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QUARTERLY

Incorporation of stilbazolium merocyanines into resting and stimulated mononuclear leukocytes*

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Stimulated and resting mononuclear leukocytes were incubated with a stilbazolium merocyanine dye 1-(6'-hydroxyhexyl)-4-[(4-oxocyclohexa-2,5-dienylidene)ethylidene]-1,4-dihydropyridine and immobilized in isotropic and stretched polyvinyl alcohol film. Polarized absorption, fluorescence and fluorescence excitation spectra were collected and the anisotropy of absorption and emission were calculated. Analysis of the spectra pointed to: i. the occurrence of perturbation of the membrane structure by incubation with the dye, and ii. influence of the blood serum addition, during the process of incubation with the dye, on the efficiency of incorporation of merocyanine into the cells and the degree of the dye orientation in the membrane. A small fraction of the dye molecules introduced into resting cells was found oriented to a higher degree than a large fraction incorporated into stimulated cells. The incubation time longer than 15 min caused strong changes in the membrane structure both of the resting and stimulated cells.

Previous results [1–8] suggest that stilbazolium merocyanines are promising dyes for application in photodynamic therapy. We have previously investigated the spectral properties of these dyes located in several different model matrices such as liquid crystal cells [7], polymer films [3, 8] and photosynthetic bacterial cells [6].

In this paper we compare the incorporation of the stilbazolium merocyanine 1-(6'-hydroxyhexyl)-4-[(4-oxocyclohexa-2,5-dienylidene)-ehylidene]-1,4-dihydropyridine (Fig. 1), referred to as merocyanine, into resting and stimulated mononuclear cells. It has previously been shown [6] that this dye can be efficiently incorporated into bacterial membranes.

The samples were immobilized in polyvinyl alcohol (PVA) film [9]. Polarized absorption and fluorescence spectra of anisotropic

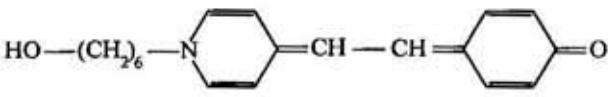

Fig. 1. Scheme of used stilbazolium merocyanine.

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(stretched) films using polarized light were then investigated to establish the orientation of the dye in membranes.

MATERIALS AND METHODS

Merocyanine was synthesized and characterized as described earlier [1, 5, 10].

Stimulated and resting mononuclear cells were prepared as follows.

Freshly drawn samples of heparinized venous blood from normal donors were centrifuged at 350 × *g* for 10 min. The buffy coats of the mononuclear cells were then removed, the samples diluted (1:4, v/v) with Hanks solution (Polfa, Poland) and centrifuged at 400 × *g* at 4°C for 30 min in a Gradisol (Polfa, Poland) gradient, according to Bøyum [11]: The mononuclear cells were collected from the interphase, washed three times with an isotonic salt solution (0.9% NaCl solution) and adjusted to a concentration of 8×10^6/ml in an NaCl solution containing 2% autologous serum. The cells were stimulated with phytohemagglutinin (PHA, HA 17, Wellcome, England, 10 μg/ml) for 1 h at 37°C [12], washed twice more with the NaCl solution, and then resuspended in an NaCl solution (concentration 36×10^7 cells/ml) containing 2% autologous serum. The unstimulated cells were prepared in a similar manner but were not stimulated by PHA. Next, 1 μl of 0.01 M ethanol merocyanine solution was added to samples of both stimulated and resting cells which contained 10^6 cells suspended in a volume of about 5 ml. Reference samples were incubated without merocyanine. The incubation (15 min, 37°C) was done in the dark. After incubation the cells were washed three times with an isotonic salt solution and immobilized in PVA film, so that the serum was not present during the last step.

Our preliminary experiments showed that the addition of a small amount of blood serum improves efficiency of incorporation of the dye and diminishes the perturbation (change in the structure which is reflected in polarized spectra) that the membrane structure undergoes during cell incubation. It is possible that the serum causes a decrease of the dye aggregation, therefore 2% of serum was added to part of the samples.

The absorption and fluorescence spectra were measured with a Zeiss Specord M 40 and with a home made arrangement, respectively. Both instruments were equipped with polarizers and holders used for precise arrangement of a sample during the measurments of polarized spectra. The instruments were connected to a computer for data processing. The fluorescence measurements were performed with the samples incubated at a lower concentration of the dye (0.01 M) in order to avoid the effect of merocyanine aggregation [3]. Absorption spectra are influenced by aggregation to a lower extent. Higher concentrations (0.03 M) have to be used for absorption measurments, because of overlapping of the bands from the dye and from mononuclear cells.

All spectra were very well reproducible when leukocytes from the same donor were used. The measurments were repeated for three sets of samples obtained from different donors. The results were qualitatively similar but quantitatively not identical. Therefore only the results obtained with one set of samples are shown as the example of observed changes.

RESULTS AND DISCUSSION

The absorption maximum at about 400 nm, observed in merocyanine in PVA spectrum (Fig. 2a) corresponds to the protonated form of the dye. The second, a small one, located at 490–550 nm is related to free base form [3]. Interpretation concerning the incorporation of the dye to the cells has to be done very carefully because the unpigmented mononuclear cells also exhibit absorption in 400 nm spectral region (Fig. 2b). The absorption band of the free base form of the dye is very weak in the spectra of the resting and stimulated cells incubated with merocyanine in the presence of serum and immobilized in PVA (Fig. 3). This is unfortunate, because this band is not overlapped with the cell's absorption band. It is much easier to measure the fluorescence of the pigmented leukocytes because the dye fluorescence bands only slightly overlap the long wavelength edge of the cell's emission (Fig. 2).

As can be seen from Fig. 3, the amount of the dye incorporated in the presence of serum into stimulated cells was higher than into resting leukocytes. For the samples incubated without

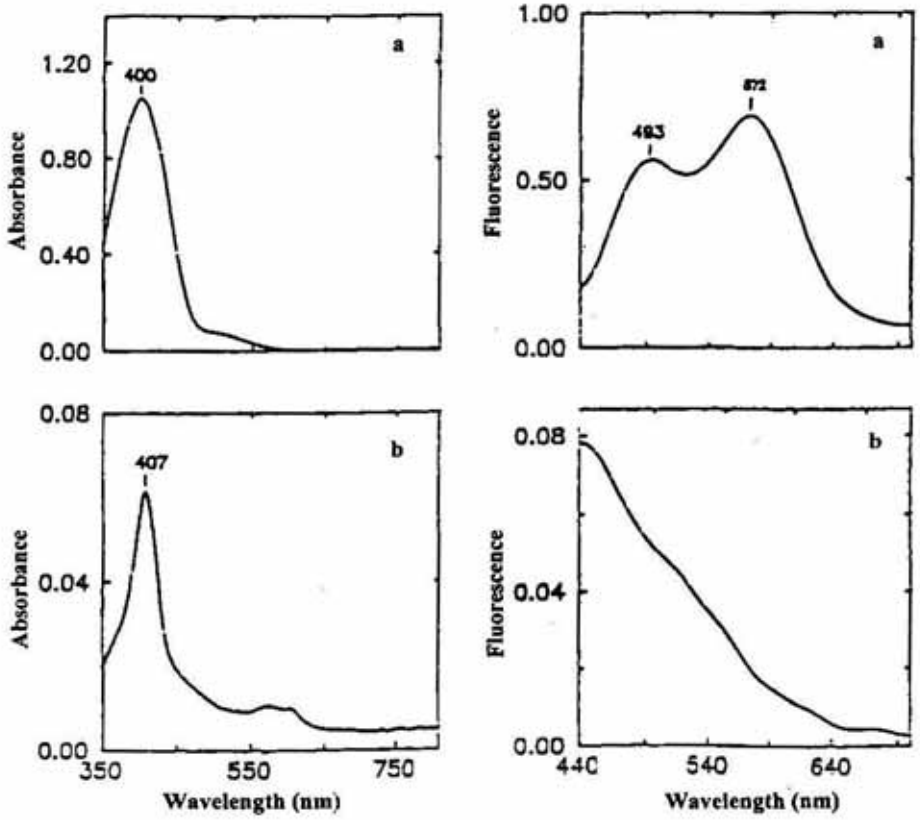
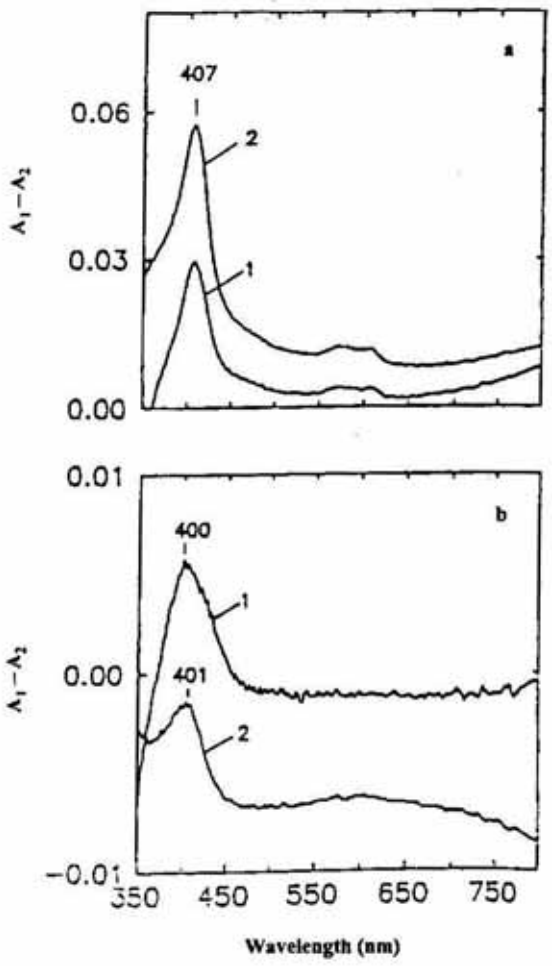


Fig. 2. Absorption and fluorescence (λ_{exc} = 400 nm) spectra of merocyanine in PVA (a) and spectra of unpigmented resting mononuclear leukocytes in PVA (b).



serum (Fig. 3b), the situation was reversed. The pattern of the difference spectrum in the region of low dye absorption (550–750 nm) is due for most part to a difference in light scattering between pigmented and unpigmented cells immobilized in PVA film. This could be caused by changes in the shape or dimensions of the scattering particles, and/or by a modification of some features of the cell surface. The arrangement of the molecules in the cell membranes can also affect the efficiency of light scattering. In both experiments, in the presence and absence of blood serum (Fig. 3a, b), the values of the difference between absorption of pigmented and unpigmented cells ($A_1 - A_2$) for resting samples (Fig. 3, curve 1) are positive and in the long wavelength region are close to zero. The absorption of the pigmented cells stimulated without serum added (Fig. 3b, curve 2) is lower than that of the unpigmented leukocytes (the difference spectrum is negative). For stimulated samples with serum — this dif-

Fig. 3. The difference absorption spectra of resting (1) and stimulated (2) leukocytes in unstretched PVA.

The results are expressed as the difference between the absorption of pigmented cells, A_1; and that of unpigmented cells, A_2; a, incubation with serum; b, incubation without serum.

ference is positive. This means that in the cases of stimulated sample the incubation with serum causes an increase in light scattering.

The information about the orientation of the dye embedded in the membrane and about the change in the arrangement of membrane molecules was obtained from polarized absorption spectra of leukocytes in the stretched PVA films (Fig. 4).

For anisotropic samples (Fig. 4) values of the $(A_1 - A_2)$ difference for resting and stimulated cells incubated with serum are negative.

A comparison of Fig. 3 with Fig. 4 strongly suggests that film stretching leads to changes in the membrane structure or/and the shapes of cells. The differences between the parallel and the perpendicular polarized components of the absorption are similar for both resting and stimulated cells. This suggests that stretching has a comparable influence on both types of cells. Therefore, the incorporation and orientation of dye molecules in the two sets of samples can be compared.

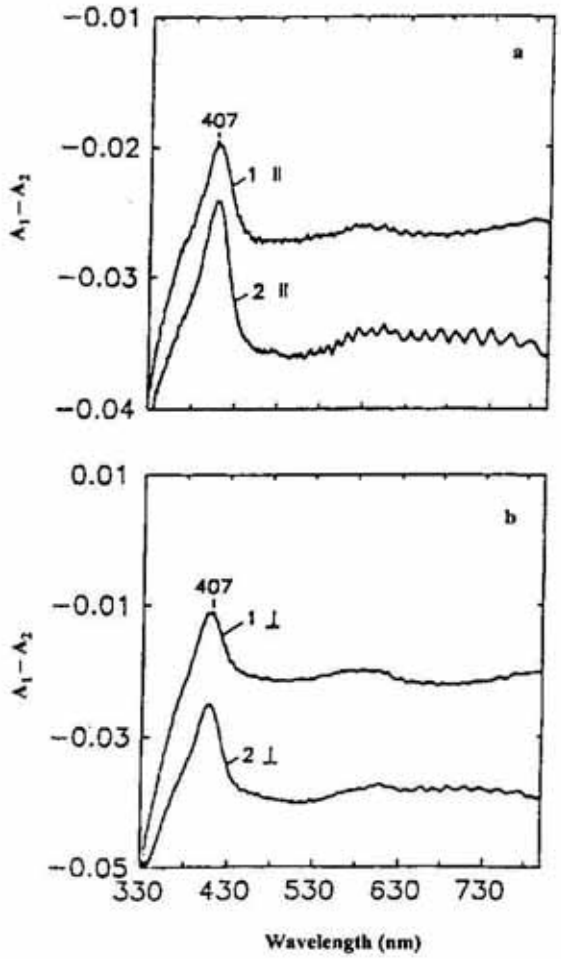


Fig. 4. Polarized difference absorption spectra of streched samples incubated with serum added (A_1 and A_2 as in Fig. 3).

1, Resting cells; 2, stimulated cells; a, incubation with serum; b, incubation without serum. Polarization of components is marked on the graphs.

From the polarized absorption spectra data the absorption anisotropy parameter s was calculated (Table 1); it describes the degree to which the absorbing molecules are oriented with respect to a given axis.

The difference in s between resting (s_r) and stimulated (s_s) unpigmented cells is negative. This means that in the resting cells the membrane molecules are almost randomly distributed around the stretching axis whereas in the stimulated cells they have some tendency to be uniaxially oriented with respect to the film stretching direction.

For pigmented cells the difference ($s_r - s_s$) is positive showing that the degree of dye orientation in the case of resting cells is higher than in the case of stimulated leukocytes. It is also higher in a region of the basic merocyanine form absorption (580 nm) than in that of the protonated dye form (400 nm).

More definite information about the orientation of incorporated dye molecules was obtained from the same parameter calculated from the difference between the absorption components $(A_1 - A_2)$ shown in Fig. 4.

The orientation parameters obtained for whole absorption of pigmented cells depend on the superposition of the orientations of all the molecules of both the membrane components and the incorporated dye molecules. The

Table 1

Difference in absorption anisotropy between resting (r) and stimulated (s) cells.

$$s = \frac{A_{\parallel} - A_{\perp}}{A_{\parallel} + 2A_{\perp}}$$

Sample	γ (nm)	Difference in absorption anisotropy $(s_r - s_s)$
Unpigmented cells	410	-0.018 ± 0.005
	580	-0.027 ± 0.005
Pigmented cells	410	0.034 ± 0.005
	580	0.048 ± 0.005
Dye in the cells from $(A_1 - A_2)$ spectra	410	0.307 ± 0.005
	580	0.188 ± 0.005

coefficient of orientation s calculated from (A_1–A_2) is predominantly dependend on the dye orientation. Data in Fig. 4 and Table 1 show that the orientation of the dye molecules is higher in the resting cells than in the stimulated cells. The positive s values show a preferential orientation of the absorption transition moments of the molecules along a direction close to the film stretching axis. Negative s values point to preferential orientation under large angle with respect to film axis. Without taking photographs of the cells in stretched film we cannot prove that the deformation of their shape was not caused by PVA stretching [13]. This deformation might be not identical for resting and stimulated cells but in a first approximation it could be assumed similar. Therefore the differences between the order parameters for the resting and the stimulated cells would be caused by a different molecular arrangement in the membranes rather than by the difference in a cell deformation. When the order parameter is calculated from difference spectra (pigmented minus unpigmented) a large part of the contributions from the membrane molecules is eliminated and the s value of the resting cells is positive and reaches very high values up to 30% at the maximum of dye absorption (410 nm) (Table 1). The fact that the stimulated cells exhibit low values of s suggests that the degree of orientation of the dye is much lower in this case. The high value of the degree of orientation in the region of dye absorption in the case of the resting cells shows that the dye is to a higher degree oriented in the resting cells than in the stimulated cells. The positive value of s means that the dye molecules must be oriented at small angles with respect to film axis. Although the amount of the dye introduced into the resting cells is lower than that introduced into the stimulated cells, the dye molecules are oriented to a higher degree. The orientations of both the membrane molecules and the introduced dye differ strongly between stimulated and resting leukocytes (Table 1). The anisotropy of unpigmented samples should be systematically investigated because of the strong influence of the membrane structure on photodynamic therapy. On comparing wavelength dependencies of s with the absorption of the dye (Fig. 2) one can clearly see that the rather small fraction of the dye introduced into resting cells is highly oriented, while a larger one incorporated into stimulated leukocytes exhibites a lower anisotropy.

Comparison of the fluorescence spectra of resting and stimulated cells incubated with serum and incorporated in isotropic and anisotropic films (Fig. 5) measured in natural light shows that fluorescence intensity of the stimulated cells was higher than that of the resting cells. This is not surprising since the concentration of the incorporated dye was found higher in the stimulated cells than in the resting cells (cf. Fig. 3).

Fluorescence intensity of resting and stimulated leukocytes incubated without serum (Fig. 6) can also reflect the difference in the merocyanine concentration between the two physiological cell states. The width of the fluorescence spectra suggest different aggregation of the introduced dye in these two states of the cells, or differences in its interactions with the macromolecules. The same dye in an isotropic

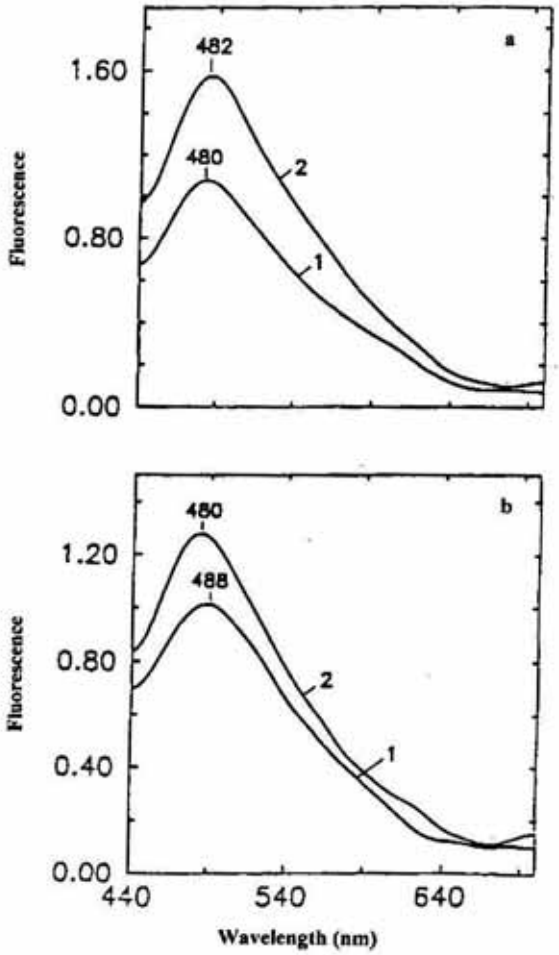


Fig. 5. Fluorescence spectra (λ_{exc} = 400 nm) of cells incubated with serum measured in natural light.
a, Isotropic film (0% stretching); b, anisotropic sample (300% elongation). 1, Resting cells; 2, stimulated cells.

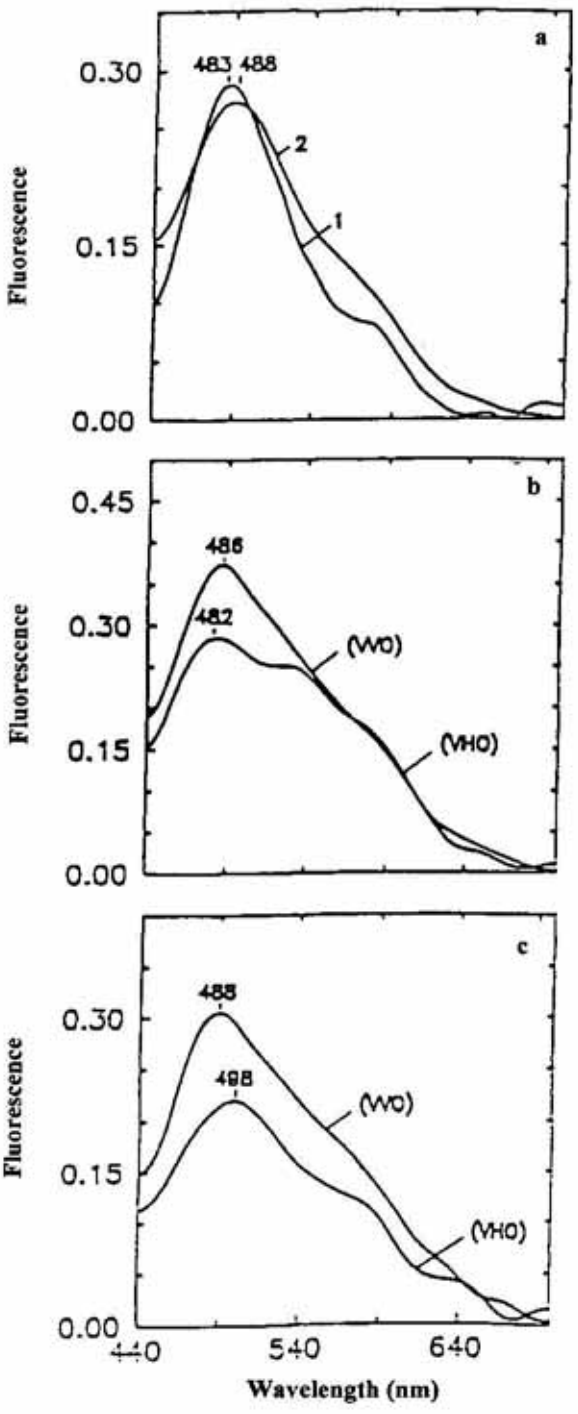


Fig. 6. Fluorescence spectra (λ_{exc} = 400 nm) of leukocytes in PVA.
Sample incubated without serum. a, Unstretched sample, natural light; curve 1, resting cells; 2, stimulated cells; b, and c, stretched samples: b, resting cells; c, stimulated cells. All the polarized fluorescence spectra are marked as previoulsy [3]: the first and last letters in the parentheses refer to the polarization of the exciting and the fluorescence light beams, respectively, and the middle letter refers to the position of the streching axis of the polymer matrix: V, vertical; H, horizontal; O, natural light or the unstreched sample. Polarization of components are marked on the graphs.

polymer film exhibited only one fluorescence band in the 570–580 nm range, whereas in stretched polymer two bands of emission were observed [3]. The perpendicular component of fluorescence was almost identical to the emission of the isotropic sample, whilst the parallel component exhibited two maxima of comparable intensity, one at 600 nm and another at 560 nm. In the present system, the observed emission is a superposition of these two bands, and can be related to various aggregated forms of the dye in the ground or excited state [3].

Polarized fluorescence spectra of resting and stimulated cells in stretched PVA film (Fig. 6 b, c) were obtained by photoselection using polarized light (this means that polarizer was located only in the excitation beam and all the emission was collected at the receiver). All the polarized fluorescence spectra in Figs. 6 and 7 are marked as previously [3]. The polarized fluorescence component VVO and VHO of resting and stimulated cells (Fig. 6 b and c) exhibit different shapes. The emission of the perpendicular polarized components (VHO) in the 540–640 nm region in which emission of aggregated dye appears [3], was especially high with respect to the main maximum. As it was shown previously [3], the monomers and aggregates of merocyanines in model membranes are oriented differently. Figure 6 suggests that a similar effect also occurs in leukocytes.

The coefficient of the anisotropy of emission, r_0 (Table 2) was calculated from the polarized fluorescence data for resting cells incubated for 15 min with and without serum added. The addition of serum had but little influence on the degree of emission anisotropy of the dye: r_0 was positive and equal to from 0.15 to 0.19 ± 0.005 over the whole region of merocyanine emission (440–640 nm, not shown).

Pajor *et al.* [14] reported a similar value of emission anisotropy for stimulated lymphocytes. The degree of emission anisotropy was dependent not only on the degree of dye orientation but also on the microviscosity which is known to influence the rotational depolarization of fluorescence. In their experiments the values of the fluorescence anisotropy (r_0) were lower than the absorption anisotropy (s) which could suggest some influence of depolarizing agents on the observed r_0 values.

The anisotropy of emission is comparable in the vicinity of both merocyanine maxima (at

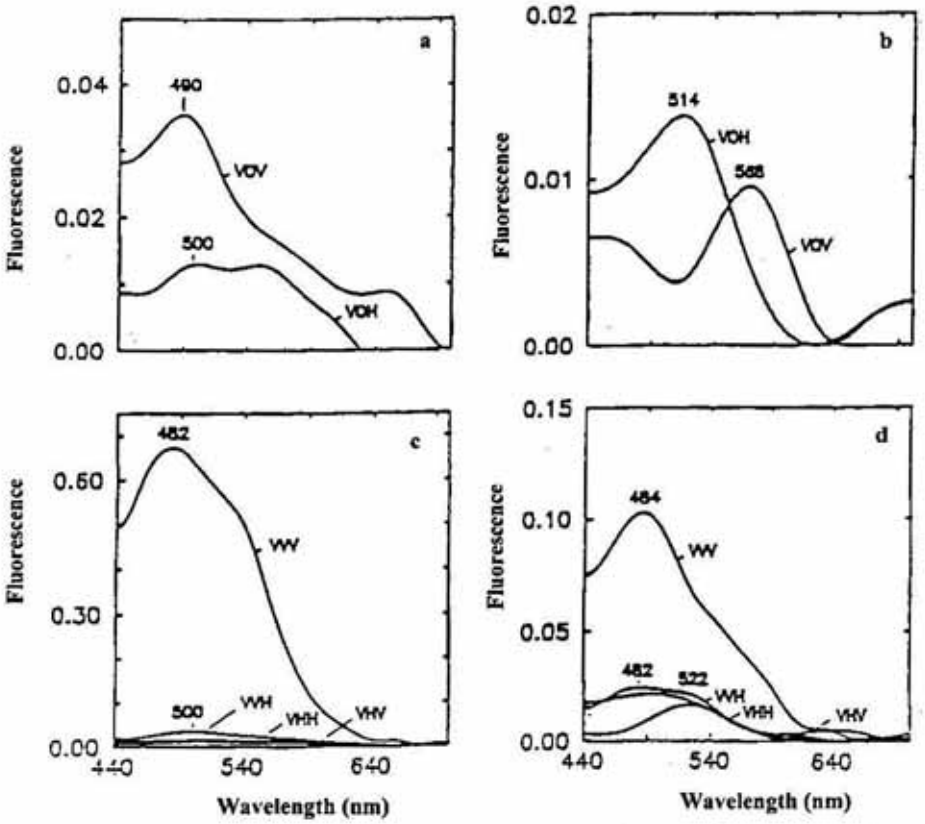


Fig. 7. Polarized fluorescence spectra (λ_{exc} = 400 nm) of leukocytes in PVA. a, and b, Unstretched films; a, resting cells; b, stimulated cells; c, and d, stretched films; c, resting cells; d, sti- mulated cells. Pola- rization of components is marked on the graphs as explained for Fig. 6.

490 nm and at 570 nm) which does not seem to be in agreement with the spectral dependence of the values of s (Table 1). The anisotropies obtained from absorption and emission spectra for samples containing several aggregates of dye, are identical only when all forms of the dye molecules give similar fluorescence yields. An increase in the time of incubation with merocyanine over 15 min caused a decrease in the anisotropy of emission in the region of merocyanine fluorescence. The value of r_o for an incubation time of 30 min was about 1% at 440 nm. The long incubation time also changed markedly the emission anisotropy spectrum. It seems that the incubation time longer than 15 min should be avoided because it introduces large changes in the membrane structure of both the resting and stimulated cells.

Polarized spectra of leukocytes in unstretched PVA films measured with polarizers in the exciting and the fluorescence beams (Fig. 7) exhibited for both the resting and stimulated cells different shapes and intensities of the fluorescence components VOV and VOH (cf. Fig. 7a and b). The degree of orientation of the fluorescent molecules must be very high as in stretched film a dramatic difference between the VVV and VHV components was observed (Fig. 7c and d). This difference shows that the emitted light is highly polarised and that depolarisation of fluorescence in both types of cell is low. This means that the microviscosity must

Table 2

Fluorescence anisotropy of leukocytes in streched PVA film.

$$r_o = \frac{F(VVO) - F(VHO)}{F(VV) + 2F(VHO)};\quad r_c = \frac{F(VVO) - F(VHO)}{F(VVV) + 2F(VHV)};\quad r_d = \frac{F(VVH) - F(VHH)}{F(VVH) + 2F(VHH)}$$

Sample		λ (nm)	r_o	r_c	r_d
Resting cells with dye	with serum	490–590	0.156	0.851	0.000
	without serum		0.150	–	–
Stimulated cells with dye	with serum	490–590	–	0.620	0.187

Accuracy of emission anisotropy: ± 0.005.

be high. From Table 2 it follows that the anisotropy r_c of the resting cells is higher than that of the stimulated cells, but both are high. The fluorescence observed in polarization perpendicular to excitation, r_d is practically unpolarized and independent of the film axis position. It is about nul in the case of the resting cells, whereas in the case of less oriented stimulated cells it equals about 0.19 ± 0.005 in the region of 490 nm fluorescence and decreases at longer wavelengths.

The presented results show that the investigated dye can be a promising candidate for application in photodynamic therapy because it is incorporated into resting and stimulated cells with different efficiency. This selective incorporation is observed only in the presence of blood serum in the incubated sample. The time of incubation has to be carefully adjusted because on longer incubation the structure of the membranes of both resting and stimulated cells becomes perturbed.

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