FLUORESCEIN EXCITATION AND EMISSION POLARIZATION SPECTRA IN LIVING CELLS

CHANGES DURING THE CELL CYCLE

L. CERCEK, B. CERCEK, AND C. H. OCKEY, *Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England*

ABSTRACT Changes in the fluorescein fluorescence emission and excitation polarization spectra in synchronized cultured S3 fibroblasts at G_1, mid-S, and mitosis, as well as in human lymphocytes before and after stimulation with mitogens, were studied. In contrast to those measured in aqueous solutions the emission and excitation polarization spectra in living cells exhibit a wavelength dependence characteristic for the state of the cell cycle. Changes in the temperature and in the amount of intracellular water result in quantitative wavelength-independent changes in the polarization spectra. Possible mechanisms for the qualitative wavelength-dependent changes in the fluorescein emission and excitation polarization spectra during the cell cycle are discussed.

INTRODUCTION

Changes in the physical state of the cytoplasmic matrix, i.e., the structuredness of cytoplasmic matrix (SCM), in living cells can be detected with the technique of fluorescence polarization (1, 2). The technique is based on the excitation of fluorescein molecules, produced by enzymatic hydrolysis of the nonfluorescing substrate fluorescein diacetate (FDA) in the cytoplasm (3), with polarized light and measurement of the degree of polarization of the emitted fluorescence.

The cytoplasmic matrix is a heterogeneous and polyphasic system in which the physicochemical properties, such as pH, dielectric constant, polarity, and viscosity differ between the microdomains (4). Therefore, in the cytoplasm the fluorescein molecule can be in its neutral (quinonoid or lactonic forms) and/or in the mono- or dianionic molecular structure (5). These molecular structures have different excitation and emission spectra as well as lifetimes of the excited states (5–9). The heterogeneity in the spectroscopic properties of these molecular structures is increased by differences in the dielectric constant and polarity between the microdomains in the cytoplasm. The fluorescein molecules in living cells, therefore, represent an ensemble of different fluorophores. In this case, the average degree of fluorescence polarization measured depends on the fraction of light contributed by each group of fluorophores emitting photons of the same polarization (10). On the basis of the above considerations, the fraction of light contributed by each group of fluorophores and, therefore, the degree of fluorescence polarization in living cells, is expected to be a function of the excitation and

emission wavelength. To verify this hypothesis, and to explore changes in the polarization spectra at different stages of the cell cycle, we have carried out a comparative study of the fluorescein excitation and emission polarization spectra in synchronized skin fibroblasts and in human lymphocytes before and after stimulation with a mitogen.

MATERIALS AND METHODS

Synchronization of Fibroblast Cultures

Sorex oroneus (common shrew) S3 cells were plated in Eagle's modified minimal essential medium (MEM; 20% fetal calf serum) into large roller bottles. 24 h later 0.06 μg/ml colcemid (Ciba Corp., Summit, N.J.) was added to some of the bottles to accumulate cells in metaphase. Metaphases or mitotic cells (without colcemid pretreatment) were selectively removed from the bottles by gentle agitation either with medium or with 0.1% trypsin in Hanks' medium without Ca^{++} and Mg^{++}. Between 89 and 94% of the selected cells were in metaphase in the colcemid-treated samples. Those where colcemid was omitted showed 57% mitotic cells contaminated mainly by G_1 cells. The selected cells were centrifuged at 200 *g* and resuspended in MEM (without serum) for the SCM measurements on metaphase and mitotic cell samples. The remainder of the metaphase cells were distributed into T30 Corning flasks (Corning Glass Works, Corning, N.Y.) in medium containing serum, gassed with 5% CO_2 in air, and incubated at 37°C. Flasks were removed at intervals corresponding to various stages (G_1 and mid-S) of the mitotic cycle. Cells were removed after a quick rinse with 0.1% trypsin and suspended in serum-free MEM. Before polarization measurements, cells were washed twice in phosphate-buffered saline (PBS; cat. no. 404, Grand Island Biological Co., Grand Island, N.Y.) and resuspended in PBS. The S3 cells have a cycling time of 12.5 h divided into 3.25 (G_1), 7 (S), 1.5 (G_2), and 0.75 h (M).

Isolation of Human Lymphocytes

Lymphocytes were prepared from blood collected in Vacutainer sodium heparin tubes (no. 4792, Becton-Dickinson, Rutherford, N.J.) 10-ml samples were transferred into glass vials containing 0.1 g of iron powder type 8365 (Koch-Light Ltd.) and rotated at 30 rpm at 37°C for 30 min. Vials were then placed on a magnet for 10 min. Lymphocytes were isolated by the Ficoll-Triosil (Pharmacia Fine Chemicals, Discataway, N.J.) gradient technique (11), but using a modified gradient with a *d* of 1.0810 g/cm^3 and osmolality of 0.320 osmol/kg. Details of the technique were described earlier (12). Only lymphocytes that band on the interphase between blood plasma and the Ficoll-Triosil solutions were collected: these lymphocytes are characterized by a high polarization value at 510 nm ($P > 0.185$) and by a decrease in the degree of fluorescein fluorescence polarization on stimulation with mitogens or antigens. Cells that separate inside the gradient were discarded. These cells are the nonresponding lymphocytes that are characterized by about 20% lower polarization value at 510 nm (12). The lymphocytes were washed twice with 0.9% saline and once or twice with complete Dulbecco's PBS and resuspended in PBS at the concentration of 5×10^6 cells/ml. To trigger lymphocytes into the cell cycle, the mitogen phytohemagglutinin (PHA) was used (13). 0.1 ml of the reconstituted and five times-diluted reagent grade PHA (The Wellcome Foundation, Ltd., The Wellcome Research Laboratories, Kent, England) was added per milliliter of lymphocyte suspension, and the suspension was placed into the incubator at 37°C.

Measurement of the Fluorescein Fluorescence Polarization Spectra

The degree of fluorescein fluorescence polarization, *P*, of fluorescein molecules in living cells was measured on cell suspensions following procedures described earlier (2, 12). The technique is based on the excitation of fluorescein molecules, produced by enzymatic hydrolysis of the

nonfluorescing substrate FDA in the cytoplasm, with polarized light and measurement of the degree of polarization of emitted fluorescence. Aliquots of cell suspensions were resuspended at concentrations of not > 3×10^5 cells/ml in 3 ml of 0.7×10^{-6} M FDA in complete PBS, containing calcium and magnesium, of pH 7.4 and osmolality of 0.333 osmol/kg. This suspension was rapidly transferred into a 1-cm cuvette and put into the thermostated cuvette holder of the Perkin-Elmer MPF-4 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) fitted with polarizers, which under the experimental conditions used exhibit no intrinsic fluorescence and transmit on crossing < 1% of light. Measurements were made at 27°C. The emission polarization spectra were measured with the excitation monochromator set at 470 nm, spectral slit width 10 nm. The emission monochromator wavelength was varied from 500 to 550 nm in steps of 5 or 10 nm using a spectral slit width of 5 or 10 nm, respectively. When the excitation polarization spectra were measured, the emission monochromator was set at 510 nm, spectral slit width at 10 nm, and the excitation wavelength was varied from 450 to 480 nm in steps of 5 nm.

At the moment when cells were suspended in the FDA solution, the recorder of the fluorescence spectrophotometer was started. The intensities of emissions parallel, $I_{\parallel}(T)$, and perpendicular, $I_{\perp}(T)$, to the vertically plane-polarized exciting light beam were recorded for about 6 min. Because some of the fluorescein from inside cells leaks out into the solution, this fluorescence has to be subtracted from the total intensities (T). Therefore, the cells were filtered away on Millipore (0.22-μm pore size) paper mounted in a Millipore filter head (25-mm diameter; Millipore Corp., Bedford, Mass.). The filtration was performed by suction applying not > 40 cm of mercury vacuum using a hand vacuum pump (Mytival, Neward Die and Manufacturing, Upland, Calif.). The times of the beginning and the end of filtration were marked on the chart of the recorder and the fluorescence intensities of the components $I_{\parallel}$ (F) and $I_{\perp}$ (F) in the filtrate (F) measured. An example of the recordings obtained is shown in Fig. 1. Fluorescence intensities $I_{\parallel}$ and $I_{\perp}$ emitted from the cells were obtained by subtracting values for the filtrate from the total fluorescence intensities $I_{\parallel}$ (T) and $I_{\perp}$ (T) extrapolated to the half-time of filtration (12). This subtraction of the filtrate values (F) is also a correction for any background fluorescence introduced by the PBS solutions. At the concentrations of cells used in these experiments no corrections for light scattering were necessary (see next paragraph). Corrections for the filtrate were between 20 and 30% of the fluorescence in-

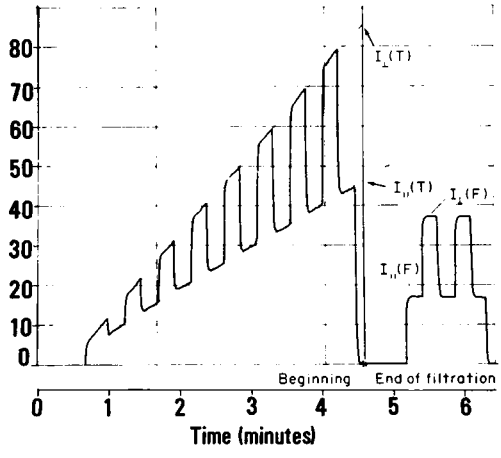

FIGURE 1 An example of the recording obtained on human lymphocytes with a Perkin-Elmer type MPF-4 fluorescence spectrophotometer.

tensities $I_\parallel$ (T) and $I_\perp$ (T). The polarization values, P, were calculated from the relationship $P = (I_\parallel - GI_\perp)/(I_\parallel + GI_\perp)$, where $I_\parallel$ and $I_\perp$ denote the corrected components of fluorescence intensities and G is the correction factor for the amount of polarization induced by the emission optical system and monochromator (14). The total fluorescence intensities emitted by the lymphocyte suspensions, I, were calculated using the relationship $I = I_\parallel + 2GI_\perp$ (10) from the polarization recordings obtained at constant cell concentrations of 4×10^5 cells/ml and times of FDA hydrolysis of 8 min. The measurements at each wavelength were corrected for the unequal transmission of the vertical and horizontal component of polarized light through the emission-optical system of the fluorescence spectrophotometer. The correction factor, G, was 0.451 at 500 nm and decreased to 0.395 at 550 nm. The low value of the G factor is predominantly the result of the 1,200 line/mm grating used in the MPF-4 fluorescence spectrophotometer. The G factor was estimated using the spectrum of light emitted from cells.

Fluorescence polarization measurements can be affected by artifacts caused by scatter of the exciting or emitted light (10). In the present measurements at cell numbers of $< 6 \times 10^5$ cells/ml (in the cuvette) the degree of fluorescence polarization was constant. Therefore, depolarization effects caused by scatter of the exciting or emitted light are negligible. A further artifact could arise if there were insufficient resolution of the exciting and emitted light by the monochromators. Using the same settings on the fluorescence spectrophotometer and cell concentrations as in the experiment, there was no detectable difference between the signals obtained with parallel polarizers on cells suspended in PBS and on the PBS solution without cells, indicating that artifacts due to scatter of the exciting light as well as the intrinsic fluorescence of cells are negligible. Furthermore, the degree of fluorescence polarization was the same whether the measurements were made at any time between 3 and 15 min after the cells were suspended in the FDA substrate solution, i.e., changes in the bulk concentration of fluorescein from 6×10^{-10} M to 3×10^{-9} M had no effect on the polarization spectra. Independence of the degree of fluorescence polarization over a 10-fold change in intracellular fluorescein concentration was also observed in fluorescence polarization measurements on single cells (15). The degree of fluorescence polarization of the same cell did not change even though the fluorescence intensity decreased 10-fold. The polarization values at the different wavelengths were independent of the order of the measurements, i.e., the polarization values were constant over the time-span of the individual experiments.

In experiments in which the effects of osmolality on the polarization spectra were investigated, cells were suspended in 0.7×10^{-6} M FDA solution in complete PBS of pH 7.4 and osmolalities of 0.100, 0.333, and 0.586 osmol/kg. The osmolality (osmol per kilogram) was adjusted by varying the concentration of sodium chloride and measured with the Advanced Instruments osmometer model 3D (Advanced Instruments, Inc., Needham Heights, Mass.).

RESULTS AND DISCUSSION

The fluorescein emission polarization spectra in living cells (Figs. 2–5) differ profoundly from those in homogeneous aqueous solutions (reference 14 and Fig. 3, line A). Whereas the fluorescein emission polarization spectra in aqueous solutions are independent of the wavelength between 500 and 550 nm, those in living cells exhibit a wavelength dependence characteristic of the physiological state of these cells.

The resting phase, G_0 or G_1, is characterized by a sharp peak at 510 nm and a broader maximum at 525 nm (Figs. 2 and 3). When cultured synchronized cells progress beyond the G_1 phase, the sharp peak in the fluorescein emission polarization spectrum at 510 nm disappears. In human, resting phase lymphocytes the 510-nm polarization

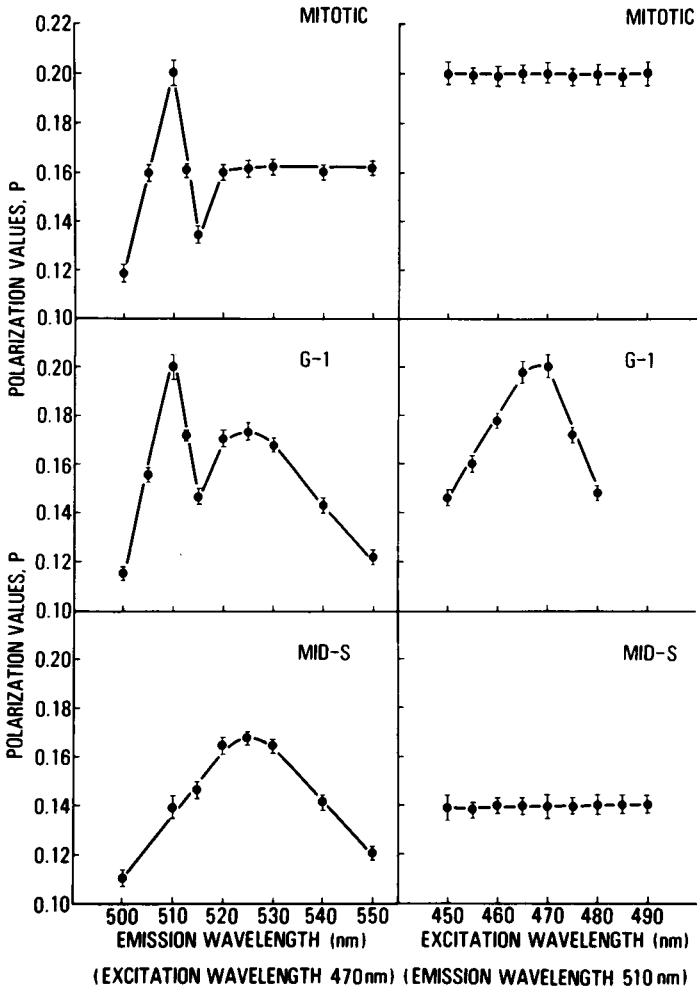

FIGURE 2 Fluorescein emission (left) and excitation (right) polarization spectra in synchronized cultured S3 fibroblast cells in mitosis (top), G_1 phase (middle), and mid-S phase (bottom). The error limits represent maximal deviations from the mean values of three independent experiments.

peak disappears within minutes after the cells are triggered into the cell cycle by mitogen or antigen stimulations. These results indicate that the disappearance of the peak precedes the onset of the S phase. However, the polarization values at 510 nm become progressively smaller when cells proceed through the S phase (1, 2, 13). In contrast, there is no significant change in the region of the broader maximum at 525 nm (Figs. 2 and 3) when cells progress from the G_1 into the S phase of the cell cycle.

In the G_2 phase the degree of fluorescence polarization increases (2) and in mitosis the emission polarization spectrum again exhibits the sharp peak at 510 nm (Fig. 2). However, in mitotic cells the broad maximum at 525 nm is extended into a plateau from 520 to 550 nm.

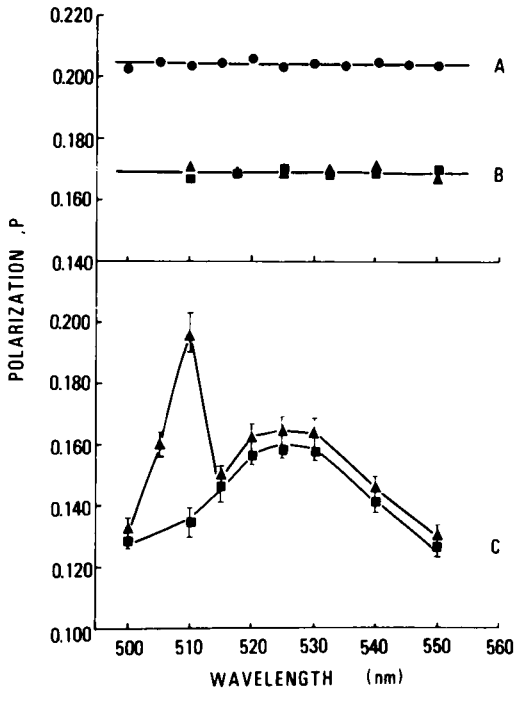

FIGURE 3

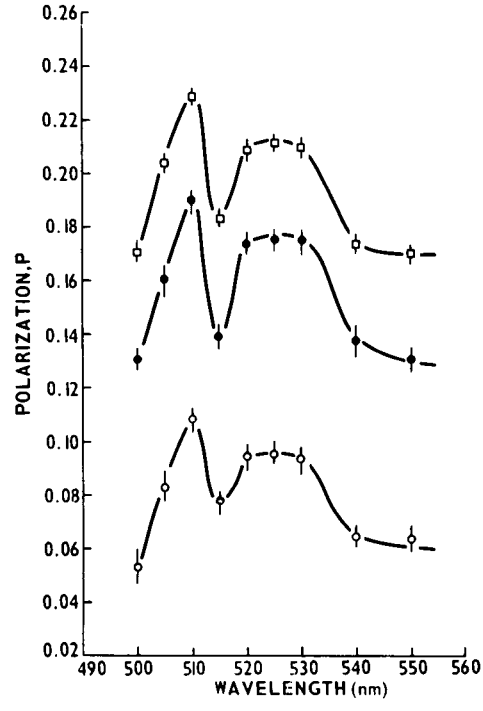

FIGURE 4

FIGURE 3 Fluorescein emission polarization spectra: (A) In aqueous glycerol solution (●); excitation wavelength 470 and 488 nm. (B) In human lymphocytes before (▲) and after (■) stimulation with PHA; excitation wavelength 488 nm. (C) In human lymphocytes before (▲) and after (■) stimulation with PHA; excitation wavelength 470 nm. The error limits represent maximal deviations from the mean value of five independent experiments using lymphocytes from donors A, B, C, D, and E. The values at 510 nm before and after stimulation, have been confirmed in over 2,000 experiments (12, 24).

FIGURE 4 Effect of osmolality on the fluorescein emission polarization spectrum of human lymphocytes: (○) 0.104 osmol/kg; (●) 0.333 osmol/kg; (□) 0.586 osmol/kg. Excitation wavelength 470 nm. The error limits represent maximal deviations from the mean value of two independent experiments on lymphocytes from donors F and G.

A dependence on the physiological state of cells is also evident in the fluorescein excitation polarization spectra (Figs. 2 and 6). The characteristic of the resting (G_0 or G_1) phase cells is a maximum in the excitation polarization spectrum at 470 nm, whereas in cells triggered into the cell cycle, during the S phase and in mitosis, the degree of fluorescence polarization becomes independent of the exciting wavelength between 450 and 480 nm and similar to that in aqueous solutions (Fig. 6, line A). The difference between the S phase and mitosis is in the magnitude of the degree of fluorescence polarization. For example, in the mid-S phase of the S3 fibroblast cells, or in PHA-stimulated lymphocytes, the polarization values are about 30% lower, whereas in mitosis the degrees of polarization are as high as their G_1 phase maximum values at 470 nm (Fig. 2).

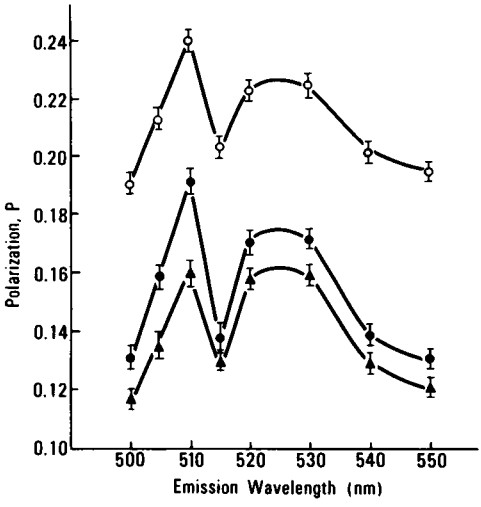

FIGURE 5

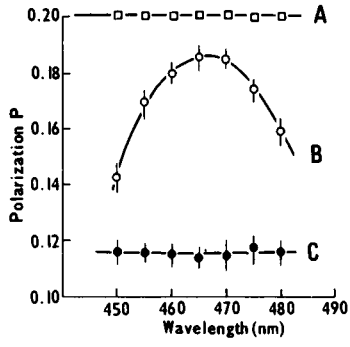

FIGURE 6

FIGURE 5 Effect of temperature on the fluorescein emission polarization spectrum in human lymphocytes: (▲) 37°C; (●) 27°C; (○) 13°C. Excitation wavelength 470 nm. The error limits represent maximal deviations from the mean value of three independent experiments on lymphocytes from donors F, G, and H.

FIGURE 6 Fluorescein excitation polarization spectra: (A) In aqueous glycerol solution. In human lymphocytes before (B) and after (C) stimulation with PHA. Emission wavelength 510 nm. The error limits represent maximal deviations from the mean value of three independent experiments on lymphocytes from donors G and K.

It should be pointed out that the lymphocytes isolated under our experimental conditions (12) contain about 50% of cells that do not respond to mitogenic or antigenic stimulation with changes in the intracellular fluorescein fluorescence polarization (15) and do not have the sharp fluorescence polarization peak at 510 nm. The fluorescence emitted from lymphocyte suspensions contains a large fraction of photons from nonresponding lymphocytes. Therefore, any changes in the fluorescence polarization spectra observed are underestimated. This is not the case when the intracellular fluorescein fluorescence polarization is measured on single lymphocytes under the microscope, when either a responder or a nonresponder lymphocyte is measured. With the single-cell technique we could detect changes in the polarization histograms of human lymphocytes after antigenic or mitogenic stimulations even when the total intracellular fluorescein fluorescence spectrum above 505 nm was measured (15). These results indicate that in the single responder-lymphocyte a much larger fraction of the intracellular fluorescein fluorescence than would appear from the polarization spectra obtained on cell suspensions (Fig. 3) changes its polarization on stimulation.

Changes in the fluorescein emission polarization spectra observed when cells progress into the cell cycle are not caused simply by changes in the content of intracellular water. The results in Fig. 4 indicate that with increasing osmolality, i.e., decrease in content of intracellular water (16), the degree of fluorescence polarization increases at each wave-

length, resulting in a quantitative, but not qualitative change in the emission polarization spectra. This, however, does not exclude the participation of water molecules in the physiological changes of the physical state of the cytoplasmic matrix, as these are likely to involve changes in the conformation of macromolecules, which participate in changes of the physical state of organization of the cytoplasmic microdomains, and concomitant variations of free-to-bound water (1, 16–18). Similar quantitative changes in the emission polarization spectra are detected when the degree of fluorescence polarization is measured at different temperatures (Fig. 5). In this case the decrease in fluorescence polarization can be ascribed to increased Brownian motion of fluorescein molecules with increasing temperature (19). By analogy the observed decrease in the degree of fluorescence polarization with increasing content of intracellular water can be explained by an increase in the rotational motion of fluorescein molecules. This is in agreement with electron spin resonance measurements which show that the tumbling rate of free radical probes in single barnacle muscle cells increases with increasing content of intracellular water (20). Inasmuch as the overall changes in the rotational motion of fluorescein molecules result in a quantitative wavelength-independent change in the fluorescein emission polarization spectra, the qualitative wavelength-dependent changes observed during the cell cycle, therefore, cannot be explained by a similar overall change in the rotational motion of fluorescein molecules. Other factors, such as changes in fractions of light contributed by photons of the same degree of polarization, have to be considered. This may involve changes in the fractions of the various molecular structures of fluorescein molecules (e.g., neutral and anionic forms), lifetimes of their excited states, rotational motion (19), and/or changes in the transfer of energy between fluorescein molecules (21) caused by variations in the physicochemical properties of the microdomains, their size distribution, and/or rates of FDA hydrolysis in these domains in the cytoplasm as a result of changes in the physiological state of living cells (4). However, unless there are microdomains of fluorescein concentrations $> 5 \times 10^{-4}$ M, changes in the transfer of energy are not likely to be a major factor (22, 23) as the average intracellular concentration of fluorescein is about 5×10^{-6} M. This value was calculated from the bulk fluorescein concentration of 10^{-9} M, 3×10^{8} cells/liter and a cell volume of 6.7×10^{-13} liter. Changes in the fluorescein emission spectra observed on mitogen (Fig. 7) and antigen (24) stimulation in human lymphocytes support the hypothesis that changes in the emission polarization spectra involve changes in the fractions of the various intracellular fluorescein species. This interpretation, however, does not exclude the possibility that changes in the fluorescence polarization of fluorescein molecules in a particular cytoplasmic microdomain are due to changes in the rotational mobility of the specific fluorescein species. In fact our previous results on the relationship between Michaelis-Menten constants for the intracellular FDA hydrolysis and the polarization values during the cell cycle (1, 2) as measured at 510 nm suggest that changes predominantly in the rotational mobility of a particular fluorescein species were observed.

Variations in the degree of fluorescence polarization as a function of the emission and excitation wavelength observed in living cells and absence of such a dependence in

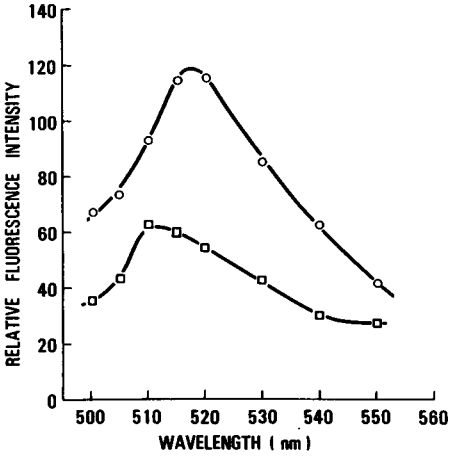

FIGURE 7

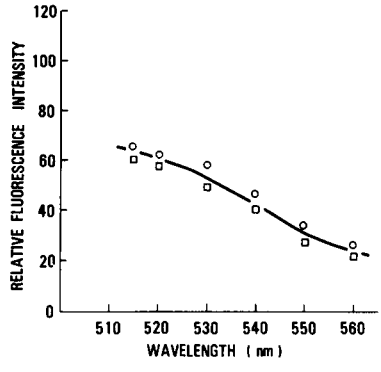

FIGURE 8

FIGURE 7 Fluorescein emission spectra in human lymphocytes: before (○) and after (□) stimulation with PHA. Excitation wavelength 470 nm; cell concentration 4×10^5/ml; time of FDA hydrolysis 8 min. Each point is the mean value of measurements on lymphocytes from donors A, B, and C. Spectra are corrected for the relative quantum response using rhodamine B as a quantum counter.

FIGURE 8 Fluorescein emission spectra in human lymphocytes: before (○) and after (□) stimulation with PHA. Excitation wavelength 488 nm; cell concentration 4×10^5/ml; time of FDA hydrolysis 8 min. Each point is the mean value of measurements on lymphocytes from donors A, C, and L. Spectra are corrected for the relative quantum response using rhodamine B as a quantum counter.

aqueous solutions (Fig. 6; line A) support the hypothesis that in the cytoplasm the fluorescein molecules exist as several spectroscopically different species. The average degree of fluorescence polarization measured, therefore, depends on the fraction of light contributed by each group of fluorophores emitting photons of the same degree of polarization (10). On the basis of this model, the emission polarization spectra are expected to be a function of the excitation wavelength. For example, we find that when the excitation wavelength is 488 instead of 470 nm, in the mitogen-responding lymphocytes, the fluorescein emission polarization spectra become independent of the wavelength and no changes are observed on stimulation with PHA (Fig. 3). Similarly no changes in the fluorescein emission spectra were observed under the above spectroscopic conditions (Fig. 8). Furthermore, in synchronized cultured S3 fibroblasts we did not observe any changes in fluorescein fluorescence polarization between the G_1, mid-S, and mitotic phase of the cell cycle when the excitation wavelength was 488 nm and the emission wavelength 525 nm. The dependence of the emission polarization spectra on the excitation wavelength can explain why no changes in the degree of fluorescence polarization were observed between middle G_1 and S phase in synchronized cultured NHIK 3025 cells in a flow-cytopolarization meter when the fluorescence was excited with 488 nm light of an argon laser and the emissions were measured at 525 nm (25). However, in agreement with the present results (see Fig. 4) the osmolality

effects, which change the rotational motion of fluorophores and result only in a quantitative change in the polarization spectra, were observed in NHIK 3025 cells also under the above spectroscopic conditions (25).

In conclusion, the observed emission and excitation wavelength dependence of the degree of fluorescein fluorescence polarization in living cells shows that changes in the polarization during the cell cycle (2), or those induced by mitogens (13), antigens (12), growth inhibitors, or stimulators (26, 27) can only be observed under spectroscopic conditions of preferential excitation of fluorescein molecules which probe domains in which changes of the physical state of organization of the cytoplasmic matrix occur (12). Further studies are in progress to find out if these domains could be correlated to any particular cell structures.

This work was supported by grants from the Cancer Research Campaign and the Medical Research Council.

Received for publication 16 September 1977.

REFERENCES

1. CERCEK, L., and B. CERCEK. 1973. Relationship between changes in the structuredness of cytoplasm and rate constants for the hydrolysis of FDA in Saccharomyces cerevisiae. *Biophysik.* **9**:109.
2. CERCEK, L., B. CERCEK, and C. H. OCKEY. 1973. Structuredness of the cytoplasmic matrix and Michaelis-Menten constants for the hydrolysis of FDA during the cell cycle in Chinese hamster ovary cells. *Biophysik.* **10**:187.
3. ROTMAN, B., and B. W. PAPERMASTER. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluoregenic esters. *Biochemistry.* **55**:134.
4. DE ROBERTS, E. D., P. NOWINSKI, and F. A. SAEZ. 1965. Cell Biology. W. B. Saunders Company. Philadelphia. 129.
5. LINDQVIST, L. 1960. A flash photolysis study of fluorescein. *Ark. Kemi.* **16**:79.
6. UDENFRIEND, S. 1962. Fluorescence Assay in Biology and Medicine. Academic Press, Inc., New York. 17.
7. MARTIN, M. M. 1975. Hydrogen bond effect on radiationless electronic transitions in xanthene dyes. *Chem. Phys. Lett.* **35**:105.
8. LINDQVIST, L., R. LOPEZ-DELGADO, M. M. MARTIN, and A. TRAMER. 1974. Lifetime and time-resolved fluorescence measurements with ACO synchrotron radiation. *Optics Commun.* **10**:283.
9. GUYOT, G., R. ARNAUD, J. LEMAIRE. 1975. Emission des differentes formes de la fluoresceine en solution aqueuse. *J. Chim. Phys.* **72**:647.
10. WEBER, G. 1952. Polarization of the fluorescence of macromolecules. *Biochem. J.* **51**:145.
11. HARRIS, R., and E. O. UKAEJIOFFO. 1969. Rapid preparation of lymphocytes for tissue typing. *Lancet.* **II**:327.
12. CERCEK, L., and B. CERCEK. 1977. Application of the phenomenon of changes in the structuredness of cytoplasmic matrix (SCM) in the diagnosis of malignant disorders: a review. *Eur. J. Cancer.* **13**:903.
13. CERCEK, L., B. CERCEK, and J. V. GARRETT. 1974. Biophysical differentiation between normal human and chronic lymphocytic leukaemia lymphocytes. *In* Lymphocyte Recognition and Effector Mechanisms. K. Lindhal-Kiessling and K. Osoba, editors. Academic Press, Inc., New York and London. 553.
14. CHEN, R. F., and R. C. BOWMAN. 1965. Fluorescence polarization: Measurement with ultraviolet polarizing filters in a spectrophotometer. *Science (Wash. D.C.).* **147**:729.
15. CERCEK, L., and B. CERCEK. 1976. Changes in the structuredness of cytoplasmic matrix (SCM) in human lymphocytes induced by PHA and cancer basic protein as measured in single cells. *Br. J. Cancer.* **33**:539.
16. CERCEK, L., and B. CERCEK. 1976. Effects of osmomolarity calcium and magnesium ions on the structuredness of cytoplasmic matrix (SCM). *Radiat. Environ. Biophys.* **13**:9.

17. LING, G. N. 1972. Hydration of macromolecules. *In* Water and Aqueous Solutions: Structure, Thermodynamics and Transport Processes. R. A. Horne, editor. Wiley Interscience. John Wiley & Sons, New York. 692.
18. LING, G. N. 1962. A Physical Theory of the Living State. Ginn & Company, New York.
19. PERRIN, F. 1929. La fluorescence des solutions. *Ann. Phys.* **10**:169.
20. SACHS, F., and R. LATORRE. 1974. Cytoplasmic solvent structure of single barnacle muscle cells studied by electron spin resonance. *Biophys. J.* **14**:316.
21. WEBER, G. 1966. Polarization of the fluorescence of solutions. *In* Fluorescence and Phosphorescence Analysis. D. M. Hercules, editor. Wiley-Interscience, John Wiley & Sons, New York. 217.
22. WEBER, G. 1954. Dependence of the polarization of the fluorescence on the concentration. *Trans. Faraday Soc.* **50**:552.
23. DALE, R. E., and R. K. BAUER. 1971. Concentration depolarization of the fluorescence of dyestuffs in viscons solution. *Acta Physiol. Pol.* **A40**:853.
24. CERCEK, L., and B. CERCEK. 1977. Detection of malignant diseases by changes in the structuredness of cytoplasmic matrix of lymphocytes induced by phytohaemagglutinin and cancer basic proteins. *In* Proceedings of the VIth Tenovus Workshop on Tumour markers, Determination and Clinical Role, Cardiff, U.K., 21–23 April 1977. In press.
25. LINDMO, F., and H. B. STEEN. 1977. Flow cytometric measurement of the polarization of fluorescence from intracellular fluorescein in mammalian cells. *Biophys. J.* **8**:173.
26. LORD, B. I., L. CERCEK, B. CERCEK, G. P. SHAH, T. M. DEXTER, and L. G. LAJTHA. 1974. Inhibitors of haemopoietic cell proliferation: Specificity of action within the haemopoietic system. *Br. J. Cancer.* **29**:168.
27. CERCEK, L., P. MILENKOVIC, B. CERCEK, and L. G. LAJTHA. 1975. Induction of PHA response in mouse bone marrow cells by thymic extracts as studied by changes in the structuredness of cytoplasmic matrix. *Immunology.* **29**:885.