components to dc components reaching the receiver of the CD apparatus [11]. The intensity of light related with ac and dc electrical

components are :

$$I_{dc} = e^{-A}\left\{1 + \frac{1}{2}(LD^2 + LD'^2 + CD^2)\right.$$

$$\left. + J_0(\delta_0)\left[-LD + \frac{1}{2}(-LB' \times CD + CB \times LD')\right]\right\},$$

$$\langle I_\omega \rangle = \frac{4}{\pi} J_1(\delta_0) e^{-A}\left[CD + \frac{1}{2}(LB \times LD' - LB' \times LD)\right],$$

where:
$A = (\ln 10)(E_{0°} + E_{90°})/2$,
$LD = (\ln 10)(E_{90°} - E_{0°})/2$,
$LD' = (\ln 10)(E_{45°} - E_{135°})/2$,
$LB = 2\pi(n_{90°} - n_{0°})l/\lambda_{vac}$,
$LB' = 2\pi(n_{45°} - n_{135°})l/\lambda_{vac}$,
$CD = (\ln 10)(E_L - E_R)/2 = (\ln 10) \times \Delta E/2$,
$CB = 2\pi(n_L - n_R)l/\lambda_{vac}$,
l — path length,
J — Bessel function.

As a result of the uniaxial orientation of the long chain of polymer in the direction of stretching, the LB of the film has to be much higher than LB'. Therefore the $LB \times LD'$ has to be larger than $LB' \times LD$ at a similar orientation order of absorbing pigments in both 0° and 45° film positions. We can neglect in first approximation terms with LB' and CB. Values of LD, LD' and CD (rea) (contribution from the real circular dichroism of measured systems) are much smaller than one. Therefore, squares of these quantities are much smaller than the first powers. As a result, the $(-LD)$ is important in I_{dc} and the CD(rea) is important in I_{ac}. LD, LB, LD' and LB' are changing their signs with the 90° rotation of the film. At the positions of 0° and 90°, $LB \times LD'$ is large in the case of pigmented samples, whereas at the 45° and 135° positions the following terms have to be considered: CD, $(-LB' \times CD)$, CD^2, LD'^2 and $CB \times LD'$.

The second and the last terms can only be responsible for the sign change of the measured CD following sample rotation. The CB of the film has not vanished, since there is some contribution to the CD signal by the unpigmented film which is independent of film rotation. Also the biological matrix to which pigments are attached have some CB. However, at this stage of investigation contributions to LB and LB' from this biological matrix can not be excluded. Especially the LB of this part of the sample seems to be important, because it has large value which is changing the sign with the rotation of the sample at 45° and 135°. This suggests a large $LB \times LD'$ term.

In this discussion we have formally neglected the CDS effects, but they are present in the CD terms of our formula together with intrinsic and textural CD contributions from the photosynthetic pigments.

The presented results show that the CD apparatus can be used as a sensitive tool to investigate the organization of pigments in biological samples oriented by

stretched polymer matrix. The LD of such a sample is rather low. Therefore, LD measurements are not sensitive enough for the investigation of the small differences or changes in pigment organization due to some physiological or physicochemical processes. By using the proper location of anisotropic film it is possible to trace predominantly the changes occurring in the structure of photosynthetic apparatus. The further investigations of the simpler model system will possibly provide more information about molecular processes responsible for the CD signal formation. But even at this stage of work it is possible to use this simple method in investigations of the changes in photosynthetic apparatus texture due to some physical or chemical perturbations.

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