JOURNAL OF BIOMEDICAL OPTICS 2(2), 186–194 (APRIL 1997)

2′,7′-BIS-(CARBOXYETHYL)-5-(6′)-CARBOXYFLUORESCEIN (BCECF) AS A PROBE FOR INTRACELLULAR FLUORESCENCE POLARIZATION MEASUREMENTS

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(Paper JBO-091 received May 10, 1996; revised manuscript received Aug. 26, 1996; accepted for publication Sep. 5, 1996.)

ABSTRACT

The utility of 2′,7′-bis-(carboxyethyl)-5-(6′)-carboxyfluorescein (BCECF) for the execution of the structuredness of the cytoplasmic matrix (SCM) measurement for lymphocyte activation is investigated. Cells were incubated with BCECF/AM [2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethylester], a nonfluorescent lipophilic acetoxymethylester that readily enters cells and is enzymatically hydrolyzed to fluorescent BCECF once inside. Leakage of BCECF out of cells is negligible in comparison to that observed with fluorescein, greatly reducing one source of background fluorescence. However, spontaneous hydrolysis of BCECF/AM in aqueous solution does contribute significant background fluorescence, which can be minimized by staining at relatively high concentrations of cells and subsequent dilution. As is the case with fluorescein, the polarization spectrum of intracellular BCECF shows a wavelength dependence not seen in the spectrum of the dye in homogeneous media of various viscosities. The more pronounced wavelength dependence of the polarization observed with BCECF compared with fluorescein suggests that BCECF might be preferable to fluorescein as a marker for the SCM test.

Keywords BCECF; fluorescence; polarization; cancer test.

1 INTRODUCTION

The structuredness of the cytoplasmic matrix (SCM) test for cancer was devised by Cercek, Cercek and Franklin in 1974.[1] The test detects differences between individuals with and without cancer by patterns of early lymphocyte activation following stimulation with polyclonal mitogens such as phytohemagglutinin (PHA) and tumor antigen extracts (TAE). The measure of activation is a change in the polarization of the fluorescence of fluorescein produced intracellularly by enzymatic hydrolysis of fluorescein diacetate (FDA).[2] While the test has yielded variable results in the hands of different investigators,[3–7] there have been enough published confirmations of the Cerceks' original work to suggest that the effect is real[8–17] and might reflect malignancy-associated changes.[18] The validity of the SCM method has been confirmed in our laboratory for about a thousand cases.[19–24]

In addition to the diagnostic aspect of the SCM phenomenon, one striking fact is that the intracellular fluorescein fluorescence polarization (IFFP) depends on the emission wavelength.[25] Such wavelength dependence is uncommon for fluorescent molecules in homogeneous solutions. Fluorescence emission normally takes place from the lowest excited electronic level. Therefore, it is not clear why the direction of the emission dipole varies with the emission wavelength, which is determined by the particular vibronic transition associated with the return of the molecule to its electronic ground state. After examining the Cercek phenomenon, four major findings were reported:[19–24]

1. The intracellular fluorescence polarization of fluorescein is indeed dependent on the emission wavelength.
2. This wavelength dependence is not found in every lymphocyte population.
3. The wavelength dependence can be changed or eliminated by an adequate biological stimulation of the lymphocyte population.
4. In many cases, it has been found that the SCM test distinguishes between lymphocytes from healthy donors and those from patients with a malignant disease.

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Assuming that the wavelength dependence of the polarization would not be confined to a single compound, and in order not to deviate excessively from the original compound (FDA), closely related compounds like carboxyfluorescein diacetate (CFDA) and 2′,7′-bis-(carboxyethyl)-5-(6′)-carboxyfluorescein acetoxymethylester (BCECF/AM) were chosen to study the phenomenon. As is the case with FDA, these nonfluorescent, lipophilic esters readily enter cells and are enzymatically hydrolyzed to fluorescent materials. BCECF is a well-known and widely used fluorescent marker for intracellular pH,[26] and for determining cell membrane characteristics.[27] Absorption and emission spectra of BCECF have also been studied under various conditions[28] and were used in Ca^{2+} transport studies.[29]

BCECF/AM and CFDA have a potential technical advantage over FDA, since their leakage is much less pronounced than that of fluorescein. This may be due to differences in their charge. At physiologic pH, BCECF holds 4 to 5 negative surplus charges and fluorescein only 1.

The fluorescence polarization properties of intracellular BCECF and the implications for their use in SCM measurements are reported. Clinical results obtained from SCM tests using this probe will be published in a later paper.

2 MATERIALS AND METHODS

2.1 PREPARATION OF SOLUTIONS

The preparation of the FDA staining solution, stimulants [PHA and encephalitogenic factor (EF) solutions], modified phosphate buffer saline (PBS), and modified Ficoll-metrozoate gradient cell separation solution; the procedures of cell separation, cell stimulation, and cell suspension filtration were all carried out as described previously.[21]

Ten micromolar stock BCECF/AM (Molecular Probes Inc., USA) solution was prepared as follows: 1 mg BCECF/AM was dissolved in 1.1 ml ethanol (99.5%). This solution was stored at −20 °C. Ten microliters of this solution were then added to 1 ml of PBS to give a concentration of 10 μM BCECF/AM. BCECF/AM in PBS solution is unstable and is hydrolyzed readily at room temperature; therefore, it is stored on ice when not in use. In order to obtain a 1-μM BCECF/AM solution for staining, the stock solution was diluted in PBS at a volume ratio of 1:10.

2.2 CELL STAINING

With BCECF/AM, two staining methods were used for two different purposes: *Method a:* For studying the staining process itself, by measuring the development in time of the fluorescence intensity in the cells, aliquots of 0.1 ml of 10^7 lymphocytes/ml were added to 3.5 ml of 1 μM of the staining solution. *Method b:* For studying the excitation and emission polarization spectra, where staining must be discontinued before the measurement, and minimum background fluorescence of BCECF (in the suspending solvent) is required, a 10-μM BCECF/AM solution was added to a cell suspension containing 2.0×10^7 cells/ml at a ratio of 1:5. After 15 min of staining at room temperature, the suspension of stained cells was diluted 24 times by adding modified PBS to eliminate further cell staining. In this way, background fluorescence due to spontaneous hydrolysis was reduced to a negligible level.

2.3 FLUORESCENCE MEASUREMENTS

Three spectrofluorimeters were used for the fluorimetric measurements:

1. A Perkin-Elmer MPF-44 with a polarization measurement setup as described previously.[21]
2. An SLM-4800 spectrofluorimeter. This apparatus permits the simultaneous measurement of the p- and s-polarized fluorescence. The G-factor of the SLM detection grating was electronically set to 1 (explained later).
3. A Perkin-Elmer MPF-66 with a polarization accessory. This is used for polarization excitation and polarization emission spectra measurements.

All measurements were carried out at 23 °C. For the determination of the background fluorescence, cells were removed from the solution by filtration with a 0.45-μm Millipore filter made from a mixture of cellulose acetate and nitrate (TAMAR, Jerusalem, Israel). The fluorescence intensity of the filtrate was measured using the same meticulously washed (under a strong flow of hot tap water, followed by three saline rinsings) cuvette placed in the same position as that used for the cell suspension measurements.

2.4 CELL DISRUPTION

Cells and their organelles were disrupted by sonication with a Soniprep 150 (MSE, England) apparatus for 90 s in a 3.5-ml volume cuvette. After sonication, no whole cells or their fractions could be observed using light microscopy. In order to prevent the rise in temperature caused by sonication, the cuvette containing the cells was maintained in cold water during the sonication procedure.

3 RESULTS AND DISCUSSION

3.1 ESTABLISHMENT OF THE STAINING PROCEDURE

The kinetics of the hydrolysis of BCECF/AM in cells and the spontaneous hydrolysis of BCECF/AM in PBS were examined and the results are shown in Figure 1. Two optically identical 1-cm quartz cuvettes were filled with 3.5 ml of a 1 μM BCECF/AM solution in PBS and kept at a temperature of 23 °C for 180 s. This time period is indicated

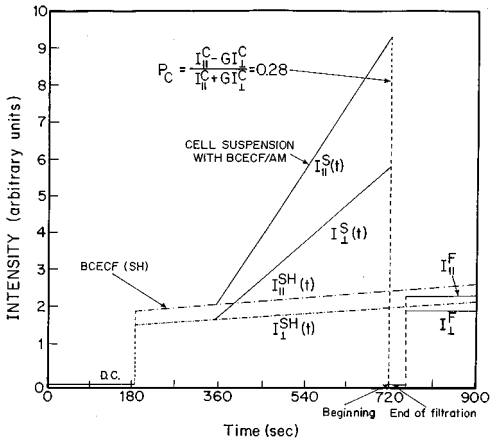

Fig. 1 Spontaneous and intracellular hydrolysis of BCECF/AM. Broken lines: Parallel ($I^{SH}_{\parallel}$) and perpendicular ($I^{SH}_{\perp}$) components of the fluorescence polarization of spontaneously hydrolyzed (SH) BCECF/AM in PBS. Full lines: Parallel ($I^{S}_{\parallel}$) and perpendicular ($I^{S}_{\perp}$) components of the fluorescence polarization of BCECF in cell suspension(s). $I^{F}_{\parallel}$ and $I^{F}_{\perp}$ are the intensities of the parallel and perpendicular polarized components of the BCECF fluorescence in the filtrate. Measurements were carried out on the PE-MPF-44 spectrofluorimeter.

by the D.C. (dark current) signal in Fig. 1. One of the cuvettes was then transferred into the thermostatted cuvette holder of the SLM 4800 and the intensities $I^{SH}_{\parallel}$ and $I^{SH}_{\perp}$ of the spontaneously hydrolyzed (SH) BCECF fluorescence polarization were measured.

The two cuvettes were then alternately measured at intervals of 30 s between time points of 180 and 360 s. Their intensities were identical.

At 360 s, 0.1 ml of 10^7 cells/ml were rapidly injected into one of the cuvettes. From this point on, the fluorescence intensity of the cell suspension(s) (first cuvette) steeply increased due to the staining of the cells (lines $I^{S}_{\parallel}$ and $I^{S}_{\perp}$), while the intensity of the spontaneously hydrolyzed BCECF (second cuvette) continued to increase slowly (lines $I^{SH}_{\parallel}$ and $I^{SH}_{\perp}$).

For the evaluation of the fluorescence background due to possible leakage of BCECF from the cells (or any background radiation due to BCECF in solution), the cells were quickly filtered out of the suspension (at 720 s) and the filtrate (F) fluorescence components $I^{F}_{\parallel}$ and $I^{F}_{\perp}$ were measured in the same meticulously cleaned cuvette.

It was also found that the filter is impermeable to BCECF/AM and almost totally permeable to BCECF, leaving only a minute residue, which is adsorbed by it. This finding is illustrated by the fact that the $I^{F}_{\parallel}$ and $I^{F}_{\perp}$ lines are horizontal, and immediately after filtration these lines are a bit lower than the $I^{SH}_{\parallel}$ and $I^{SH}_{\perp}$ lines, respectively (right side of Figure 1).

The intracellular BCECF fluorescence polarization P_c is then calculated from the relationship $P_c=(I^c_{\parallel}-GI^c_{\perp})/(I^c_{\parallel}+GI^c_{\perp})$, where the fluorescence intensities $I^c_{\parallel}$ and $I^c_{\perp}$ emitted from the cells are obtained by subtracting the filtrate intensities from those of the cell suspension and extrapolating to the half-time point of the filtration process. G is the correction factor of the emission monochromator (the reflection of unpolarized light from a grating is stronger for the normally polarized component than for the parallel polarized one). The value of $G(\lambda)$ for each wavelength is calculated from the parallel $[i_{\parallel}(\lambda)]$ and perpendicular $[i_{\perp}(\lambda)]$ polarized intensities of the unpolarized fluorescence emitted from the cell suspension, excited with horizontally polarized light, i.e., $G(\lambda)=i_{\parallel}(\lambda)/i_{\perp}(\lambda)$.

It was noted that immediately after filtration (t=720 s), the filtrate fluorescence intensity is minutely less than the original solution (the small difference is due to dye dilution caused by the introduction of the cell suspension aliquot, and to BCECF adsorption by the filter). This indicates that the background fluorescence is due to the spontaneous hydrolysis of BCECF/AM to BCECF in the suspending solution, rather than to BCECF leakage out of the cells. For the sake of clarity, the intermissions in the measurements, due to the alternations between the cuvettes, are not indicated in the figure. Figure 1, therefore, represents a schematic presentation of the real results obtained from the two cuvettes.

The fluorescence polarization of BCECF in PBS at room temperature was found to be unexpectedly high (0.1) compared with that of fluorescein (about 0.02). This is likely to be the result of two properties of the BCECF molecule. The much greater length and mass of the BCECF molecule, compared with those of fluorescein, give rise to a considerably greater moment of inertia and hence to a longer rotational relaxation time. In addition, BCECF has 4 to 5 negative charges, while fluorescein has only 1. This may create a larger cage of water molecules around the BCECF molecule, impeding its rotation.

Therefore, when measuring the cell suspension fluorescence polarization $P(\lambda_{em})$, the contribution of the substrate fluorescence polarization cannot be neglected. The overall cell suspension fluorescence polarization is given by:[30]

$$P_s=\frac{(I_cP_c)}{(I_c+I_F)}+\frac{(I_FP_F)}{(I_c+I_F)}, \qquad (1)$$

where I_c and I_F are the sum of the measured cell fluorescence intensities $I_{\parallel}$ and $I_{\perp}$, and of the substrate background in the absence of leakage, respectively, and P_c and P_F are their respective polarizations. P_F is smaller than P_c so the term on the right-hand side of Eq. (1) may be neglected for fluorescein. This term cannot be neglected for BCECF because of the particularly high spontaneously hydrolyzed BCECF fluorescence polarization.

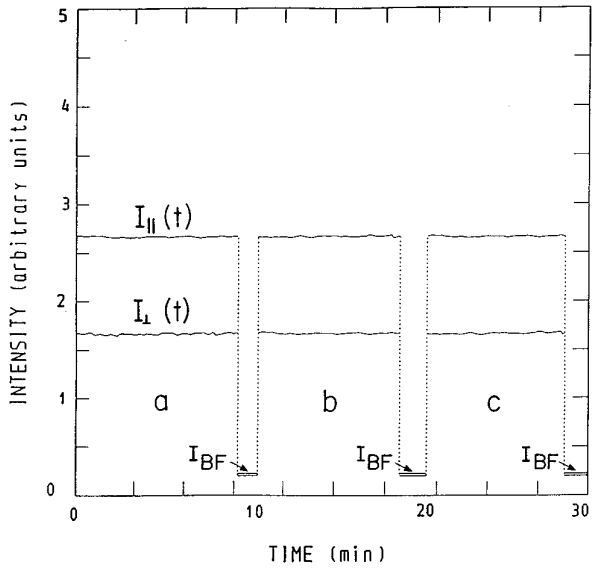

Fig. 2 Stability of fluorescence intensity and polarization of intracellular BCECF over 30 min. Three samples of cell suspension (a,b,c, parts of the same suspension stained according to method b) were sequentially measured for 9 min, filtered, and the background filtrate fluorescence (I_{BF}) measured again. Measurements were carried out on the SLM-4800 spectrofluorimeter.

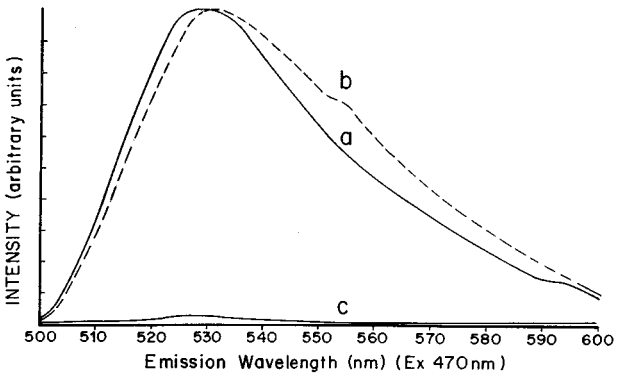

Fig. 3 Fluorescence spectrum of BCECF as measured by the PE-MPF-66 spectrofluorimeter. Curve a (full line): 5 μM BCECF in PBS solution. Curve b (dashed line): cell suspension stained according to method b with BCECF/AM. Curve c (full line): spontaneously hydrolyzed BCECF background (both duration of hydrolysis and concentration of BCECF/AM molecules were the same as those used in cell staining of curve a).

In order to overcome this problem for polarization spectra measurements, method b was used for staining (see section on cell staining).

Figure 2 shows the results of the dilution procedure for method b. These experiments were carried out to explore the possibility of performing fluorescence spectrum and polarization spectrum measurements undisturbed by background radiation, which did not vary with time. After the dilution procedure, the polarization of a 3-ml sample from a 10-ml stock suspension was measured (at 515 nm; excitation at 470 nm) for 9 min. The cells were then filtered out; the background (I_{BF}) measured and found to be negligible (Fig. 2). This same procedure was repeated for a second and third sample (3 ml) of the stock suspension. The polarization was unchanged for 30 min, which is ample time in which to carry out polarization spectrum measurements without the use of filtration.

All this does not work with fluorescein. Several investigators in the past have tried to measure the fluorescein polarization spectrum, albeit with great difficulty.[21,31] Some of them did not observe the peak behavior of the polarization as a function of the emission wavelength.[32–34]

3.2 MEASUREMENT OF THE POLARIZATION SPECTRUM OF BCECF IN LYMPHOCYTES

In Figure 3, curve a shows the spectrum of a solution of 5 μM BCECF in PBS; curve b shows the fluorescence spectrum of BCECF in lymphocytes; and curve c shows the spectrum of the spontaneously hydrolyzed BCECF in PBS (originally containing the same concentration of BCECF/AM and hydrolyzed spontaneously under the same conditions as for the cell staining).

Figure 3(b) shows an approximate 7-nm red shift of the fluorescence peak upon introducing BCECF into cells. It also shows that spontaneous hydrolysis is slow, giving an insignificant background interference with the spectrum measurement.

Figure 4 shows BCECF fluorescence polarization spectra (in cells stained according to method b) obtained by a Perkin-Elmer MPF-66 spectrofluorimeter. The degree of polarization was calculated according to the relationship

$$P(\lambda)=\frac{[I_{\parallel}(\lambda)-G(\lambda)I_{\perp}(\lambda)]}{[I_{\parallel}(\lambda)+G(\lambda)I_{\perp}(\lambda)]}. \qquad (2)$$

Figure 4 shows the results for lymphocytes from three different samples (1, 3, 5) of normal individuals. Curve a (the upper curve in each figure in the left column) shows the polarization spectrum of the untreated lymphocytes (i.e., the control values P_o); curve b shows the polarization spectrum after stimulation of the lymphocytes with PHA (P_{PHA}). The curves on the right side of the figures show the ratios P_O/P_{PHA} of the respective figures of the left column as a function of wavelength. From these curves it appears that intracellular BCECF exhibits its own unique pattern of polarization dependence on the emission wavelength.

In order to show, at least qualitatively, that the observed effect of the wavelength dependence of the polarization is independent of the measuring apparatus, the measurements shown in Figures 4(G) and 4(H) were carried out by the SLM spectrofluorimeter (sample 6). The measurements performed on BCECF in homogeneous glycerin solutions (Figure 5) should be enough proof for the initiation of the observed effect. The polarization

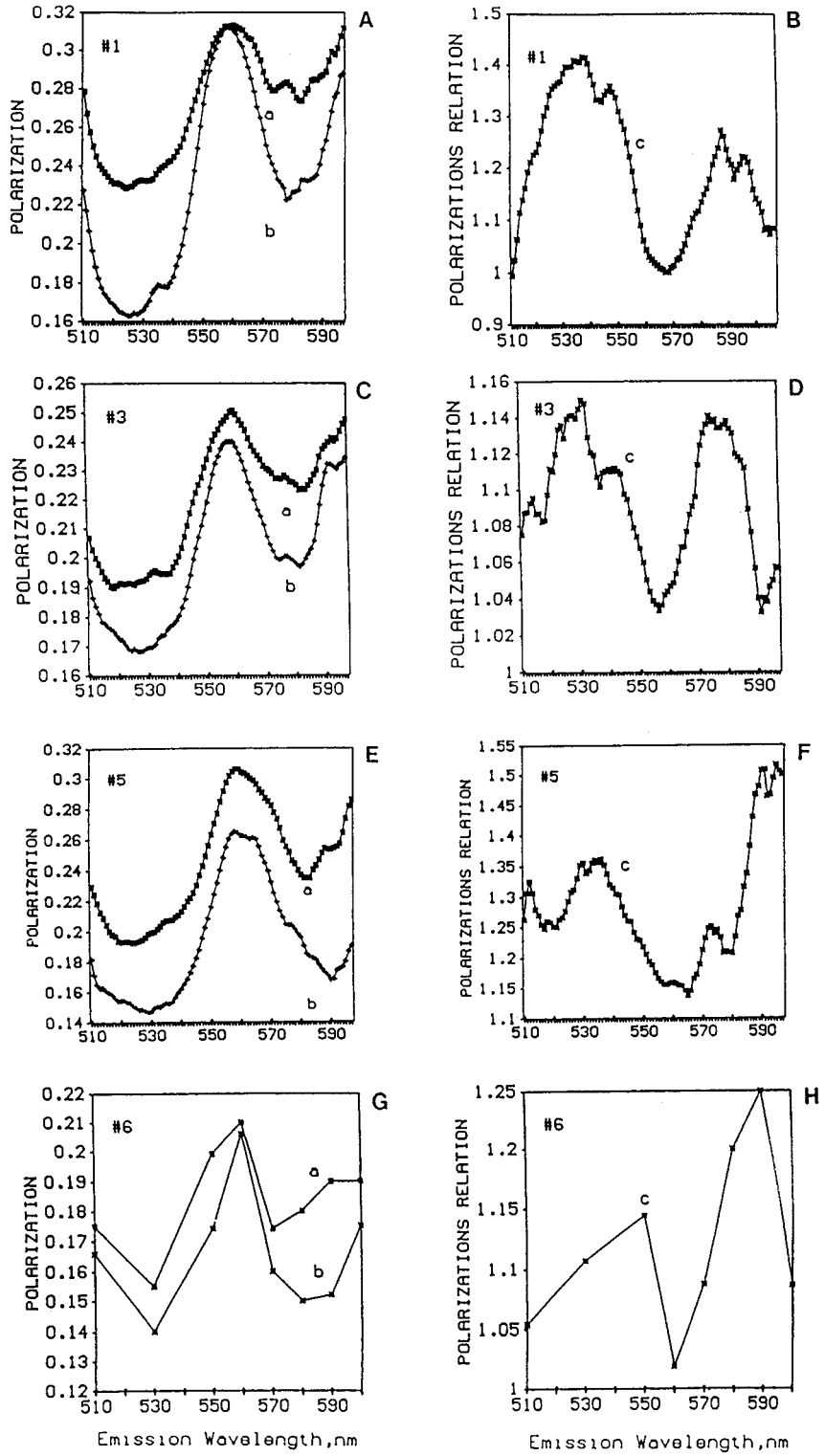

Fig. 4 Left column: Polarization spectra of BCECF in lymphocytes from healthy individuals. The a curves describe P_o, i.e., polarization before stimulation. The b curves show spectra after stimulation with PHA. The c curves in the right column show the respective ratios of P_o/P_{PHA}. (A to F measured by PE MPF-66, G and H by SLM).

spectra of BCECF in three solvents of different viscosities (two water-glycerine mixtures and PBS) are, within the limits of the measurement accuracy, absolutely flat. No dependence of the degree of polarization on the emission wavelength was observed.

In order to examine the possibility that the dependence of the fluorescence polarization on emis-

Table 1 Lifetime measurements: τ_ϕ lifetime as measured by the phase shift method; τ_m lifetime as measured by the demodulation ratio method.

	τ_ϕ	τ_m	Polarization	τ_ϕ/τ_m
Fluorescein in PBS	3.99±0.050	3.93±0.03	0.015	1.015
BCECF in PBS	3.72±0.090	3.70±0.10	0.080	1.005
BCECF in cells (0.320 osmol/kg)	3.52±0.062	4.09±0.11	0.230	0.86
BCECF in cells (0.254 osmol/kg)	3.43±0.050	4.04±0.08	0.170	0.85
BCECF in cells after stimulation with PHA	3.33±0.070	4.49±0.19	0.164	0.74

sion wavelength is due to turbidity effects produced by light scattering by the cells,[35] hydrolyzed BCECF was added to a suspension of unstained lymphocytes and the fluorescence polarization of the suspension was measured. The cell concentration and the fluorescence intensity were the same as in the experiments presented in Figure 4. The polarization was found to be independent of the emission wavelength, as seen in Figure 5 (dashed line). The ease of obtaining the above polarization spectra should be compared with the heroic efforts of the Cerceks to obtain the polarization spectrum of fluorescein in lymphocytes.[36]

A comparison of the polarization spectrum of BCECF in cells (Figure 4) and that of fluorescein shown by the Cerceks[25] with both dyes excited at 470 nm shows large differences between the two. While the polarization peak of BCECF lies at 560 nm, that of fluorescein lies at 510 nm. Polarization values of BCECF are 50% greater than those of fluorescein (0.3 versus 0.2) for the same osmolality and temperature.

In addition to stimulation experiments with PHA, stimulation with EF was tried. As expected, EF did not stimulate lymphocytes of healthy donors, and hence the polarization spectrum was not affected.

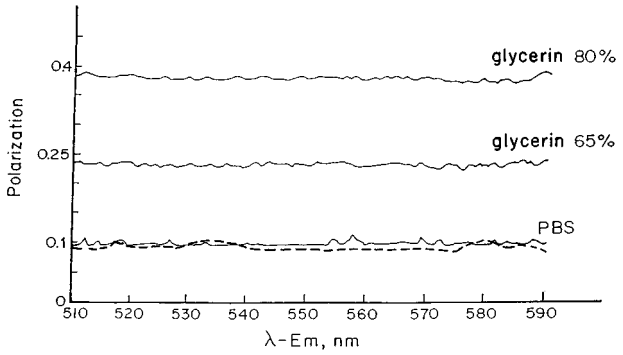

Fig. 5 Polarization spectrum of BCECF in glycerin-water mixtures (80 and 65%), in PBS (solid line), and in unstained cell suspensions (dashed line). The polarization does not depend on the emission wavelength. The measurements were carried out on the PE-MPF-66 spectrofluorimeter.

3.3 INTERACTION BETWEEN BCECF AND THE SURROUNDING MEDIUM IN THE CELLS

The rather high degree of fluorescence polarization of BCECF in cells indicates a somewhat strong interaction between the probe and its intracellular environment. The absence of significant leakage out of the cells is also suggestive of such an interaction. Different mechanisms that might explain this behavior were considered; namely, caging or attachment of dye molecules to macromolecules. If BCECF is tightly trapped in a cage of the surrounding molecules, its motions will be limited although not bound to them. In this case, the embedding of BCECF is assumed to be strongly dependent on the structure of the surrounding medium. On the other hand, if some kind of binding of BCECF to the surrounding macromolecules[37] (e.g., H-bonding, charge attraction, or even covalent bonding) prevails, the motion of each macromolecule is more relevant to the degree of fluorescence polarization than to the viscosity effect of the fluid domains formed by the structure of the cytoplasmic matrix.

In order to check which of the two mechanisms is dominant, or possibly their cooperation, cells were disrupted ultrasonically after staining with BCECF. The fluorescence polarization of the cell fragment suspension was then measured. Figures 6(A) and 6(B) show results illustrating the same phenomenon for two different blood donors (samples 1 and 2). Curve a in each figure shows the polarization spectrum of BCECF in the intact cells, while curve b in each figure shows the polarization spectrum after disruption of the cells by sonication.

The high polarization values observed after cell disruption suggest that at least part of the BCECF molecules are bound to sites on macromolecules, rather than trapped within the cytosol. The size of these macromolecules, as well as their interaction with the liquid phase, causes a slow rotational diffusion that could produce a polarization value on the order of 0.3 in PBS after cell disruption, a value similar to that found in the intact cell. On the other hand, BCECF molecules that are free within the cell or that break loose from their binding sites upon disruption should yield a polarization value of 0.1,

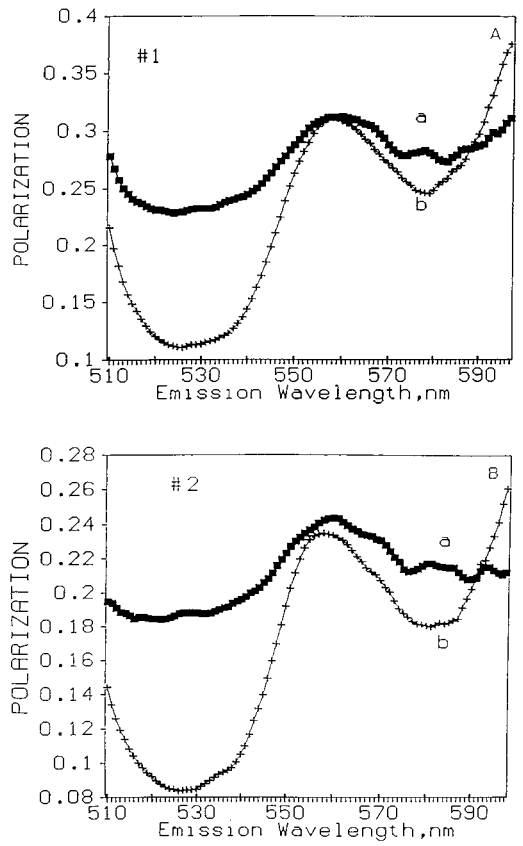

Fig. 6 Polarization spectrum (P_o) of BCECF in cells of two healthy donors (1 and 2) (a) before and (b) after sonication as measured by the PE-MPF-66 spectrofluorimeter.

which is similar to that observed for the dye in PBS.

Figures 6(A) and 6(B) both show an increase in the polarization values at the longest wavelengths after cell disruption. Two tentative explanations are suggested for this effect. (1) The number of sites for the binding of the marker could increase with the unfolding of the macromolecules after cell disruption. The contribution of the newly bound marker molecules would be seen particularly in the long wavelength part of the spectrum because of the red shift in the emission spectrum upon binding. (2) Also, upon disruption of the cells, the transfer of excitation energy between marker molecules might be reduced due to the unfolding of the macromolecules to which the marker molecules are bound, thus augmenting the polarization in this region.

3.4 DEPENDENCE OF THE DEGREE OF POLARIZATION ON THE EMISSION WAVELENGTH

The dependence of the degree of polarization on the emission wavelength in fluorescein[25] and BCECF introduced into the cells via FDA and BCECF/AM, respectively, is not a trivial phenomenon. Several mechanisms that might explain this dependence are discussed:

1. Local environmental properties such as pH, polarity, and polarizability may influence the spectroscopic characteristics of fluorescent probes. Therefore, fluorescent molecules may differ from each other in their excitation and emission spectra as well as in their lifetime and polarization. This might be due to their location at different cellular organelles or microdomains having different properties. The average polarization measured at a given wavelength will therefore be the result of a sum of the polarizations of all domains weighted by their fluorescence intensities, yielding a polarization spectrum that peaks at a given emission wavelength. Such an explanation was suggested by the Cerceks[25] for fluorescein. This explanation is not believed to hold, at least for BCECF. It has previously been shown (Fig. 6) that the polarization spectra of disrupted cells still show a strong dependence of the polarization on wavelength. Under the reasonable assumption that the disrupted cell suspension is homogeneous in its environmental properties, the explanation of the wavelength dependence by additive environmental properties seems unlikely in the case of BCECF.

2. There may be additive phenomena based on the BCECF-macromolecule bond. The high polarization values observed after cell disruption (Fig. 6) suggest that at least part of the BCECF molecules are bound to macromolecules. This bond is also associated with a red shift of the BCECF emission spectrum (Fig. 3). Now, let us assume that only free and bound BCECF molecules exist and that the binding interaction uniformly influences the emission spectra and polarization of all bound molecules. For a given ratio of free to bound molecules, one should expect a polarization emission spectrum consisting of a relatively low polarization section at the shorter wavelengths that continuously and moderately increases toward the longer wavelength section, which is mainly determined by the bound molecules. In the intermediate region, the slope will be determined by the ratio of free to bound molecules and by the magnitude of the red shift due to binding. Indeed, such behavior has previously been observed;[32–34] while other work,[21,25,31] including that described in this article, showed a peak behavior of $P(\lambda_{em})$, which cannot be explained by this model.

Still, in order to explain the peak behavior by the bound/unbound mechanism, the prevalence of a variety of BCECF bonds to a variety of sites on the same macromolecule (or on other macromolecules) is suggested. This will result in different emission properties for each type of bond, as well as in different degrees of rotational freedom of BCECF, which will lead to different degrees of polarization of the bound BCECF molecules. The average polarization may therefore depend on the emission wavelength in a ''peaklike'' behavior determined by the fluorescence intensity and polarization of

each fraction of the differently bound and unbound BCECF molecules.

3. There may be an intrinsic dependence of the polarization of a bound molecule on its emission wavelength. The emission spectrum is composed of different vibronic (vibrational-electronic) transitions from the first excited electronic state to the ground state. Generally, it is assumed that the electronic transition does not depend on the vibrational transition (Born-Oppenheimer approximation). Assume, however, that the binding of a marker molecule to a macromolecule destroys the symmetry restrictions of the electronic transition so that different vibrational transitions (hence different emission wavelengths) will be affected differently by the binding forces. It is then possible that different vibrational transitions will be enhanced, depending on the site of binding, which will also influence the rotational freedom of the fluorescent molecule at the site and thus yield a relation between the emitted wavelength and the degree of polarization.

The proper solution to this problem is still unresolved. It seems however, that a sensible theoretical solution might require abandoning the Born-Oppenheimer approximation, while considering the implications of the binding of the marker molecule to the macromolecules of the cell and the influence of such binding on the electronic transitions via the vibrational system of the marker.

3.5 FLUORESCENT LIFETIME MEASUREMENTS

After the spectroscopic investigation of BCECF, we deemed it advisable to see whether some meaningful information on the behavior of the system could be obtained by lifetime measurements of the fluorescent marker. For this purpose we used the SLM-4800 lifetime measuring accessory. These measurements are based on the modulation of the exciting beam (SLM 4800 Operators Manual, Sept. 1980). There are two ways of extracting lifetime. The first is by the phase shift between the modulated exciting beam scattered from a scattering solution (e.g., glycogen solution) and the radiation emitted by the fluorescent marker (excited by the same excitation beam). This decay time is denoted by τ_ϕ:

$$\tau_\phi = \frac{1}{\omega} \tan\ \theta,$$

where ω is the angular frequency of excitation and θ the phase shift in degrees. The other way is based on measuring the demodulation ratio:

$$\tau_m = \frac{1}{\omega}\left(\frac{1}{D^2} - 1\right)^{1/2},$$

where D is the ratio M_f/M_s and M_f and M_s represent the relative modulation of the fluorescent and scattering solution, respectively. This lifetime is denoted by τ_m. It can easily be shown that if $\tau_\phi = \tau_m$, there is only one fluorescent decay time for the marker. If, however, $\tau_\phi \neq \tau_m$, the marker has two (or more) components with different lifetimes (in the case of two components, the ratio of their values can be found). Although we performed a multitude of measurements, we consider the results still preliminary; however we believe that they show an interesting trend which we will elaborate on after discussing the results shown in Table 1.

From the table it can be seen that for the solution of the marker (fluorescein and BCECF) in PBC, $\tau_\phi = \tau_m$ within the limits of error (lines 1 and 2 of the table). This means that in this case only one decay component is present. The situation is very different for the marker in the lymphocytes. The two lifetime components are now quite different. We can hypothesize that this difference is due to the fact that the marker is embedded in two or more environments within the cell, or that one part of the marker is more tightly bound to one cellular component while the other is in a more aqueouslike environment. Each component will then contribute differently to the overall measured polarization of the marker fluorescence. Measured polarization values are given in the third column of the table. Indeed, decreasing the osmolality from 0.320 to 0.254 osmol/kg, which causes a reduction in the overall cell viscosity (lines 3 and 4), is accompanied by a decrease in the overall polarization. We see, however, that this change is not followed by a change in the τ_ϕ/τ_m value. Line 5 of the table shows the influence of cell stimulation (by PHA) on the τ_ϕ/τ_m ratio. The overall polarization value is decreased by stimulation, as expected for cells from healthy donors, but the longer lifetime component (τ_m) is enlarged and the shorter (τ_ϕ) shortened.

This finding may indicate that the mechanism involved in intracellular BCECF fluorescence depolarization induced by decreasing osmolality and mitogenic stimulation might be different. It is suggested that the latter can be caused by a change in the distribution of the marker molecules so that more of them are transferred from the bound to the free phase, while the former is caused by overall intracellular viscosity.

In the many experiments we performed, we observed differences between the absolute values of τ_m and τ_ϕ; the general pattern was, however, always the same, so that the results of Table 1 should be considered as a true representation of the essential features.

Acknowledgments

Dr. Howard M. Shapiro was supported in part by National Institutes of Health Grant AI30853.

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