INVOLVEMENT OF MITOCHONDRIA IN CHANGES OF FLUORESCEIN EXCITATION AND EMISSION POLARIZATION SPECTRA IN LIVING CELLS

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ABSTRACT The comparison of fluorescein fluorescence polarization spectra in living cells and in isolated subcellular structures identified the mitochondria as the cytoplasmic domain in which on excitation at 470 nm the sharp fluorescein emission polarization peak at 510 nm is formed. Changes in the emission polarization peak during the cell cycle or those induced by growth stimulators and inhibitors reflect structural changes in the mitochondria on their transition from the resting, orthodox into the active, ATP-generating, condensed conformation and vice versa. Possible mechanisms for the formation of the sharp emission polarization peak are discussed.

INTRODUCTION

In living cells the intracellular fluorescein fluorescence polarization spectra exhibit wavelength-dependent changes that are characteristic for the physiological state of these cells. These changes are observed only under spectroscopic conditions of preferential excitation of fluorescein molecules that probe domains in which changes in the physical state of organization occur (1). A characteristic feature in normal resting, G-O or G-1 phase, cells is a sharp fluorescein emission polarization peak at 510 nm which disappears on triggering of cells into the cell cycle (1, 2). To elucidate the underlying biological mechanisms and to find out if any particular cell structure is implicated in the formation of the sharp fluorescein emission polarization peak at 510 nm we have now studied the fluorescein fluorescence polarization spectra in isolated subcellular components, i. e., in the cell sap, nuclei, microsomes, lysosomes, and mitochondria.

MATERIALS AND METHODS

Isolation of the Cell Sap

The cell sap was prepared from liver cells of Wistar outbred rats according to an established technique (3). All procedures were carried out at 0-4°C. Cells, washed with Dulbecco's phosphate-buffered saline (PBS, catalogue No. 404, Gibco, Biocult Ltd., Glasgow, Scotland) were packed by centrifugation at 145,000 *g* for 15 min. The supernate was decanted and the pellet of cells was dried with a wad of filter paper. The pellet was transferred into a flat-bottom stainless steel tube, and a stainless steel plunger of 0.1 mm smaller diameter than the internal diameter of the tube was forced down into the cell mass with a manually operated press. The cells were extruded through the gap between the plunger and the cylinder, their external membranes being broken during extrusion. All metal surfaces in contact with cells were siliconized. The mass of disrupted cells was centrifuged at 145,000 *g* for 20 min. The

supernatant fluid was taken off and clarified by centrifugation at 175,000 *g* for 2 h. Four layers were formed. The second clear layer representing the cell sap was used in our studies.

Preparation of Other Subcellular Fractions

Nuclei, microsomes, lysosomes, and mitochondria were isolated from liver cells of overnight-starved, 12-wk-old Wistar outbred rats according to established techniques (4).

All procedures were carried out at 0-4°C. The liver of 10-12 g was cut into small pieces, washed with PBS, suspended in 3 vol of 0.25 M phosphate-buffered sucrose (pH 7.4), and homogenized in a Potter-Elvehjen type homogenizer (5) using 4 strokes with a pestle speed of 980 rpm. The strokes were applied slowly to prevent formation of vacuum which damages mitochondria. The homogenate was filtered through two layers of 14-N nylon bolting cloth to remove the connective tissue. Differential centrifugation was then used to separate the various subcellular fractions. First the homogenate was centrifuged for 10 min at 600 *g*. The sediment was used to isolate the nuclear fraction by centrifugation on 2.2 M sucrose solution at 50,000 *g* for 1 h (6). The isolated nuclei were washed and resuspended in PBS. The supernate after centrifugation at 600 *g* was centrifuged again at 10,000 *g* for 10 min, and the supernate obtained was then centrifuged at 100,000 *g* for 30 min. The sediment consisting of microsomes was washed and resuspended in PBS. To separate lysosomes and mitochondria, the sediment after centrifugation at 10,000 *g* was taken up in PBS and layered on a phosphate-buffered (pH 7.46) sucrose gradient of densities between 1.18 and 1.24 g/cm^3 at 4°C. After centrifugation at 100,000 *g* for 3 h the mitochondria and lysosomes that band out between densities of 1.18–1.20 g/cm^3 and 1.21–1.23 g/cm^3, respectively, were collected. Each fraction was washed 3 times and suspended in complete PBS of pH 7.46.

Fluorescein Labeling

The cell sap was labeled with fluorescein by adding 2 μl of a 10^{-4}M aqueous solution of di-sodium fluorescein, (BDH Chemicals Ltd., Poole, England) pH 7.5, to 0.5 ml of the cell sap preparation and gently stirring to obtain a homogeneous distribution. The final bulk concentration of fluorescein in the cell sap was 4×10^{-7} M.

The nuclear, microsomal, lysosomal, and mitochondrial fractions were labeled with fluorescein iso-thiocyanate (FITC), isomer 1 (BDH Chemicals,Ltd.). 10 mg of FITC were suspended in 10 ml of complete PBS, pH 7.46, by vigorous shaking at room temperature for ~1 h. The suspension was centrifuged at 2,000 *g* for 20 min to obtain a saturated solution free of FITC particles. The optical density at 495 nm of the 1:200 diluted FITC solution was 0.6 at 21°C. Before labeling the concentrations of subcellular fractions were adjusted so that 1:10 diluted suspensions had an optical density at 520 nm of ~0.5 at 21°C. Equal volumes of the cleared FITC solution and of the subcellular fractions to be labeled were mixed and left standing for 30 min at 35°C. The labeled fractions were centrifuged at 10,000 *g* for 30 min and the supernate containing the excess FITC was decanted. The labeled fractions were washed 3 times with complete PBS, suspended in ~10 ml of complete PBS (pH 7.46) and kept for 2 h before use at 4°C. Immediately before use the labeled fractions were further diluted with complete PBS to obtain a suspension that at 520 nm had an optical density of ~0.4 at 21°C. In the labeled fractions 25–30% of the FITC was irreversibly bound as determined by 24 h of dialysis at 4°C against complete PBS, 200 times the volume of labeled fractions, with one change of PBS and under constant stirring.

The mitochondria and lysosomes were labeled also by fluorochromasia (7) using the intraorganelle enzymatic hydrolysis of the fluorescein diacetate (FDA; Riedel-deHaën, AG, Seelze-Hannover, West Germany) to introduce fluorescein into the organelles. In these experiments 0.5-ml aliquots of organelle suspensions were resuspended in 2.5 ml of 0.7×10^{-6} M FDA in complete PBS, pH 7.46. Details of the procedures were the same as in experiments in which the intracellular fluorescein fluorescence polarization of cell suspensions was measured (1, 2). Mitochondria and lysosomes retain only ~25% of the fluorescein produced by intra-organelle FDA-hydrolysis. However, on FITC labeling 40–50% of the fluorescein label was retained. We, therefore, used labeling by FDA-hydrolysis only to ascertain that the same results were obtained as by the FITC labeling.

Mitochondria damaged during the preparation retain the fluorescein and FITC labels to a lower, or negligible extent; they also do not exhibit the described characteristic fluorescein polarization spectra.

Preparation of Cell Suspensions

Liver cell suspensions were prepared from livers of overnight-starved 12-wk-old, Wistar outbred rats. Livers were broken into single cell suspension in PBS by gentle teasing of the tissue followed by aspiration through syringe needles. The connective tissue was removed by filtration through 14-N nylon bolting cloth. Cells were washed twice in PBS and resuspended in PBS at a concentration of 3×10^6 cell/ml. Rat liver cells can be easily damaged during the preparation of cell suspensions. Since the retention of intracellular fluorescein in damaged cells is impaired (7), the accuracy of polarization measurements is decreased. We have, therefore, used synchronized shrew fibroblasts and "SCM-responding" lymphocytes to compare the effects of ATP, ADP, succinate, pyruvate, and pH on cells and mitochondria.

Synchronized shrew S3 fibroblast cells (1) and human "SCM-responding" lymphocytes (2, 8) were prepared in the same way as described before.

Measurements of the Fluorescein Fluorescence Polarization Spectra

All measurements were carried out at 27°C with a Perkin-Elmer type MPF-4 fluorescence spectrophotometer equipped with a thermostatted cuvette holder and an automatic emission-polarizer changer (Perkin-Elmer Corp., Norwalk, Conn.). Polaroid type HNP'B polarizers (Polaroid Corp., Cambridge, Mass.) which under our experimental conditions did not exhibit any intrinsic fluorescence and transmitted on crossing <0.5% of light were used. To improve the resolution of the exciting and emitted light by the monochromators and to eliminate stray light artifacts, a primary blue FITC filter (Barr and Stroud, Ltd., Glasgow, Scotland) was mounted between the excitation monochromator and polarizer as well as a GG495 cut-off filter (Barr and Stroud, Ltd.) between the cuvette and the emission polarizer. A twofold increase in the intensity of the vertically polarized light was achieved by inserting a polaroid type HNCP 37 half-wavelength plate (Polaroid Corp.) between the FITC filter and the excitation monochromator oriented with its optical axis at an angle of 45° to the horizontal plane.

Details of the experimental conditions, procedures, and calculations of the intracellular, or of the intraorganelle, fluorescein fluorescence polarization values, P, of cell suspensions, or of organelles labeled via FDA, were the same as described before (1). To obtain the fluorescein fluorescence polarization values of the FITC-labeled subcellular fractions (nuclei, microsomes, lysosomes, and mitochondria), 0.5 ml of the labeled suspension was injected into 2.5 ml of complete PBS, pH 7.46 and 0.290 osmol/kg. This suspension was transferred in a 1-cm cuvette and put into the thermostatted cuvette holder of the fluorescence spectrophotometer. The intensities of the emissions parallel, $I_{\parallel}$ (T), and perpendicular, $I_{\perp}$ (T), to the vertically, plane-polarized, exciting light beam were recorded for 3–4 min. To correct for the free FITC-label in the PBS solution the suspensions were filtered on Millipore paper (Millipore Corp., Bedford, Mass.) of 0.025-μm pore size, using the same set-up and technique as described before (1, 2). The parallel, $I_{\parallel}$ (F), and perpendicular, $I_{\perp}$ (F), emission intensities of the filtrate were recorded for ~3 min. The polarization values were calculated from the net intensities, I, obtained by subtracting the corresponding values of the filtrate values from the total fluorescence intensities, both extrapolated to the half-time of filtration, using the following relationship: $P = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + GI_{\perp})$. The filtrate values were between 40 and 50% of the total fluorescence intensities. Details of the calculations of the polarization values, P, were the same as described before (1). 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 (9). The correction factor, G, was estimated using the spectrum of light emitted from the labeled subcellular fractions. 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 500–550 nm in steps of 5 nm using a spectral slit width of 5 nm. When the excitation polarization spectra were measured, the emission monochromator was set at 510 nm, spectral slit width 10 nm, and the excitation wavelength was varied from 450 to 485 nm in steps of 5 nm using a spectral slit width of 5 nm.

Fluorescein fluorescence polarization measurements of the fluorescein labeled cell sap were carried out in a 0.4-cm cuvette. A Hitachi (Hitachi Chemical Co. Ltd., Tokyo, Japan) cell holder adapter of high thermal conductivity was used to fit the 0.4-cm cuvette into the 1-cm, thermostatted cuvette holder of the Perkin-Elmer MPF-4 fluorescence spectrophotometer. The measurements of polarization values were the same as described above, except that the filtration step was omitted. Since the cell sap is not a suspension but a homogeneous viscous fluid in which the fluorescein molecules are dissolved, no corrections for free fluorescein were required.

Biochemicals

To study changes in the fluorescein fluorescence polarization spectra in subcellular fractions and in living cells, the following reagents were used: ATP, disodium salt, Sigma grade (Sigma Chemical Co., St. Louis, Mo.); ADP disodium salt, grade 1 (Sigma Chemical Co.); sodium pyruvate puriss (Koch-Light Laboratories, Ltd., Colnbrook Bucks, England); disodium succinate, pure A. R. (Koch-Light Laboratories Ltd.); encephalitogenic nanopeptide (Beckman Instruments, Inc., Bioproducts Dept., Palo Alto, Calif.). The pH of the PBS or of the FDA substrate solutions was varied by addition of either acetic acid, aristar grade (BDH Chemicals, Ltd.) or disodium hydrogen orthophosphate dodecahydrate, analar grade (Hopkin and Williams, Chadwell Heath, Essex, England). The pH was measured with a PHM 63 Digital pH-meter (Radiometer, Copenhagen, Denmark).

RESULTS AND DISCUSSION

In living cells the fluorescein molecules represent an ensemble of spectroscopically different fluorophores, a subgroup of which exhibits a characteristic sharp emission polarization peak at 510 nm in normal resting, G-0 or G-1 phase, cells (1). This polarization peak was used as a "fingerprint" in the search for the cell structure involved in its formation. The average intracellular polarization value depends on the fraction of light contributed by each subcellular domain (12, 1) and is, therefore, proportional to the fraction of the total cell volume occupied by any cell structure. On this basis the plasma membrane, which constitutes $<1\%$ of the cell volume, cannot be the domain that contributes significantly to the intracellular fluorescein fluorescence polarization. However, the cell sap, nuclei, lysosomes, microsomes, and mitochondria, which all constitute a significant fraction of the cell volume, had to be considered on this basis as possible candidates for the domain in which the observed polarization peak is formed.

The results in Fig. 1 show that the fluorescein-labeled cell sap, nuclei, microsomes, and lysosomes do not show any fluorescein emission polarization peaks. In contrast, the fluorescein emission polarization spectrum in the mitochondria exhibits a characteristic sharp peak at 510 nm (Fig. 2) similar to that found in rat liver cells (Fig. 3) from which the mitochondria were isolated. Furthermore, the fluorescein excitation polarization spectrum in the mitochondria shows a wavelength dependence with a maximum at 470 nm (Fig. 4) resembling that observed in resting phase cells (1).

Within minutes after the cells are triggered into the cell cycle the 510-nm emission polarization peak disappears (1). This phenomenon could, therefore, reflect the ultrastructural alterations known to occur in the mitochondria when the ATP-generating system is switched-on to sustain the increased energy requirements of the cycling cells (10). It is known that in solutions of phosphate ions, such as PBS, the mitochondria are in the switched-off state (11), i. e., in the orthodox conformation (10). The addition of ATP to these mitochondria

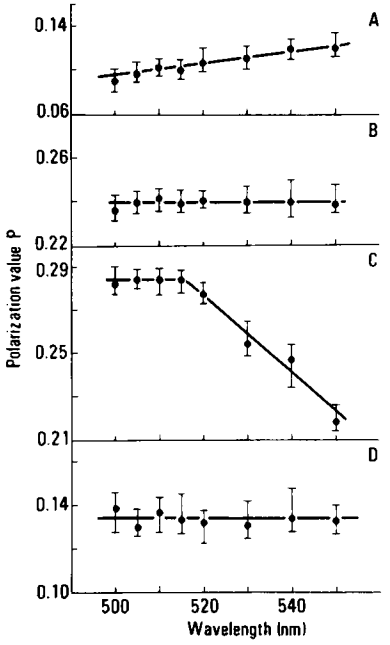

FIGURE 1

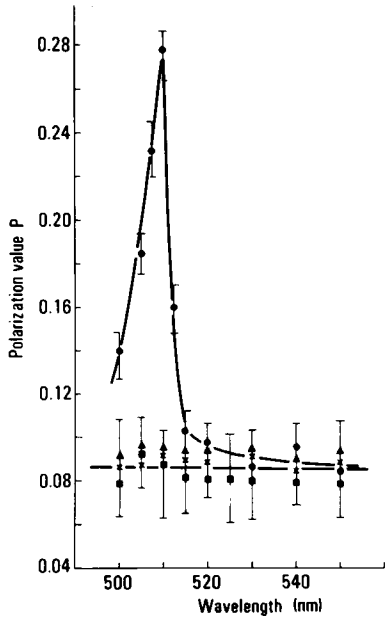

FIGURE 2

FIGURE 1 Fluorescein emission polarization spectra in: (A) fluorescein-labeled cell sap; (B) FITC-labeled nuclei; (C) FITC-labeled microsomes; (D) FITC and FDA-labeled lysosomes. Excitation wavelength, 470 nm. The error limits represent maximal deviations from the mean value of two independent experiments.

FIGURE 2 Fluorescein emission polarization spectra in isolated rat liver mitochondria suspended in PBS (pH 7.46): ●, control incubated for 30 min in the solvent, i. e., PBS only. After 30 min incubation with 10^{-4} M:■, ATP; ▲, ADP; X, succinate and pyruvate. Samples were incubated at room temperature. Excitation wavelength, 470 nm. The error limits represent maximal deviation from the mean value of four independent experiments including both FDA and FITC labeling techniques.

causes their transition into the switched-on state (11) in which the inner-membrane-matrix compartment is contracted into the condensed conformation. The same transition is induced by ADP, succinate and pyruvate, a substrate and precursor substrate, respectively, of the tricarboxylic acid cycle (10). The results in Fig. 2 show that on addition of 10^{-4} M ATP, ADP, succinate, or pyruvate to mitochondria suspended in PBS the sharp peak in the fluorescein emission polarization spectrum at 510 nm disappears. A similar effect was observed on the excitation polarization maximum at 470 nm (Fig. 4). To correlate these observations with changes in the polarization spectra in intact cells we treated synchronized, G-1 phase, fibroblasts (1) and human SCM-responding lymphocytes (2, 8) with 10^{-4} M ATP, ADP, succinate, or pyruvate. The results confirmed that as observed in isolated mitochondria the sharp emission polarization peak at 510 nm and the maximum in the excitation polarization spectrum at 470 nm disappeared. Examples of the effects are illustrated on fibroblasts in Figs. 5 and 6. These changes are analogous to those observed on triggering of cells into the cell cycle (1). The above results explain why the 510-nm peak disappears before the onset of the S phase (1).

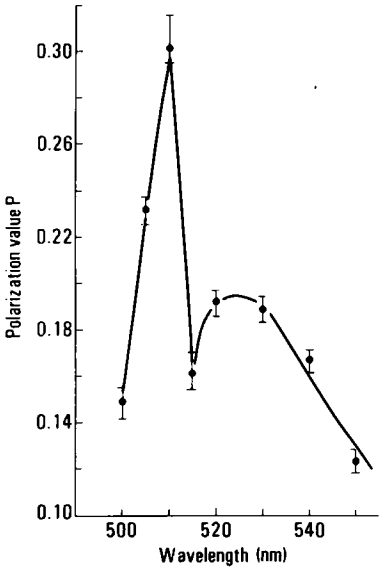

FIGURE 3

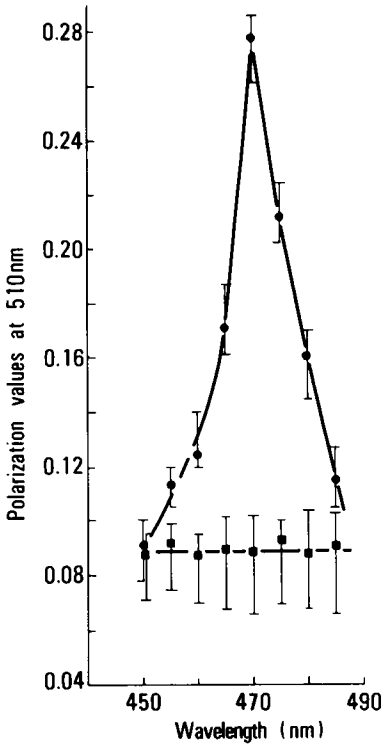

FIGURE 4

FIGURE 3 Fluorescein emission polarization spectra in resting , G-0 phase, rat liver cells. Excitation wavelength, 470 nm. The error limits represent maximal deviations from the mean value of three measurements.

FIGURE 4 Fluorescein excitation polarization spectra in isolated rat liver mitochondria suspended in PBS (pH 7.46): ●, control incubated for 30 min with the solvent, i. e., PBS only; ■, mean values after 30 min of incubation with 10^{-4} M ATP, ADP, succinate, and pyruvate. Samples were incubated at room temperature. Emission wavelength, 510 nm. The error limits represent maximal deviations from the mean value of three independent experiments including the FDA and FITC labeling techniques.

A further analogy can be seen in the effect of pH. In both the intact cells (2) and in isolated mitochondria (Fig. 7), the emission polarization peak at 510 nm progressively decreases when the pH of the suspensions decreases from 7.46 to 6.4. In addition biochemicals that do not induce a change in the fluorescein emission polarization peak at 510 nm in intact cells also do not have an effect on the fluorescein polarization spectra in isolated mitochondria. For example, 10^{-5} M encephalitogenic nanopeptide, which has no effect on the emission polarization peak at 510 nm in the SCM-responding subpopulation of lymphocytes from healthy donors or in G-1 phase fibroblasts, also does not affect the fluorescein polarization peak in isolated mitochondria.

The present results imply that the "phenomenon of changes in the structuredness of cytoplasmic matrix" induced in living cells by growth stimulators or inhibitors (1, 2, 13–15) de facto reflects alterations in the structuredness of the mitochondrial inner-membrane-matrix compartment as a result of their transition into the active ATP-generating state and

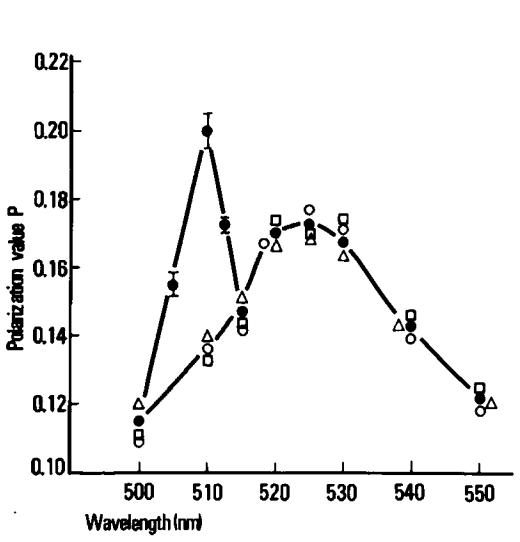

FIGURE 5

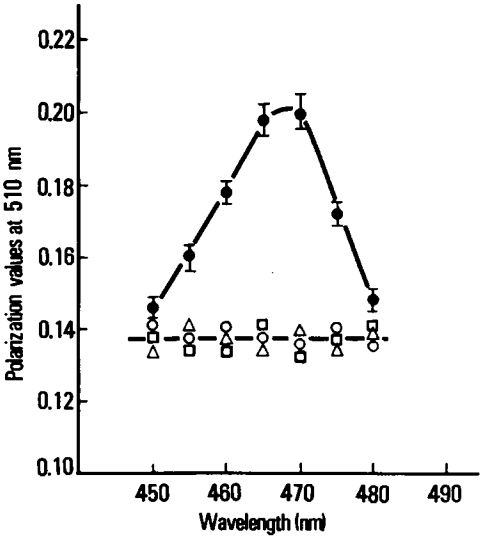

FIGURE 6

FIGURE 5 Fluorescein emission polarization spectra in G-1 phase fibroblasts. ●, control incubated for 30 min with the solvent, i. e., PBS only. After 30 min incubation with 10^{-4} M: ○, ATP; △, ADP, □, succinate and pyruvate. Samples were incubated at 37°C. Excitation wavelength, 470 nm. The error limits are indicated or are within the spread of experimental points. The results are mean values of three independent experiments.

FIGURE 6 Fluorescein excitation polarization spectra in G-1 phase fibroblasts. ●, control incubated for 30 min with the solvent, i. e., PBS only. After 30 min of incubation with 10^{-4} M: ○, ATP; △, ADP; □, succinate and pyruvate. Samples were incubated at 37°C. Emission wavelength, 510 nm. The error limits are indicated or are within the spread of experimental points. The results are mean values of two independent experiments.

vice versa. We, therefore, propose that the term, SCM (1, 2, 13–15), should specifically refer to "structural changes in the mitochondria" and not as up to now "structuredness of the cytoplasmic matrix" in general.

The sharp peak at 510 nm in the intracellular fluorescein emission polarization spectrum suggests that there is a similar narrow peak in its fluorescence spectrum. Although fluorescence spectra are generally broad, it has been reported that in aqueous solutions of 7×10^{-3} M cyanine dyes the association of single molecules into stacks, or molecular aggregates, results in the formation of a sharp optical absorption and fluorescence peak of only 5 nm half-band width (16). Furthermore, it was observed that the presence of proteins catalyzes the aggregation of cyanine dye molecules into stacks already at very low concentrations of dyes (17). Mechanisms for the formation of such molecular aggregates and spectroscopic properties of the resulting "excitons" have been discussed (18). In excitons the band-width of the allowed electronic transition can be very narrow. It is therefore possible that a similar aggregation of fluorescein molecules occurs when the inner-membrane-matrix compartment is in the orthodox conformation (10) resulting in the formation of the narrow fluorescein emission polarization peak. Further studies are needed to characterize the spectroscopic properties of this intracellular fluorescein species and the submitochondrial structure responsible for its formation.

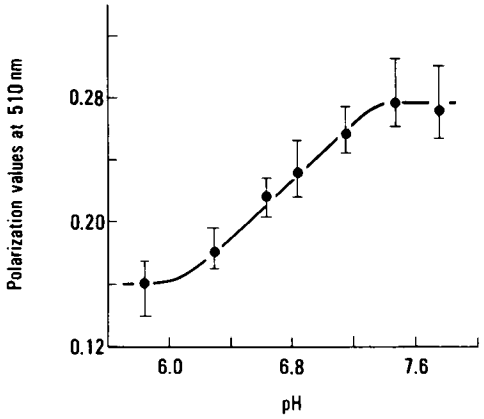

FIGURE 7 Effect of pH on polarization values in mitochondria. Emission wavelength, 510 nm; excitation wavelength 470 nm. The error limits are maximal deviations from the mean of two independent experiments.

In isolated mitochondria (Fig. 2) the fluorescein emission polarization spectrum above 515 nm does not resemble that observed in living cells (1) (Figs. 3 and 5). Therefore, in cells the broad maximum at 525 nm and the changes observed in this wavelength region during the cell cycle (1) are the result of contributions from fluorescein molecules in as yet unidentified subcellular domains. However, the involvement of the microsomes seems likely since a decrease in the polarization values of the emission spectrum above 520 nm is observed in both intact cells and microsomes (Figs. 1C, 3 and, 5). In theory, a complete matrix scan of the intracellular fluorescein excitation and emission polarization spectra could establish spectroscopic conditions for the preferential excitation and emission of fluorescein molecules which probe different subcellular structures.

In conclusion, this study shows that on excitation at 470 nm the intracellular fluorescein fluorescence polarization at 510 nm reflects structural changes in the mitochondria (SCM). The sharp fluorescein emission polarization peak appears when the mitochondria are in the resting, orthodox conformation, and it disappears when they are triggered into the active, ATP-generating state. This technique offers a unique possibility to study the function of mitochondria in their natural milieu.

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