AZIDOFLUORESCEIN DIACETATE – A NOVEL INTRACELLULAR PHOTOLABELLING REAGENT

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1. Introduction

Many colloidal properties of the cell, such as those essential to sol–gel transformation, intracellular motion (cyclosis), ameboid movement, spindle formation and cell cleavage, depend for the most part, on the cytoplasmic matrix. Furthermore, the cytoplasmic matrix is the site of many fibrillar differentiations such as keratin fibers, myofibrils, microtubules and filaments. The viscosity of the cytoplasm is probably dependent on environment and internal factors such as the state of the microfilaments and microtubules. Therefore, it is of utmost importance and interest to label the cell cytoplasm and monitor molecular processes through the microviscosity changes.

The viscosity of the membrane lipids [1–3] and the rotational relaxation time of lectins bound to the cellular surface membrane [4] have been measured using fluorescent probes. They were used also to study the lateral movement of membrane proteins [5,6] and the vertical displacement of membrane proteins [7]. In [8] the lipid soluble fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to study membrane microviscosity change during platelet activation.

Changes of the intracellular microviscosity of malignant and normal cells were studied by the introduction of fluorescein into the cell [9,10]. These studies suffer from the disadvantage that the free fluorescein leaks out of the cell, especially at 37°C. We report here the introduction of an intracellular photoreactive labelling probe. This probe, azidofluorescein diacetate, is trapped inside the cell by rapid hydrolysis of the ester groups then is activated by irradiation. Using this method the fluorescein derivative is covalently attached to intracellular peptides or polypeptides and therefore is unable to leak out. We were thus able to study the intracellular microviscosity in platelets before and after activation with thrombin. These intracellular microviscosity changes might be the result of cytoskeletal changes which are very important to the activation process in platelets and other cells.

2. Materials and methods

Amino fluorescein (isomer I), glutathione (reduced form), ovalbumin, Diamide (azodicarboxylic acid bis-dimethylamide) and thrombin were purchased from Sigma, St Louis MO.

Washed platelets were prepared as in [11]. Erythrocytes were prepared by centrifugation of citrated fresh blood at 100 × *g* for 10 min at room temperature. The platelet-rich plasma was removed and the erythrocytes pellet washed 3 times with modified Tyrode's buffer [11]. Fibroblasts from human foreskin, FS11, were kindly supplied by Dr D. Gurari-Rotman, Department of Virology, Weizmann Institute.

Azidofluorescein was bound to ovalbumin as follows: to a 6 ml solution containing 6 mg ovalbumin in saline, azidofluorescein (dissolved in ethanol) was added in the dark to 1 μM final conc. (final ethanol conc. 0.5%). The solution was irradiated at room temperature and at specified time intervals a sample of 1 ml was removed and kept in the dark. The protein was precipitated with acetone and the pellet was washed with ethanol to remove trapped and precipitated azidofluorescein. The protein was dissolved immediately in 0.1 N sodium bicarbonate and the fluorescence was measured.

Incorporation of azidofluorescein in cells: This was done by a modification of the method in [12]. Cells were incubated with a 10^{-5} M solution of azidofluorescein diacetate at 37°C for 15 min. The cells were separated by a quick centrifugation at 4°C (in the case of platelets or erythrocytes) or aspiration of the medium (in the case of fibroblasts). The cells were resuspended in fresh medium (~10^8 cells/ml) and irradiated for 2–4 min at room temperature. Release of unbound material was achieved by incubation of the cells in a large volume of buffer for 2–4 h.

Paper chromatography was carried out using Whatman no. 3 paper and elution with *n*-butanol: acetic acid:water (4:1:4). Fluorescence studies were carried out using a Perkin-Elmer MPF-3L fluorescence spectrometer. Fluorescence polarization measurements were carried out using a Perkin-Elmer 1000M fluorimeter. The fluorescence polarization of resting platelets was determined by measuring the horizontal and vertical emission of a washed platelet suspension (10^8 cells/ml) labelled with fluorescein azide and irradiated with polarized light. Platelets were activated by the addition of 0.5 or 1 unit thrombin/ml (final conc.) to the cell suspension in a Chronolog aggregometer. When aggregation was completed, the platelets were transferred to the fluorimeter and the degree of polarization was determined.

The light source was a Wild microscope UV source containing a 200 W mercury arc lamp (Osram, HBO); a filter omitting light below 300 nm was used.

Azidofluorescein and azidofluorescein diacetate were prepared from amino fluorescein (synthesis to be detailed elsewhere) and were dissolved in ethanol to 2–4 mM (stock solution).

3. Results and discussion

The structure of azidofluorescein diacetate (I), the alcohol obtained through enzymatic hydrolysis (II) and the active nitrene species formed in the irradiation (III) are shown in fig.1.

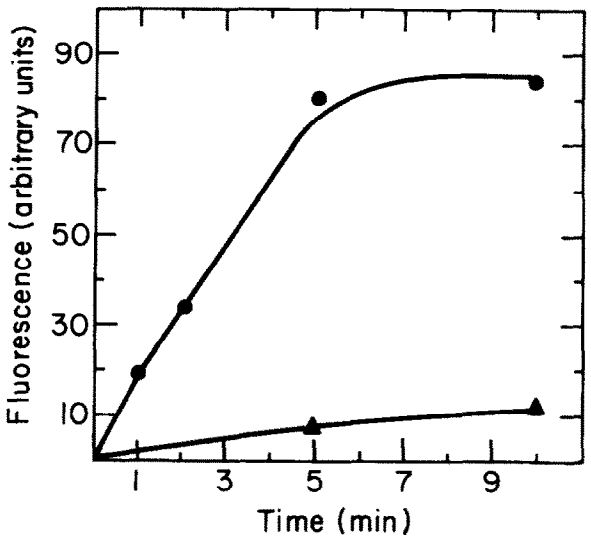

Fig.2. Covalent binding of azidofluorescein to ovalbumin: light conditions (•); dark conditions (▲).

As an example of the insertion reaction of azidofluorescein and polypeptide we irradiated ovalbumin with azidofluorescein. A highly efficient binding occurred which was maximal after 5 min (fig.2).

The partition coefficient of azidofluorescein and fluorescein derivatives are shown in table 1. The relative solubility of azidofluorescein diacetate and azidofluorescein in a hydrophobic solvent such as *n*-octanol is similar to that of fluorescein diacetate and fluorescein, respectively.

Due to the similarity in the hydrophobicity between the fluorescein and azidofluorescein the mechanism of azidofluorescein incorporation into cells can be expected to be similar to that of fluores-

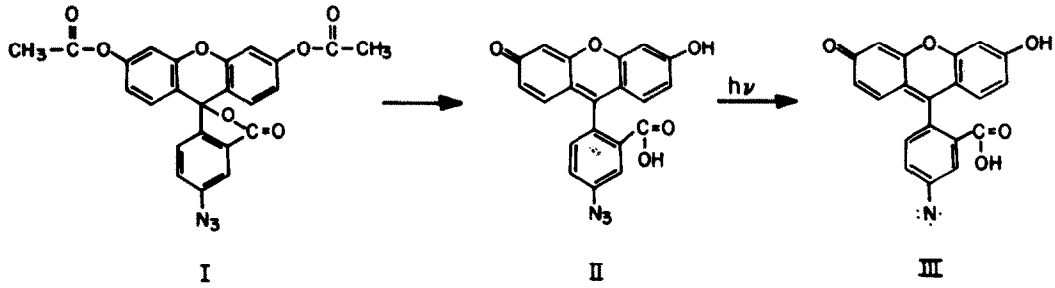

Fig.1. The structure of azidofluorescein diacetate (I), azidofluorescein (II) and the nitrene formed during irradiation (III).

Table 1
Partition coefficients of azidofluorescein, azidofluorescein diacetate, fluorescein and fluorescein diacetate in *n*-octanol–phosphate buffer (pH 7.3)

Compound	Partition coefficient octanol–phosphate buffer
Azidofluorescein	0.50
Azidofluorescein diacetate	333
Fluorescein	0.52
Fluorescein diacetate	300

cein in [12]. Azidofluorescein diacetate is a hydrophobic water-insoluble compound which will penetrate easily into the cells. As a result of intracellular enzymatic esterase hydrolysis, azidofluorescein will be formed. This compound, which is already water soluble, is much more hydrophilic than the parent compound, the diacetate. Due to the hydrophilic nature of azidofluorescein, its ability to cross the membrane is substantially reduced. At this stage the cell is irradiated and the intracellular azidofluorescein is converted to the nitrene derivative which as an active species will insert to nearby peptides or proteins [13,14].

The incorporation of azidofluorescein in cell systems was studied in 3 kinds of cells: human platelets, human erythrocytes and fibroblasts. After 15 min incubation of cells with 10–40 μM azidofluorescein in the dark, separation of the cells and irradiation for 4 min, the cells were postincubated in a large volume of buffer. The release of non-bound material was monitored by measuring the increase of fluorescence in the buffer or by the decrease of fluorescence in the cells. Fig.3 shows a comparison between cells incubated with fluorescein diacetate and those incubated with azidofluorescein diacetate. Both samples were irradiated under the same conditons. It is clear that fluorescein (generated from the fluorescein diacetate) leaks out much faster than the product obtained after the irradiation of intracellular azidofluorescein (generated from azidofluorescein diacetate). After 40 min there is practically no fluorescein in the platelets while the release of non-bound material from those cells treated with the azido derivative reached a plateau after 3–5 h and the cells retained 15% of original fluorescence even after 7 h. Labelled platelets incubated for a few hours in buffer to ensure complete release of non-bound reagent and then viewed

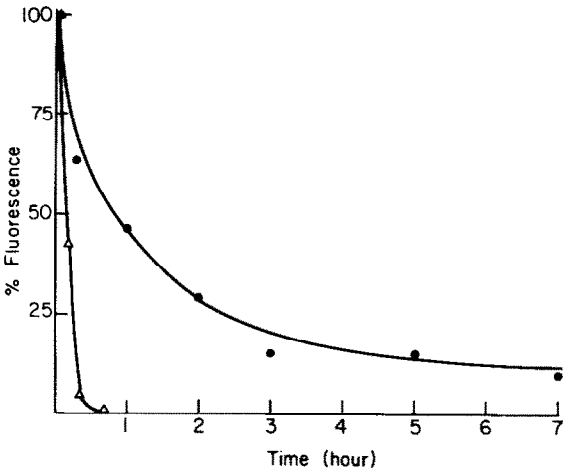

Fig.3. Release of trapped fluorescein (△) and azidofluorescein (●) from platelets after incubation of the cells with the respective diesters and irradiation for 4 min.

under fluorescent microscope showed a homogeneous population of fluorescent cells with no fluorescence in the background (not shown). Similar results were obtained with human erythrocytes. Gel filtration on Sephadex G-75 of the lysate of platelets treated with azidofluorescein diacetate showed the existence of two fluorescent peaks. The minor peak eluted at the void volume and the major one corresponded to low M_r peptides. When the low M_r fraction was paper chromatographed it showed the existence of a ninhydrin-positive spot which is an indication that the majority of the fluorescein is bound to low M_r peptides or amino acids. The main advantage of this intracellular covalent labelling lies in the possibility of performing long experiments with constant levels of intracellular fluorescence. Thus, we measured the fluorescence polarization values of resting platelets and those activated by thrombin. It is well known that platelets undergo quite dramatic intracellular changes during activation by various reagents. These

Table 2
Change of fluorescence polarization in labelled human platelets as a result of thrombin activation (av. 10 typical expt., each expt. is a mean of 3 measurements)

Resting platelets	Thrombin (1 unit/ml)	Thrombin (1 unit/ml) EDTA 2 mM
0.23 ± 0.013	0.16 ± 0.010 Full aggregation	0.17 ± 0.009 No aggregation

include disappearance of the microtubules ring, the movement of the granule towards the center of the cell, appearance of pseudopods, change in the state of actin and other events [15]. It is, therefore, only logical to assume that these processes involve changes in the intracellular microviscosity which can be monitored by the use of the fluorescence polarization technique. According to results in table 2, there is a very significant decrease in the polarization (p) value after aggregation of platelets by thrombin. That this change in the p value is not due to clumping of the cells is evident from the fact that in platelets activated with thrombin in the presence of EDTA (where no aggregation occurs) a similar decrease in the polarization value was observed. The reason for this decrease in intracellular microviscosity might be the conversion of the soluble G-actin into the insoluble F-actin which will lead to a less viscous cytoplasm. The decrease in the p value is certainly not due to release of fluorescent material from the cell during the aggregation process as $\leqslant 5\%$ of the intracellular fluorescent material was released and this did not affect the polarization value of the incubation buffer. The fact that there is practically no release of fluorescence into the medium after the aggregation and release reaction of the platelets indicates that the fluorescent label is not concentrated in the granules but rather is covalently bound to proteins in the cytoplasm.

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References

[1] Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657.
[2] Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603–615.
[3] Shattil, S. J. and Cooper, R. A. (1976) Biochemistry 15, 4832–4837.
[4] Inbar, M., Shinitzky, M. and Sachs, L. (1973) J. Mol. Biol. 81, 245–250.
[5] Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. and Webb, W. W. (1976) Biophys. J. 16, 1055–1069.
[6] Schlessinger, J., Webb, W. W., Elson, E. L. and Metzger, H. (1976) Nature 264, 550–552.
[7] Borochov, H. and Shinitzky, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4526–4530.
[8] Nathan, I., Fleischer, G., Livne, A., Dvilansky, A. and Parola, A. (1979) J. Biol. Chem. 254, 9822–9828.
[9] Cercek, L. and Cercek, B. (1977) Eur. J. Cancer 13, 903–915.
[10] Cercek, L., Cercek, B. and Ocker, C. H. (1978) Biophys. J. 23, 395–405.
[11] Peerschke, E. I., Zucker, M. B., Grant, R. A., Johnson, M. A. and Egan, T. J. (1980) Blood 55, 841–842.
[12] Rotman, B. and Papermaster, B. W. (1966) Proc. Natl. Acad. Sci. USA 55, 134–141.
[13] Knowles, J. R. (1972) Acc. Chem. Res. 5, 155–160.
[14] Bercovici, T. and Gitler, C. (1978) Biochemistry 17, 1484–1489.
[15] Gordon, J. L. and Milner, A. J. (1976) in: Platelets in Biology and Pathology (Gordon, J. L. ed) pp. 3–20, Elsevier/North-Holland, Amsterdam, New York.