Fluorescence Patterns of Chromatin and Cytoplasm by Hematoxylin Solutions

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Treatments of chicken blood and Ehrlich ascites tumor smears with hematoxylin solutions give a fluorescence reaction in chromatin, basophilic cytoplasm and leukocyte granules. In these structures the fluorescence emission increases upon dye aging and prolonged staining times. We present a preliminar spectral analysis suggesting the possibility to employ hematoxylin as a fluorochrome.

Hematoxylin is a natural, weak acid dye, which develops colour upon standing due to spontaneous oxidation into hematein [1]. When used as aluminium or transitional metal lakes, hematein shows high selectivity for nucleic acid containing structures [2–4]. As far as we know, fluorescence properties of hematoxylin solutions seem to have been overlooked. During the course of staining experiments with this dye, we have found an unexpected fluorescence reaction by hematoxylin, which is the aim of the present communication.

Smears of chicken blood and Ehrlich ascites tumor cells were fixed in methanol for 2 min and air dried. Hematoxylin from several suppliers (Merck; Ernst Leitz, Berlin; Schering, Kahlbaum, Berlin) was applied at room temperature as 0.1% solutions in distilled water after different aging periods. Smears were briefly washed in distilled water, air dried, and observed under oil immersion. Extraction procedures were the following: DNase I (Serva, 1 mg/ml in tris-HCl buffer at pH 6.8 containing 10^{-3} M $MgCl_2$) at 37 °C for 2 h; RNase (Boehringer, 1 mg/ml in distilled water) at room temperature for 2 h; 5% trichloroacetic acid (TCA) at boiling temperature for 20 min; 5% perchloric acid (PCA) at 4 °C for 18 h; 5 N HCl at room temperature for 30 min; 0.2 M EDTA solution for 30 min. Extraction after enzymatic digestions were controlled by staining preparations with 10^{-4} M acridine orange (BDH) in distilled water. Post-treatments consisted in washing preparations with absolute ethanol for 30 min or in treatment with a 5% aqueous solution of potassium aluminium sulfate for 5 min. Hematoxylin was also applied as solutions either containing 1 M NaCl or 2 M urea. Observations were made in a Zeiss Photomicroscope III equipped with an epi-fluorescence condenser III RS and the filter sets for violet-blue (436 nm) or green (546 nm) exciting light. Cytofluorometric measurements were performed as previously described [5]. Spectral analysis was made with Perkin-Elmer instruments (Spectrophotometer 551-S and Fluorescence Spectrophotometer 650-10 S).

After 15–30 min hematoxylin treatment, nuclei of chicken erythrocytes and the basophilic cytoplasm of lymphocytes and Ehrlich cells showed a yellow-green or red fluorescence emission under violet-blue or green excitation, respectively. Ehrlich cell nuclei appeared with a lower fluorescence intensity than cytoplasm. A striking fluorescence reaction was present in the cytoplasmic granules from chicken heterophil leukocytes. All the fluorescent structures showed a very low degree of fading. Washing with ethanol did not decrease the fluorescence reaction, although aluminium postreatment resulted in a strong obliteration. Aging experiments demonstrated that this fluorescence pattern was dependent on dye ripening and commercial supplier (Fig. 1 A), being in agreement with the known

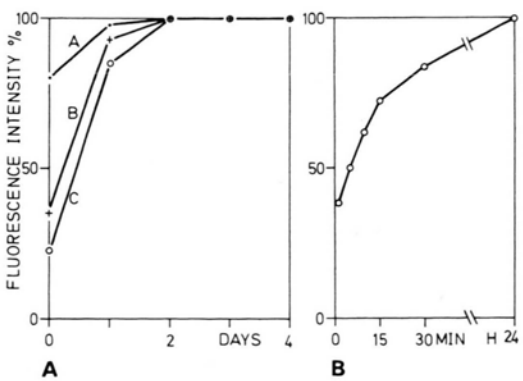

Fig. 1. A: Relation between the intensity of nuclear fluorescence in chicken erythrocytes and the different aging of hematoxylin from 3 commercial suppliers. A: Schering; B: Ernst Leitz; C: Merck. Staining time was 30 min; violet-blue exciting light.
B: Effect of the staining time on the intensity of nuclear fluorescence in chicken erythrocytes by hematoxylin solution (Merck, 10 days aged); violet-blue exciting light.

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Table I. Extraction procedures and experimental treatments applied on smears of chicken blood. Hematoxylin (H), (Merck or Ernst Leitz, 0.1% aqueous solution, from 3 to 30 days aged), was applied for 30 min at room temperature; violet-blue exciting light.

Experimental design	Relative intensity of fluorescence in erythrocyte nuclei (in percent)
1. H (control)	100
2. TCA, H	0
3. DNase, H	0
4. HCl, H	80
5. EDTA, H	100
6. H, ethanol	100
7. H, aluminium	0
8. H in 1 M NaCl	64
9. H in 2 M urea	42

variable amount of hematoxylin and its oxidation products in different commercial samples [6]. After aging of 1 – 2 month, hematoxylins from Merck or Ernst Leitz were still found quite suitable to produce the fluorescence reaction, but with more prolonged aging times the emission intensity of cell structures decreased slowly. 15 – 30 min of hematoxylin treatment showed to be adequate for the development of the reaction in erythrocyte nuclei; however, increasing staining times resulted in stronger fluorescence (Fig. 1 B).

Extraction procedures indicated that DNA could be the responsible chromatin component for the fluorescence by hematoxylin (Table I). However, the emission of heterophil granules and the basophilic cytoplasm seems to depend on other substrates. The fluorescence of the RNA rich cytoplasm proved to be resistent to RNase and cold PCA extraction. According to the results in Table I, a dye binding mechanism based on ionic and/or hydrogen bonds could be implicated in the chromatin fluorescence. This mechanism may use the same dye groupings as for metal complexing. It is interesting to note that hydrogen bonding seems also to occur in glycogen [7] and elastic fiber [8] staining by hematoxylin. In addition, hematoxylin stains basic nuclear proteins [9]. A covalent binding between the *o*-quinone hydroxyl grouping of this dye and the guanidyl group of arginine has been proposed for the hematoxylin staining of keratohyalin and eosinophil leukocyte granules [1]. Next, a peliminar spectral study of the absorption and emission characteristics of hematoxylin was made to substantiate the fluorescence reaction of the dye at the cytologic level (Fig. 2). Absorption maxima were observed at 290 and 405 nm; two emission maxima at 476 and 546 nm were found for the free dye under excitation at 410 nm. Aqueous hematoxylin shows an absorp-

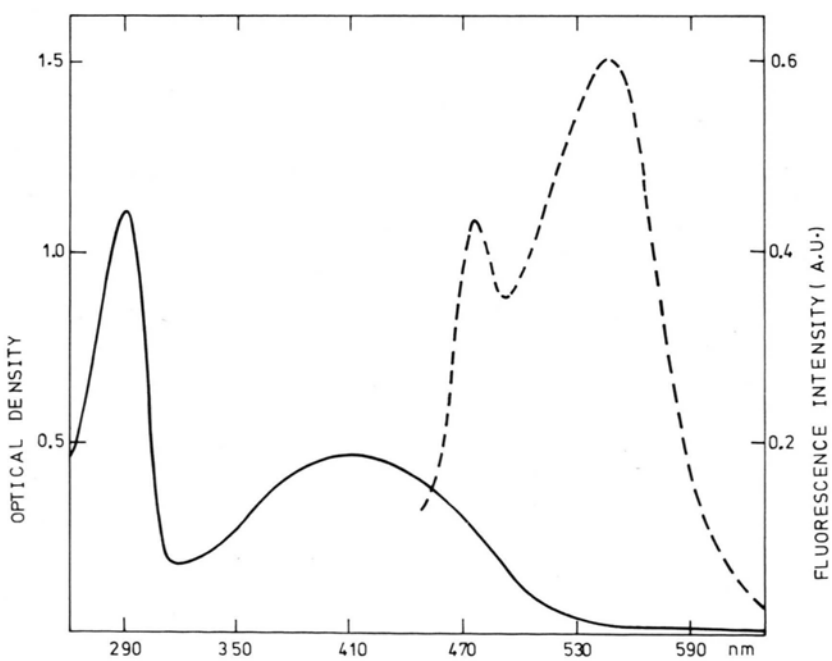

Fig. 2. Absorption (——) and fluorescence emission (– – –) spectra of hematoxylin (Merck, 0.066 mg/ml in acetate buffer at pH 4.3) after 9 days aging. Excitation wavelength was 410 nm.

tion spectrum somewhat different from that of the methanolic solution, which possesses maxima at 292 and 445 nm [10].

Hematoxylin is considered among the most widely used biological stains, but some properties and binding mechanism are still unknown. Although the present work is far from complete, the observations described above suggest that this dye has the potential to serve as an interesting fluorochrome for some cell components. Further investigations to analyze the fluorescence reaction induced by hematoxylin solutions are in course.

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