

## Neutral Red Fluorescence of Chromatin: Specificity and Binding Mechanism

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After treatment with neutral red at low concentrations ( $10^{-6}$ ,  $10^{-5}$  M), the chromatin of chicken erythrocytes shows an intensive red fluorescence, which is reduced or practically abolished when nuclei become stained by using the dye at concentrations higher than  $10^{-4}$  M. Both the fluorescence and staining reactions are dependent on the DNA content of chromatin. Neutral red fluorescence of nuclei increases considerably after treatment with inorganic cations ( $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ), while previous treatments with methylene blue reduce the fluorescence intensity. The possibility that chromatin fluorescence depends on the intercalative binding of neutral red is suggested.

Neutral red is a basic azine dye which is widely used in numerous staining techniques because of its affinity for chromatin [1, 2] and its potential as a vital dye [3, 4]. Although the fluorescence properties of neutral red are known [5], the use of this dye as a direct fluorochrome has been scarcely explored.

The chemical structure of neutral red shows interesting similarities with other planar cationic dyes belonging to the acridine, thiazine, and xanthene groups [2]. Like these dyes, neutral red [6] as well as other azine derivatives [7, 8] were shown to interact with DNA. Basic planar dyes bind to nucleic acids by two different mechanisms, namely, an external interaction with phosphate groups, and the intercalative binding mode, in which the dye monomer is located between two base pairs [9–11]. The aim of this work is to describe the chromatin fluorescence and staining characteristics by neutral red in terms of binding mechanisms.

Smears of chicken blood were fixed in methanol for 5 min and air dried. Staining was performed at room temperature by using neutral red (Merck, without further purification) solutions in distilled water, diluted from a  $10^{-3}$  M stock solution. Staining time was 5 min after which slides were briefly washed in distilled water and air dried. Before

staining, the following extraction procedures were applied: 5% trichloroacetic acid (TCA) at boiling temperature for 30 min; DNase I (Serva, 0.5 mg/ml in 1 mM  $\text{MgCl}_2$ ) and RNase A (Sigma, 1 mg/ml in distilled water), both used at room temperature for 2 h. Some stained smears were subjected to posttreatments with 2% aqueous solutions of potassium aluminium sulfate and barium chloride for 3 min. In other cases, neutral red staining was carried out after treatment with methylene blue (Fluka). Observations were made in a Zeiss Photomicroscope III equipped with an epifluorescence condenser III RS and the filter set for green exciting light (546 nm). Cytofluorometric and cytophotometric measurements were carried out according to the previously described procedures [12, 13].

At high concentration ( $> 10^{-4}$  M) neutral red stained the chromatin from erythrocyte nuclei in a deep red color. On the contrary, smears subjected to lower dye concentrations ( $< 10^{-4}$  M) showed unstained nuclei under bright field illumination but an intensively red chromatin fluorescence. Extraction procedures such as TCA and DNase abolished the staining and fluorescence reaction of nuclei by neutral red, while treatment with RNase was without effect. These results indicate that DNA is the chromatin component responsible for dye binding.

The intensity of chromatin staining and fluorescence after treatment with different concentrations of neutral red is shown in Fig. 1. At dye concentration higher than  $10^{-4}$  M, the erythrocyte cytoplasm

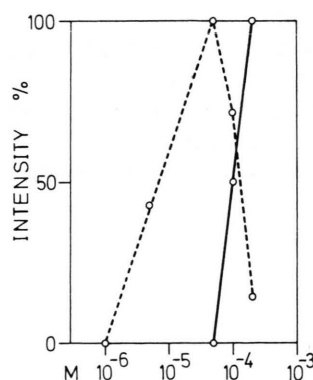


Fig. 1. Staining (—) and fluorescence (---) intensities of chicken erythrocyte nuclei after treatment with different concentrations of neutral red. Staining intensity was analyzed at 510 nm [13]; fluorescence measurements were made within the first 5 seconds on at least 3 microscopic fields [12].

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showed an increasing red emission, meanwhile nuclei appeared with a decreasing fluorescence. It is already known that the emission characteristics of fluorochromes are strongly dependent on the concentration and the type of binding mechanism to nucleic acids [14–16]. The fluorescence intensity increases up to the intercalation sites by monomeric dyes become saturated, and then quenching appears as result of the external binding of dye aggregates to phosphate groups. As revealed for other dyes, both binding modes have been shown to occur in the case of neutral red [6]. Similar correlations between the dye concentration and the intensity of chromatin staining and fluorescence were also observed by using pyronin Y [17] and safranin O [18].

The differential dependence of the external and intercalative binding mode on the ionic strength is a well known feature for several dye-DNA complexes [11, 16]. The relation between neutral red staining or fluorescence, and the effect of inorganic cations was investigated by using posttreatments with potassium aluminium sulfate and barium chloride (Fig. 2A). The staining degree of nuclei subjected to neutral red at high concentration was abolished after treatment with inorganic cations, while chromatin fluorescence increased considerably. Since at this concentration fluorescence quenching occurs, the competition of  $Al^{3+}$  or  $Ba^{2+}$  for the phosphate groups could result in the enhancement of fluorescence by removal of most externally-bound dye molecules.

Competition experiments between neutral red and methylene blue on the chromatin fluorescence are shown in Fig. 2B. Previous treatment with methylene blue caused either reduction or extinction of the neutral red fluorescence when the thiazine dye was applied at concentrations  $2.5 \times 10^{-5}$  or  $10^{-4}$  M, respectively. Under these conditions, the effect of the intercalating dye methylene blue [19, 20] on the neutral red fluorescence suggests compe-

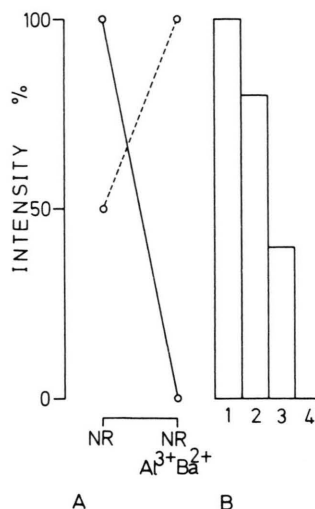


Fig. 2. A: Effect of the posttreatment with 2% solutions of either potassium aluminium sulfate ( $Al^{3+}$ ) or barium chloride ( $Ba^{2+}$ ) on neutral red fluorescence (---) or staining reaction (—) of erythrocyte nuclei. The dye was used at  $6.8 \times 10^{-4}$  M. B: Effect of the previous staining with methylene blue on the fluorescence reaction of chromatin by neutral red ( $3.4 \times 10^{-5}$  M). 1: Control smear treated with neutral red alone ( $3.4 \times 10^{-5}$  M). Methylene blue was used at the following concentrations, 2:  $10^{-5}$  M; 3:  $2.5 \times 10^{-5}$  M; 4:  $10^{-4}$  M.

tition for the same binding sites on chromatin [18]. Taking these results into account, it seems logical to assume that chromatin fluorescence by neutral red at low concentrations is essentially caused by intercalation, while external interactions resulting in dye stacking are responsible for the staining reaction.

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