Fluorescence fading in quantitative fluorescence microscopy: a cytofluorometer for the automatic recording of fluorescence peaks of very short duration

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Synopsis. Rates of photodecomposition were studied in some fluorophores during short (milliseconds) and longer (minutes) illumination periods. A xenon burner served as light source, and care was taken to obtain optimum conditions for activation. The fluorophores studied included (i) the formaldehyde-induced fluorescent product from 5-hydroxytryptamine in mast cells, (ii) Berberine sulphate bound to mast cell polyanions, (iii) Feulgen-Pararosaniline-stained DNA, and (iv) Fluorescein isothiocyanate-conjugated IgG in an antinuclear factor test. All fluorophores showed a significant fading during 3 min illumination. The Fluorescein isothiocyanate-conjugate faded the most rapidly; its fluorescence intensity was reduced to 50% of the initial value after 2 sec continuous illumination. No fluorophore faded significantly during the initial few milliseconds of illumination. On the basis of these findings, an inexpensive measuring device was constructed. It contained a peak-reader and memory circuit triggered by the flash synchronization tap of a camera shutter positioned in the activation beam. The peak-reader has a response time of about 2 msec. Repeated measurements on the various fluorophores indicate that this peak-reading device may be used to measure fluorescence intensity without fading.

Introduction
Cytofluorometry has several advantages over absorption cytophotometry, such as freedom from distributional error and high sensitivity. There is a vast field of application for cytofluorometry in cell physiology and in experimental and clinical pathology. However, fluorescence measurements may be complicated by the photodecomposition of the sample, resulting in fading of the fluorescence at a rate dependent on the intensity of the activating light. With the high activation energies which may be necessary to ensure a homogeneous activation of dense biological objects, or with particularly susceptible fluorophores, maximum fluorescence intensity may only be obtained within a fraction of a second after starting the excitation.

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The purpose of the experiments described in this paper was to study the fading rate of some fluorophores of general interest under optimum conditions of activation, and to devise a simple and inexpensive measuring equipment capable of automatically recording fluorescence peaks of very short duration.

**Material and methods**

5-Hydroxytryptamine was demonstrated by the Falck-Hillarp technique (Falck, 1962; Falck et al., 1962) in mast cells collected from the peritoneal cavity of male Sprague-Dawley rats (Anticimex AB, Stockholm) and treated with formaldehyde gas as described previously (Enerbäck, 1973).

Mast cell polyanions were stained with Berberine sulphate (C.I. 75160, Fluka A.G., Basle, Switzerland, Lot No. 562319) in peritoneal mast cells sedimented on microscope slides and fixed for 1 hr in 3:1 v/v ethanol-acetic acid. The slides were stained with Berberine sulphate (0.02% in redistilled water acidified to pH 4 by the addition of 1% citric acid) for 20 min, and, after 10 min wash in acidified redistilled water (to remove dye bound non-specifically), mounted in water under cover-slips sealed with varnish (Enerbäck, to be published).

Nuclear DNA was estimated quantitatively by the Feulgen-Pararosaniline reaction, as described by Böhm & Sprenger (1968), on liver imprints from 1-year-old Swiss mice.

Fluorescein isothiocyanate-conjugated immune globulin was measured in an antinuclear factor test on imprints from livers of Sprague-Dawley rats, fixed in acetone for 10 min and incubated in human serum with an antinuclear antibody titre of 1/800 diluted with phosphate-buffered saline (Nairn, 1969). This was followed by incubation in Fluorescein isothiocyanate-conjugated sheep antihuman IgG. After washing in phosphate-buffered saline the imprints were mounted in phosphate-buffered saline under cover slips sealed with varnish.

**Instrumentation**

A Leitz MPV I microscope photometer equipped for fluorescence microscopy with incident-light illumination as described previously (Enerbäck, 1973) was used. Excitation light was provided from an Osram XBO 75 xenon burner equipped with a stabilized power supply (Siemens Type VXIVH) and connected to the mains via a voltage stabilizer. A mechanical camera shutter ('Prontor Press', Prontor-Werk, Calmbach, GFR) equipped with a flash synchronization tap was placed in the activation beam. The performance of the shutter was tested with an oscilloscope. The opening time was found to conform within 10% of the nominal values (available range, 1 to 1/125 sec). The time lapse between full closure and full opening was about 2 msec. The synchronization tap was found to trigger at full opening. The detection system comprised the peak-reading device to be described, a PDM 611 printing digital voltmeter (Practical Automation Inc, Shelton, Conn., USA) and a Yew Type 3046 potentiometric recorder (Yokogawa Ltd, Tokyo, Japan).

The activation maxima of the various fluorophores were isolated with a combination of glass filters and interference filters (Schott & Gen., Mainz, GFR). For Fluorescein isothiocyanate, a newly developed short-pass interference filter (Richards & Waters,