Short Communications

Partial recovery of fluorescence intensity after irradiation-induced fading of fluorescence, and its effects on techniques of quantitative fluorescence microscopy

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Summary. Partial recovery of fluorescence intensity after irradiation-induced fading of lipopigment autofluorescence is reported, during a subsequent period of 60 sec when each specimen was in darkness. This phenomenon may have significance for various techniques of quantitative fluorescence microscopy.

Fading of fluorescence intensity during irradiation is a well-recognized phenomenon, although its nature is unclear[1,2]. The rate of fluorescence fading, which may vary between fluorophores[1], is related to the degree of absorption of the irradiation and thus to the intensity of, and the duration of exposure to, the exciting light. A further general property of fading is that the rate shows a gradual diminution even if the intensity of exciting light remains constant[3]. Although different fading rates have been used to distinguish certain fluorophores[4], fading has produced methodological problems in quantitative studies; for example in those of neuronal lipopigment autofluorescence[5-8].

Reviews[1,3] of quantitative fluorescence microscopy do not state that recovery of fluorescence intensity after irradiation-induced fading can occur, and 1 report has claimed that there was no recovery of fluorescence intensity after what was termed 'photodecomposition'[9]. However, the present study reports partial recovery of fluorescence intensity after irradiation-induced fading of lipopigment autofluorescence.

Materials and methods. Postmortem tissue was examined from a human brain of an elderly individual with no evidence of neurological disease. Unstained 20-μm sections were examined using a Leitz Ortholux II microscope with a MPV1 photometer and EM1 9558B photomultiplier. Each specimen was irradiated from above with an HBO 100 W mercury lamp via a X 100 objective (NA 1.32) and the exciting light passed through a heat filter, a red suppression filter, and an exciting filter which allowed the passage of violet and blue light (390–490 nm). A dichroic mirror then reflected light below 510 nm towards the specimen, and part of the resulting autofluorescence (mostly above 510 nm), was transmitted by the mirror before passage through a barrier filter which transmitted wavelengths above 515 nm.

Selection and positioning of the region to be measured was carried out for a constant period of 10 sec at the lowest intensity of excitation compatible with neuronal identification. Further details of the methodology have been previously reported[5,6].

Regions of intraneuronal lipofuscin were each exposed to continuous irradiation for 60 sec, and measurements of fluorescence intensity were noted initially (0 sec) and 15, 30, 45, and 60 sec after the onset of irradiation. The irradiation was then discontinued, and the region of pigment being examined remained in darkness for a subsequent 60 sec. At the end of this period a final measurement was taken using the automatic shutter and recording system which provided the mean of 10 readings of fluorescence intensity over approximately 0.3 sec[5]. Other regions of pigment were exposed to continuous irradiation for 120 sec, and measurements of fluorescence intensity were again obtained at 0, 15, 30, 45, 60 and 120 sec after onset of irradiation.

Results. In the table it can be seen that, when neuronal lipofuscin was irradiated for 60 sec, fading occurred; but, in each of the 8 consecutively-examined regions of pigment, the measured intensity at the end of a subsequent 60 sec in darkness was higher than the previous reading. The increases between the readings at 60 and 120 sec are significant at $p = 0.008$ (Sign test, 2-tailed)[10].

The table also shows that when a further 8 consecutively-examined regions of pigment from the same brain were continuously irradiated for 120 sec, the mean intensity for the 8 regions at 120 sec was lower than at 60 sec. Although 1 region gave an identical reading at 60 and 120 sec, the decreases between the readings at 60 and 120 sec are significant at $p = 0.016$ (Sign test, 2-tailed)[10]. Inconsistencies in the table may have been due to several methodological factors including relatively small variations in the intensity of the light source[5]. Partial recovery of lipofuscin fluorescence intensity after irradiation-induced fading has been confirmed by the author on many occasions.

Discussion. Partial recovery of fluorescence intensity after fading must be taken into account in quantitative studies involving lipopigment autofluorescence[6], and may have relevance to other areas of research such as the fluorescence photobleaching recovery technique[11] which investigates the diffusion and/or flow of unbleached fluorescent molecules into a region whose fluorescence has been reduced by irradiation.

The effects of A) continuous irradiation for 60 sec followed by 60 sec in darkness on the measured intensity of autofluorescence from 8 consecutively-examined regions of neuronal lipopigment, and B) continuous irradiation for 120 sec on the measured intensity of autofluorescence from a further 8 consecutively-examined regions of neuronal lipopigment

A) Time (sec)	Region 1	2	3	4	5	6	7	8	Mean (1–8) as percent of initial value (± SEM)
0	94	85	75	60	75	106	79	42	100
15	86	80	70	59	74	102	76	40	95 (0.92)
30	82	76	77	54	71	97	73	39	93 (1.72)
45	79	76	70	54	67	95	71	38	89 (1.34)
60	78	74	66	51	69	94	70	38	88 (1.01)
120	88	83	76	56	70	103	79	42	97 (1.17)

B) Time (sec)	Region 9	10	11	12	13	14	15	16	Mean (9–16) as percent of initial value (± SEM)
0	86	72	74	66	61	59	57	60	100
15	84	68	72	63	59	56	54	58	96 (0.50)
30	76	70	70	61	57	54	53	54	93 (0.98)
45	78	68	70	58	56	54	52	53	91 (0.88)
60	73	66	68	56	54	52	52	51	88 (1.10)
120	72	62	65	55	52	50	50	51	86 (0.63)

Each region of lipopigment was contained in a heavily-pigmented neurone in the inner third of layer III of the parietal cortex from an elderly non-diseased human brain. Values are given in the same arbitrary unit of fluorescence intensity. Time = time after onset of irradiation. (This followed the process of selection and positioning of each region which involved 10 sec of exposure to a reduced intensity of exciting light.)

It should be remembered that fluorescence fading can occur during the initial selection and positioning of each region to be examined, so it is possible that, for a series of measurements each of which is taken during a short discrete period of irradiation, partial recovery of fluorescence fading would result in an initial measurement being lower than the final value of fluorescence intensity in the series. Although such a finding could, in theory, be at least partly due to irradiation-induced decomposition of the fluorophore having led to a diminution of concentration-dependent quenching[1], this could not explain the present finding of an increase in fluorescence intensity during a period when the specimen is not irradiated.

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The defensive secretion of the tiger beetle *Cicindela flexuosa* (F.) (Cicindelinae; Carabidae)

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Summary. The pygidial gland secretion of the tiger beetle *Cicindela flexuosa* consists of tetradecylacetate and hexadecylacetate. It functions as a defense mechanism.

Tiger beetles (Carabidae, Cicindelinae) are predatory beetles living in sandy areas and marshes along streams and seacoasts. Often brilliantly colored, they are favorites among collectors although they are swift runners and fast flyers and are difficult to capture. In common with many other members of the Carabidae they possess a pair of pygidial glands which secrete a substance used for defense. The chemistry of the secretion of a number of carabid beetles has been extensively investigated and has revealed a variety of compounds ranging from hydrocarbons to carboxylic acids, esters, aldehydes, ketones, phenols and quinones[2-4]. However tiger beetle secretions are still mostly unexplored; only a few species, all of the genus *Megacephala*, have been examined. *Megacephala australis*[5] produces benzaldehyde in its pygidial glands while in *M. virginica* and *M. carolina*[6] this aromatic aldehyde is accompanied by hydrogen cyanide. Small amounts of mandelonitrile were also detected in *M. virginica* indicating a cyanogenic pathway in the glands. In this paper we report on the pygidial gland secretion of a tiger beetle of the genus *Cicindela*, a secretion entirely different from that of the *Megacephala* beetle.

Cicindela flexuosa is an ammophilous tiger beetle which is found in most of the sand dunes along the Mediterranean shores of Israel. The structure of its pygidial glands is similar to that described for *C. campestris*[7]. The 2 glands are elongated and open on the outer side of the abdomen between the 8th and 9th tergites. When disturbed or handled roughly, the beetles often protrude their abdominal tip to expose the intersegmental membrane and in this position they discharge the contents of the glands.

Materials and methods. Adult beetles were collected in early spring near Ma'agan Michael, Israel, refrigerated and brought to the laboratory where the pygidial glands of live beetles were dissected and then extracted with methylene chloride. The extracts were analyzed by combined gas chromatography-mass spectrometry on a LKB-2091 instrument using a 1.8-m 3% OV-17 column programmed from 60 to 300 °C and 1.8-m 10% SP-1000 column programmed from 60 to 240 °C. Identification of the components was based on comparison with authentic standards. Tetradecylacetate and hexadecylacetate were synthesized from the corresponding alcohols and acetyl chloride. Two compounds were detected in the extract. The first component which accounted for more than 75% of the volatiles eluted at 210 °C, the other at 230 °C on the OV-17 column. Their mass spectra were similar and consistent for acetates, with a base peak at m/z 43 and a substantial diagnostic peak at m/z 61. The highest mass peaks at 196 and 224 respectively (M-60) spoke for mol. wts of 256 and 284, and the remainder of the spectrum in each case was typical for aliphatic chain strucuture. The compounds were identified as tetradecylacetate and hexadecylacetate by comparison with authentic samples (mass spectra and retention times).

Tetradecylacetate and hexadecylacetate are quite common in the defensive secretions of arthropods. They have been found in the pygidial glands exudates of several carabids; tetradecylacetate was detected in Psydrinae and also in Panagaeinae[4] where it is accompanied by the hexadecyl homologue. Both acetates are present in large amounts in the Dufour's glands of the ants *Formica pergandei* and *F. subintegra*[8] and serve as alarm pheromones and also as disarming agents during slave raids on other ant colonies. In *Lasius niger*[9], *Camponotus ligniperda*[10] and *C. herculeanus*[11], these acetates are used in alarm and in defense and it is very probable that the pygidial gland secretion of *C. flexuosa* has this same dual function. Other *Cicindela* have yet to be studied to determine if acetates are common to this genus just as benzaldehyde apears to be characteristic of the *Megacephala* genus of tiger beetles.