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COLORIMETRIC DETERMINATION OF DIAZO-DYES AFFINITY FOR ELASTIC TISSUE AND MODEL PROTEINS

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Summary. *A model system was established which could serve for the quick and reliable estimation of the possibility to use aromatic dyes in staining elastic fibers and lamellas. Specially prepared samples of aorta wall tissue (taken from the corpse material of a healthy male, aged 31) and model proteins (collagen and elastin) were stained with acid diazo-dyes applied in water solution, 70% ethanol and dimethyl-sulfoxide. The amount of bonded dye was determined quantitatively according to the difference in absorbance of dye solution before and after the sample staining. The results of colorimetric measurements clearly showed a good correlation between the chemical structure of dyes and their binding to substrate, thus indicating the possibility that this model system cuold be used for simple and quick choice of dye solvent as well as particular aromatic dyes which should be used for elastic fibers dyeing in light microscopy.*

Key words: *Elastic tissue, elastin, collagen, diazo-dyes, colorimetric analysis*

Introduction

Displaying elastic fibers and lamellas in light microscopy is based on the application of numerous empirical methods whose evolution and modifications followed the better understanding of the chemical structure of elastic tissue components. The two component structure of elastic tissues, as well as the polar acid residues deficit in elastic tissue, account for the long-lasting efforts of researchers to establish a representative method which ensures reliable elastic fibers identification regardless of their topography.

Classical methods which use dyes of different chemical structures for elastic staining (orcein, Fe-hematoxylin, aldehyde-fuxin) usually fail to provide satisfactory results because the process of differentiation following the finalization of results greatly depends on the skills and experience of researchers (1). For that reason there is still a need to establish a representative procedure that will ensure a reliable identification of elastic fibers (2-4). Following the expansion of aromatic dyes application in histochemical analysis (5) we tried to establish a model system that would make easier the study of applied dyes affinities for elastica and their application in section dyeing in the light microscopy.

The usage of simple physical systems as the outer standard for quantitative research usually raises some doubts. Though the cell soluble fractions usually cover up the basic tissue properties (6), sometimes it is the only way to determine the conditions of selective dye binding to the substrate (7,8).

Material and methods

Samples of aorta walls from the corpse material of a healthy male, aged 31 have been taken as biological material. Cuttings were taken from the middle of the descending part of chest aorta, spherically along the whole circumference of the blood vessel. They were fixed into 10% buffered formalin and molded into paraffin by routine technique. After this, cuttings of 5 μm were made.

Elastin and collagen were obtained from Sigma Chem Co (USA). The masses of 1 g of refined proteins (either elastin or collagen) were fixed into buffered formalin and were prepared routinely without paraffin matrix. Samples have been stored at 0° C until their usage.

Before usage the prepared tissue and model proteins, elastin and collagen, were treated with xylol twice for 15 minutes, then 15 minutes with 96%, 80% and 70% ethanol and finally hydrated in distilled water. After each applied solvent, the samples were centrifuged 5 minutes at 3500 rpm in Serva II centrifuge in order for the solvent to be better removed. Viable tissue masses of elastin and collagen were then measured and stained by acid diazo-dyes for 24 hours with water, 70% ethanol (EtOH) and dimethyl-sulfoxide (DMSO), used as solvents. All the dyes used in this research were obtained from Sigma Chem Co (USA). Dye concentrations were adjusted according to the possibilities of colorimeter on which the absorption of dye solution was measured, before and after the sample treatment with dye.

An ISKRA MA 9507 colorimeter with 0.5 cm diameter sample tubes was employed for transparence measurements. Absorbance was calculated according to the expression:

$A = 2 - \log T(\%)$.

The amount of bonded dye was calculated according to the difference in absorbance before and after sample staining, according to the following expression:

$$\text{Absorbed dye}(\%) = \left(1 - \frac{\text{A (after staining)}}{\text{A (before staining)}}\right) \times 100$$

and then the amount of bonded dye was expressed as μmol per mass of tissue or refined proteins.

For some samples absorbance measurements were performed on SPECORD UV/Vis spectrophotometer too and the results obtained were in complete agreement with the results obtained by the colorimetric method.

Results

A comparative analysis of acid diazo-dyes absorption on elastic tissue and refined elastin and collagen was done. The data for transparence of dye solutions before and after the treatment of samples in different solvents, percentage (%) and the amount (μmol/g of sample) of dye bonded are given in Tables 1-3.

In Table 4 the comparative values of the amounts of dye bonded to model proteins and elastic tissue are showed. It can be seen that in most cases the total amount of dye bonded to elastin and collagen was a bit smaller than the amount of dye bonded to the tissue.

Discussion

The complex structure of elastic tissue gives few possibilities for investigating the quantitative binding of dyes to tissue components. Such results generally cannot be found in the literature except for the quantitative test of collagen (9). Some earlier studies showed significant differences in staining by common dyes and various sulfonic dyes (10). For that reason we tried to establish the possibility of quantification and evaluation of selective dye binding according to their structure based on the difference in quantitative binding of dye to elastic tissues and refined dominant constituents, collagen and elastin.

Table 1. Results of colorimetric measurements of diazo-dyes absorption on elastic tissue

Dye (concentration mmol/L)	Solvent	Mass of tissue (mg)	Solution volume (mL)	T (%) before/after	Dye absorbed (%)	Dye absorbed (μmol/g)
Acid Red 151	H_2O	2.6	3.0	54.0/64.0	27.6	14.0
(4.4×10^{-2})	EtOH	1.9	4.1	33.0/37.0	10.3	9.8
	DMSO	2.2	3.7	21.0 / 25.0	11.2	8.3
Acid Red 66	H_2O	2.9	4.0	16.5 / 18.5	6.5	3.1
(3.56×10^{-2})	EtOH	2.7	3.7	20.0 / 24.0	11.3	5.5
	DMSO	2.7	3.6	46.5 / 48.5	5.5	2.6
Acid Red 112	H_2O	3.4	3.9	43.5 / 45.0	4.1	1.7
(3.59×10^{-2})	EtOH	1.7	4.0	54.0 / 56.5	7.3	6.2
	DMSO	1.9	4.0	51.0 / 55.5	11.2	8.5
Acid Red 150	H_2O	2.3	4.2	61.5 / 66.0	14.5	4.8
(1.8×10^{-2})	EtOH	–	–	–	–	–
	DMSO	2.2	3.6	53.0 / 57.5	12.8	3.8
Acid Red 114	H_2O	1.3	3.5	46.5 / 57.5	27.7	27.0
(3.61×10^{-2})	EtOH	2.4	3.3	43.5 / 46.5	8.0	4.0
	DMSO	2.6	4.0	35.0 / 41.5	16.2	9.0

Table 2. Results of colorimetric measurements of diazo-dyes absorption on prepared elastin

Dye (concentration mmol/L)	Solvent	Mass of tissue (mg)	Solution volume (mL)	T (%) before/after	Dye absorbed (%)	Dye absorbed (μmol/g)
Acid Red 151	H_2O	4.3	5.0	59.5 / 63.5	12.5	6.4
(4.4×10^{-2})	EtOH	4.3	3.8	37.0 / 41.5	11.5	4.5
	DMSO	1.5	4.4	21.5 / 22.5	3.0	3.8
Acid Red 66	H_2O	1.5	4.0	30.0 / 31.0	2.7	2.6
(3.56×10^{-2})	EtOH	2.2	3.9	28.5 / 29.0	1.4	0.9
	DMSO	1.8	4.0	45.5 / 46.0	1.4	1.1
Acid Red 112	H_2O	2.1	4.2	21.5 / 22.5	3.0	2.1
(3.59×10^{-2})	EtOH	1.9	4.2	28.5 / 29.0	1.4	1.1
	DMSO	–	–	–	–	–
Acid Red 150	H_2O	2.9	4.0	40.5 / 45.0	11.7	2.9
(1.8×10^{-2})	EtOH	–	–	–	–	–
	DMSO	2.1	4.0	50.5 / 51.0	1.4	0.5
Acid Red 114	H_2O	2.1	4.4	58.0 / 66.0	23.7	17.9
(3.61×10^{-2})	EtOH	2.2	4.2	39.5 / 49.5	24.3	16.7
	DMSO	–	–	–	–	–

Table 3. Results of colorimetric measurements of diazo-dyes absorption on prepared collagen

Dye (concentration mmol/L)	Solvent	Mass of tissue (mg)	Solution volume (mL)	T (%) before/after	Dye absorbed (%)	Dye absorbed (μmol/g)
Acid Red 151	H_2O	1.2	4.0	42.5 / 44.0	4.1	5.9
(4.4×10^{-2})	EtOH	1.6	4.4	56.5 / 57.5	3.1	3.7
	DMSO	1.8	4.2	21.5 / 22.0	1.5	1.5
Acid Red 66	H_2O	2.9	3.9	16.5 / 17.0	1.7	0.8
(3.56×10^{-2})	EtOH	1.9	4.0	22.5 / 23.5	2.7	2.2
	DMSO	1.8	4.6	45.5 / 46.0	1.4	1.3
Acid Red 112	H_2O	4.2	3.8	59.5 / 63.5	12.5	4.1
(3.59×10^{-2})	EtOH	3.0	4.0	40.5 / 45.0	11.7	5.6
	DMSO	–	–	–	–	–
Acid Red 150	H_2O	4.0	4.2	40.5 / 43.5	7.9	1.5
(1.8×10^{-2})	EtOH	–	–	–	–	–
	DMSO	3.1	4.0	68.5 / 71.5	11.3	2.6
Acid Red 114	H_2O	4.0	4.0	58.0 / 68.0	29.2	10.5
(3.61×10^{-2})	EtOH	3.6	4.0	39.5 / 46.0	16.4	6.6
	DMSO	–	–	–	–	–

Table 4. Comparative analysis of amount of dye bonded on elastic tissue and model proteins

Dye	Sample	Dye absorbed (μmol/g)		
		H_2O	EtOH	DMSO
Acid Red 151	elastin	6.4	4.5	3.8
	collagen	5.9	3.7	1.5
	elastin + collagen	12.3	8.2	5.3
	tissue	14.0	9.8	8.3
Acid Red 66	elastin	2.6	0.9	1.1
	collagen	0.8	2.2	1.3
	elastin + collagen	3.4	3.1	2.4
	tissue	3.1	5.5	2.6
Acid Red 112	elastin	2.1	1.1	–
	collagen	4.1	5.6	–
	elastin + collagen	6.2	6.7	–
	tissue	1.7	6.2	8.5
Acid Red 150	elastin	2.9	–	0.5
	collagen	1.5	–	2.6
	elastin + collagen	4.4	–	3.1
	tissue	4.8	–	3.8
Acid Red 114	elastin	17.9	16.7	–
	collagen	10.5	6.6	–
	elastin + collagen	28.4	23.3	–
	tissue	27.0	4.0	9.0

Solutions containing formalin, ethanol (alcohol formalin) or picric acid (Bouin) as basic components are recommended as fixative agents in most of the procedures for elastica dyeing. According to the literature data (11) and our preliminary research (12) neutral solutions with high formalin content are most suitable. Neutral 10% formalin proved the most suitable for both staining of elastica with acid dyes and avoiding the risk of dissolving proteins in applied solvents.

Diazo-dyes applied in this study, Acid Red 151 (C.I.26900), Acid Red 66 (C.I.26905), Acid Red 150 (C.I.27190), Acid Red 112 (C.I.27195) and Acid Red 114 (C.I.23635), are derivatives of 2-naphtol and they all contain one or more SO_3^- groups at the benzene rings (Figure 1).

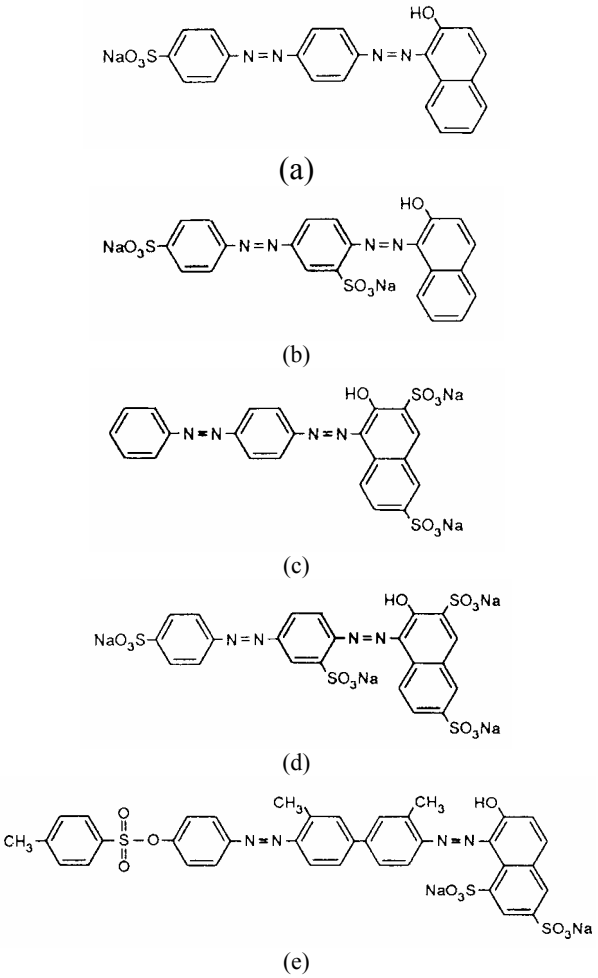

Fig. 1. Chemical structures of dyes used in this research for staining elastic tissue and refined proteins:
a) Acid Red 151 (C.I. 26900; Mr = 454.4),
b) Acid Red 66 (C.I.26905; Mr = 556.5),
c) Acid Red 150 (C.I. 27190; Mr = 556.5),
d) Acid Red 112 (C.I. 27195; Mr = 760.7),
e) Acid Red 114 (C.I. 23635; Mr = 830.8).

Acid Red 151 contains only one SO_3^- group, which is bonded at the end of the molecule. This position of the SO_3^- group can not influence the formation of the Van der Waals forces and hydrophobic binding of aromatic rings. That is why this dye having large electron delocalization had a great affinity for elastic tissues and model proteins. Acid Red 66 has one additional SO_3^- group at the second benzene ring. The addition of negative charge lowered the substrate affinity for dye, because it has an especially negative effect on hydrophobic binding, which emphasized the short-range Van der Waals forces. A dye does bind but to a lesser extent to the mentioned tissue structure and refined proteins. Two SO_3^- groups in Acid Red 150 are on the naphtol ring in positions 3 and 6 and they are directed so that the larger part of the molecule is not polar. Obviously, this can be the cause for the somewhat reinforced affinity of this dye for the substrate in comparison to Acid Red 66. Acid Red 112, tetrasulphonic homologue, stains poorly both elastica and collagen. The SO_3^- groups in Acid Red 112 are arranged in such a way that one of the SO_3^- groups is very close to the azo group, which makes the affinity of this dye lower for all tissue structures. Adding $-O-SO_2-C_6H_4-CH_3$ group significantly increases the molecular mass of Acid Red 114 and dye affinity for all tissue structures, regardless of the fact that large electron delocalization favors dye aggregation.

Between aromatic rings of dyes (or hydrated groups in dyes) and nonpolar areas of proteins, nonionic bonding appears because a large number of conjugated ring systems increases non-ionic bonding of dyes to the substrate (13). The exception in the expected increase of affinity comes from the presence of SO_3^- substituents. Introduction of SO_3^- groups decreases the affinity originating from the presence of benzene rings. These ionic groups in dyes prevent the bonding of close nonionic areas. This interference with Van der Waals forces also explains the staining properties of dyes that differ in the position of SO_3^- groups.

In model experiments with protein films Giles (14) demonstrated significant difference in the mode of binding between monosulfonated and disulfonated dyes. Monosulfonated dyes with the ionic group at the one end of the molecule penetrated the films with hydrocarbon part perpendicular to the film surface; the ionic group remained in the aqueous phase. Dyes with ionic groups at both ends of the molecule were aligned parallel to the protein film.

The results obtained from Acid Red 151 confirm the thesis that a dye orients the right part of a molecule to the long axes of collagen or elastin fibers. Clearly, the strong to intense coloration of tissue by Acid Red 151, which lacks other reactive groups must be ascribed to Van der Waals and hydrophobic bonding via the aromatic rings. The intensity of birefringerence of collagen and elastica stained with di- or polysulfonated dyes varied widely (Tables 1-3).

The expected increase of hydrophobic bonding of applied dyes by the replacement of water with ethanol solution due to the reduction of dye-water hydrogen bonding is not realized, most probably because of increased aggregation of dyes with great molecular masses.

According to the data obtained by colorimetric analysis of affinities of the applied diazo-dyes, it can be concluded that there is a connection between the structure and dye absorption and that this method can be successfully employed for a quick evaluation of whether a dye can be used in the light microscopy. Also, from Table 4 it can be seen that it is possible to follow the selectivity of dye bonding to the particular dominant tissue components elastin and collagen.

Proper selectivity of dye bonding to elastica was achieved by staining with Acid Red 151 from dye solution in DMSO, i.e. with dyes Acid Red 66 and Acid Red 150 from the water solution. Dye Acid Red 66 showed larger affinity to collagen from the solution in DMSO as well as dye Acid Red 150, while dye Acid Red 112 stained collagen from the ethanol solution well. In most cases, the investigated amount of the dye bonded to collagen and elastin was smaller than the amount of dye bonded to elastic tissue, which confirms the possibility to estimate whether or not a dye has the affinity for elastin or other tissue components.

The results were empirically confirmed by the staining of sections using the Horobin method (15) of direct staining.

Conclusions

The establishment of a model system in histochemistry usually provokes controversial opinions ranging from completely skeptical to highly enthusiastic, depending on the applicability of obtained results. We believe our model system can be used successfully for:

1. the choice of the aromatic dyes solvent which would enable selective receptivity of dyes to elastic fibers,
2. the estimation of the aromatic dye that shows better selective affinity to elastic fibers than to collagen fibers or other tissue components.

It is also worth mentioning that in all experiments a very small amount of a dye was consumed (10-20 mg for 1L of solution).

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KOLORIMETRIJSKO ODREĐIVANJE AFINITETA DIAZO-BOJA ZA ELASTIČNO TKIVO I MODEL PROTEINE

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Kratak sadržaj: *Na osnovu istraživanja uspostavljen je model sistem koji će poslužiti za brzu i pouzdanu procenu mogućnosti upotrebe aromatičnih boja za bojenje elastičnih vlakana. Posebno pripremljeni uzorci tkiva aortnog zida i model proteina (elastina i kolagena), bojeni su diazo-bojama koje su rastvarane u vodi, 70%-etanolu odnosno dimetilsulfoksidu. Količina vezane boje određivana je kvantitativno, na osnovu razlike u absorbanciji rastvora boje, pre i posle bojenja uzoraka. Rezultati sugerišu mogućnost upotrbe ovakvog model sistema za jednostavan i brz izbor rastvarača za boje, kao i diazo-boja koje bi se koristile u bojenju preseka u svetlosnoj mikroskopiji.*

Ključne reči: *Elastično tkivo, elastin, kolagen, diazo-boje, kolorimetrijska analiza*