SYNTHESIS OF DYES OF HISTOLOGICAL/HISTOCHEMICAL INTEREST

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Lucrarea de faţă dezbate patru coloranţi: albastru de metilen, roşu neutru, eozină şi Orange G, aparţinând la patru clase diferite (triazinic, phenazinic, xantenic şi monoazo, respectiv), atât din punct de vedere chimic cât şi biologic (în special de la histologice).

Au fost testate diferite metode de purificare. Calităţile IR spectrale au fost analizate în pastilă de KBr, fiind înregistrate cu un obiectiv Carl Zeiss Jena. Au fost testate de asemenea calităţile spectrale UV-VIS, împreună cu tehnica histologică, care a fost folosită pentru aplicarea noilor coloranţi.

The present paper discusses four dyes: Methylene Blue, Neutral Red, Eozine and Orange G, belonging to four different classes (triazinic, phenazinic, xantenic and monoazo, respectively) both from chemical and from biological (mainly histological) points of view.

Different methods of purification were tested. The IR spectral qualities were analyzed in KBr pill, being recorded with a Carl Zeiss Jena. Also, the UV-VIS spectral qualities were tested, together with the histological technique which was used to apply the new dyes.

Keywords: dyes, synthesis, Methylene blue, Eozine, Neutral Red, Orange G, histological applications

1. Introduction

At present, the dyes Methylene Blue, Neutral Red, Eozine and Orange G, belonging to four different classes (triazinic, phenazinic, xantenic and monoazo) can be applied in the following domains: textile, food, cosmetic, biologic, biochemical, laser, etc.

As the research in biology, mainly in the anatomical-morphological studies of the animal, plant and microorganism world, a part of the textile dyes

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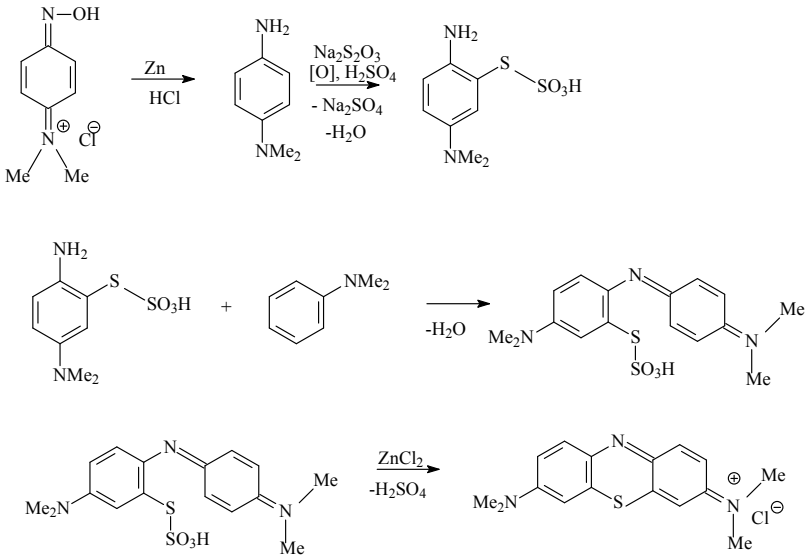
have been applied in order to evidence cells and tissues, as well as their intimate structures (stomoplasma, nucleus).

In the colouring process, the aims of these two operations differ considerably, depending on the substrate on which are applied: histological material or textile fibers. In this respect, the industrial dyeing process aims to obtain a uniform colour, while for the histological targets a differentiated colouring (in other words, different colours corresponding to different structures). Almost all dyes which are used in histological techniques have an aromatic structure. They are classified according to their chemical structure, taking into account especially the groups which have the capacity of colouring [1]. Some dyes of this type are used in studying DNA or polypeptides. Most of them belong to acridines, but there are dyes with similar properties in the tiazines class – Methylene Blue, or in the triaryl methane class – Violet Crystal [2].

This paper analyses the use of four dyes: Methylene Blue, Neutral red, Eozine and Orange G.

2. Experimental

Methylene Blue is prepared in the following succession reactions:



The reduction of 4-nitro-dimethylaniline proceeds according to literature [2]. The neutral solution of dimethyl-1,4-phenylendiamine is put in a three-necked flask equipped with stirrer, thermometer and reflux condenser. An amount of 26g $Na_2S_2O_3$ dissolved in 50 mL water and 9.6g $K_2Cr_2O_7$ into 150 mL water are rapidly added to it. The temperature is kept at 16-20^{0}C with ice. The whole mixture is continuously stirred for 2.5 hours at 18-20^{0}C, the reaction mass turning

brown. The resulted product is added to 10g dimethylaniline in 11mL HCl and 15 g ice and is vigorously stirred.

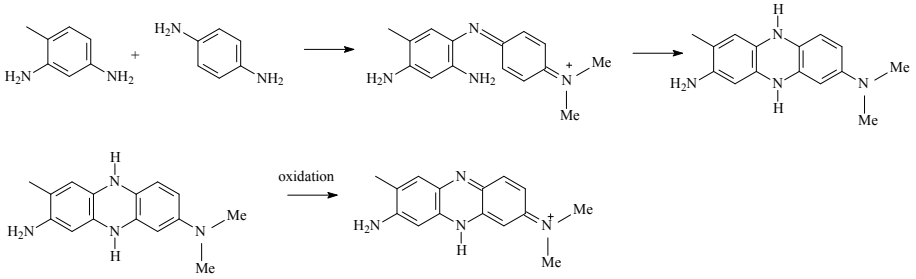
An amount of 28g $K_2Cr_2O_7$ dissolved into 280mL water and 7.2g $ZnCl_2$ dissolved into 150mL water were added upon vigorous stirring, the temperature being maintained at 10-12^{0}C.

The reaction mass is heated to boil, its colour going through various shades of green, finally becoming black-blue. The filtered product is heated in 100mL water with 10g NaCl. It is cooled and filtered off; the process is repeated 3 times, until the residue colour is dark indigo.

Small amount of HCl and $ZnCl_2$ are added at previously boiled filtrate at 80^{0}C and at the end 20g NaCl are put into it. The reaction mass is stirred for 30 minutes, cooled, pressed and filtered off. The final product is brown, with a metallic gloss [1].

In this way, 29g Methylene Blue are prepared, the reaction yield being 89%.

Neutral Red is prepared in the following succession of reactions:



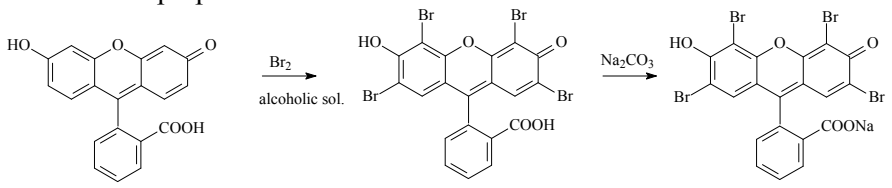
The reduction of 4-nitro-dimethylaniline and of 2,4-toluenediamine is performed according to the data in the literature [3,4].

Neutral Red is obtained by oxidizing the mixture formed of N,N-dimethyl-*p*-phenilendiamine (6g, 0.04 moles) and 2,4-toluenediamine (3.36g, 0.04 moles) in the presence of $FeCl_3$ (2mL), at low temperature (10-15^{0}C).

The indamine which is obtained as an intermediate, turns into the final product, at 76% yield. The dye is purified by turning into tin insoluble salt (with 5g $SnCl_2$ dissolved into 250mL water). The Neutral Red precipitates as an iodide with the help of 6g KI.

It is dissolved into 300mL water and reprecipitated with 5% KI solution. It is finally recrystallized from EtOH [5].

Eozine is prepared as follows:

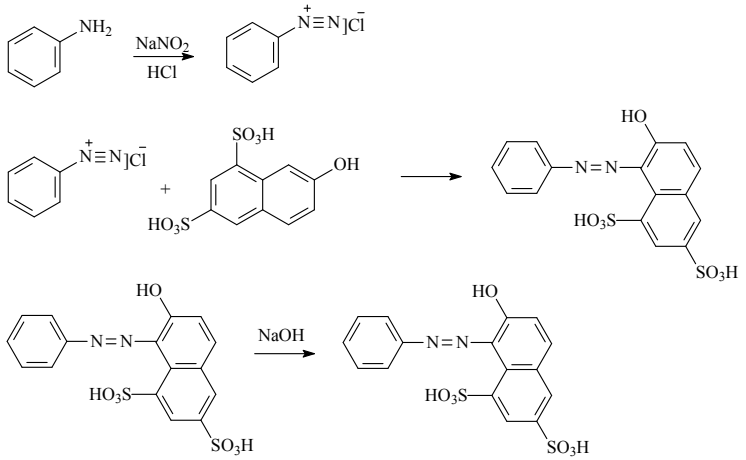


16.6g well pasted fluoresceine and 80 mL ethanol is introduced into a four-necked flask equipped with stirrer, bubber, dropping funnel and pipe of evacuation of HBr. Then 3.6g bromine is dripped while stirring the reaction mixture. After half of the bromine amount has been poured, the precipitate is formed in the solution, due to the dibrominefluoresceine, soluble in ethanol. We kept dripping bromine and Eozine started to precipitate from the solution. The stirring continued for two more hours and then it was filtered off, washed 3-4 times with 20mL alcohol and dried. The result is 30g Eozine (acid form – 93% yield).

In order to obtain Eozine disodic salt, 20g dried Eozine were mixed with 2g Na_2CO_3, some alcohol and 20mL water. The mixture was boiled to the boiling point in a round bottom flask until no more CO_2 was released. Afterwards, 50mL ethanol were added to it, the mixture was heated to the boiling point and filtered off. After 24 hours, the dye crystallized from the solution, in red-brownish crystals which were filtered off, washed with alcohol and dried at 40-50^{0}C [4]. 17g Eozine disodic salt was obtained at a yield of 85%.

Tetrabromofloresceine was treated with 15mL acetic anhydride and 15mL pyridine when the corresponding diacetate precipitated. This one turned into a disodic salt using 13mL MeONa in MeOH and the resulted salt was acidified with 10mL HCl to get a more purified Eozine [6].

Orange G was prepared in the following succession of reactions:



A solution of 5g $NaNO_2$ and 25mL water was added, while stirring, over a mixture of 7g (6.8mL) aniline, 37.8g (36mL) HCl 37% and 150mL water. The solution was poured in small amounts, every 10 minutes. To finalize the diazotization process, the stirring continued for another 15 minutes. In this time, the temperature was kept below 5^{0}C. The resulted diazonium salt was dipped in the following 15 minutes over a solution formed from 17.15g acid G, 6.5g NaOH

and 350mL water. The temperature ranged from 0-5^{0}C and was kept at this level with an ice bath, while the reaction mass was stirred continuously. When the addition was over, stirring continued for another 3 hours at 5-10^{0}C. The completion of reaction was checked by paper chromatography. Then the reaction mass was salified with NaCl, the precipitate being filtrated and dried [7], the reaction yield being 74%.

Orange G was purified in the following way: the dye was dissolved into 100 mL water and then it was filtered. The resulted solution was heated to the boiling point and another 35g NaOAc were added to precipitate the dye. The precipitate was then filtered using a Buchner funnel and then dried. Finally it was boiled in 250mL EtOH 95%. The Orange G suspension was isolated from ethanol by filtration. The treatment with ethanol was repeated several times [8].

The dyes were analysed qualitatively in KBr pill by IR, using Carl Zeiss-Jena UR 20 spectrometer. Their UV-VIS spectra were recorded on a SPECORD UV-VIS Carl Zeiss Jena spectrophotometer.

In order to apply the synthesized dyes, we went through the following steps: dissection of the animal and sampling of organs, preparation of the fixation liquid, washing, dehydration of the samples, their impregnation with hydrocarbons (inclusion into paraffin wax), sectioning at the microtone, fixing the sections on slides, colouring the slides, classifying the coloured tissues and examining at the microscope.

3. Results and discussion

The syntheses which were carried out had the purpose of deciding the best conditions in which these dyes could be prepared. As for the Methylene Blue is concerned, the factors which influence the reaction yield are: the rapid addition of dimethyl-1,4-phenylene-diamine, of the aqueous solution of $Na_2S_2O_3$ and $K_2Cr_2O_7$ respectively, a strict observance of the temperature level in all the reactions, and a vigorous stirring of the reaction mixture. The purification of Methylene Blue was done with great difficulty because of the inflammable ethanol vapours.

The preparation of Neutral Red was done in good conditions; with some impediments in its purification (crystallization from ethanol 95%).

Eozine was prepared via two different methods. In both of them, bromine was used in excess in order to form HBr.

In the synthesis of Orange G, in the first stage, an excess of $NaNO_2$ was used in the diazotizing of aniline. The yield and purity of the product depended on the rate of nitrite solution addition. For this reason, the solution was added in small portions during about 10-15 minutes. The coupling reaction with acid G was done by dripping while stirring continuously the diazonium salt over the coupling component and not vice-versa.

Different methods of purification were tested with some difficulties. By analyzing qualitatively the IR spectra in KBr pill of these dyes, the following functional groups were found, according to Table 1.

Table 1

Functional group found using IR spectra

Dye	Functional group	IR band (cm^{-1})
Methylene Blue	—C=N —C=S Ar-N $(Alkyl)_2$ m-substituted benzene	1640 1389 1369 897
Natural Red	$-NH_2$ (aril-NH_2) —C=N Ar-N $(Alkyl)_2$ m-substituted benzene	3434.07 1520 1546.9 869.03
Eozine	aromatic ether C-OH aromatic ring -COOH	1150.87 1340.67 980 1580
Orange G	-OH -N=N- $-SO_3H$ aromatic ring naphthalene ring	3250.13 1051.6 1198 970 684.80

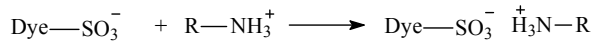
By qualitatively studying the UV-VIS spectra in water as solvent, the maximum absorption values at well-determined concentration were found, according to Table 2.

The results of the histological colourations were listed in Table 3. They correspond to the standard colours which are indicated in literature. These dyes were applied using the histological techniques for biological material and different types of tissues.

When applying on the analyzed biological material, the basic dyes (Methylene Blue, Neutral Red) containing disubstituted aminic groups are fixed on the R remained of glutamic or aspartic acid uninvolved in the peptidic link:

basic dye free group from proteins

When applying on the analyzed biological material, the acidic dyes (Eozine, Orange G) containing $–SO_3H$ or –COOH groups are fixed to the amino groups belonging to lysine, alginine, histidine:



acid dye histidine, lisine, alginine

In order to generate the histological colouring, the four dyes in question (Methylene Blue, Neutral Red, Eozine, and Orange G) require an advanced level of purity and they must meet some requirements:

- to be able to penetrate the cellular wall and create a balance between the hydrophilicity and hydrophobility of the dye, at a certain pH level by the selective cellular membrane (even when it is a dead membrane) or by the membrane they will go through.

- to keep the balance between the electric charges in the proteins and polysaccharides belonging to the membrane structure and the cellular wall.

Tests must be done at different pH levels, when the dyes are applied. The concentration of the dye is not so important, but when solution is too diluted, the colouration requires longer time. Therefore, histological colouring is no longer done with great accuracy, the kinetic conditions in this case being much different from those of a dye in aqueous solution or a solvent.

Table 2

Maximum absorption values for various concentrations of dyes in water

Dye	Concentration of the dye sol. % (w/v)	UV λ_{max} (nm)	VIS λ_{max} (nm)
Methylene Blue	(A): $5\cdot10^{-6}$ (B): $1\cdot10^{-6}$ (C): $0.5\cdot10^{-6}$	- 290 290	397 621 (shoulder) 648
Natural Red	(A): $2\cdot10^{-3}$ (B): $5\cdot10^{-4}$ (C): $1\cdot10^{-3}$	- 276 276	532 632 532
Eozine	(A): $1\cdot10^{-2}$ (B): $1\cdot10^{-3}$ (C): $5\cdot10^{-3}$	- 206 302	345 518
Orange G	(A): $2.5\cdot10^{-3}$	249-250 (plateau)	481-495 (plateau)

Table 3

Dyes application on various biological materials using various techniques

Dye	Application technique	Analyzed biological material (organism, organ)	Conclusions	
			Coloured cellular of tissular element	Colouring results
Methylene Blue	a) Histological colouring 1. Azan technique (trichromic method: -Azocarmin G -Methylene blue -Orange G)	Snail foot (Helix pomatia) Rat adrenal gland	- muscular fibers - collagen fibers - mucopoly-saccharides - conjunctive tissues	-red -vivid blue -vivid blue -blue

	2. Mallony technique (modified Azan)	Rat testicle	- gonies -seminiferous epithelie	-blue -red
	b) Vital colouring	Protozoan	cells	blue
Natural Red	a) Histological colouring trichromic technique	Rat pancreas	Glandular acini	red
	b) Vital colouring	Protozoan	cells	red
Eozine	1. Hemalaun - Mayer acid – Eozine Juanatre technique	Snail visceral mass Rat pancreas	Cytoplasm with intense secretary areas	pink
	2. Ferric hematoxiline – Eozine – Orange G	Rat cardiac muscle Carp ovary	Muscle fibres	pink
Orange G	1. Azan technique	Snail foot	Cytoplasmatic territories with secretary	orange-yellow orange - red
	2. Domeneci technique (Toluidine Blue, Eozine, Orange G)	Rat pancreas	glandular areas	yellow

4. Conclusions

Four dyes with many applications in histological and histochemical fields were synthesized. Different methods of purification were applied. The dyes were characterized by IR and UV-VIS spectra. Chemical groups and the absorption maximum values could be identified in the tested structures. By using current histological techniques, the above-mentioned dyes could be applied on a various histological material leading to different colourations – depending on the type of cell or tissue.

In order to participate in the histological colouring, the four dyes in question (Methylene Blue, Neutral Red, Eozine, and Orange G) require an advanced level of purity. The dyes should be applied at specific pH levels.

R E F E R E N C E S

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