Properties of Various Staining Solutions for Elastic Fibers —Comparison of Conventional Methods and the Alum Hematoxylin Methods for Staining Elastic Fibers—

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Summary: Elastic fibers were stained with alum hematoxylin adjusted to near neutral pH, and the characteristics of modified alum hematoxylin in the staining of elastic fibers were examined and compared with those of conventional elastic fiber stains.

Cartilage and sublingual mucous cells were strongly stained with orcein, resorcin-fuchsin, but not with modified alum hematoxylin, the latter showed an affinity for surface epithelial mucous cells of the stomach. In model tissue-experiments using synthetic polyamino acids, modified alum hematoxylin did not exhibit an affinity for tyrosine, whereas orcein, aldehyde-fuchsin, resorcin-fuchsin, and iron-hematoxylin did.

These findings and the results of various blocking reactions prior to staining, suggest that modified alum hematoxylin does not possess basic dye characteristics. The staining mechanisms of modified alum hematoxylin and conventional elastic fiber stains are discussed.

The alum hematoxylin used for nuclear staining does not exhibit an affinity for elastic fibers, whereas hematoxylin without metal mordants does, (Pizzolato and Lillie, 1969). Hematein, an oxidation product of hematoxylin, has a stronger affinity for these fibers than hematoxylin (Kaneko and Akita, 1977). We found that elastic fibers could be stained with alum hematoxylin adjusted to near neutral pH (Kaneko and Akita, 1978; Akita and Kaneko, 1981).

In 1933, Seki discussed the mechanism by which alum hematoxylin stains nuclei; cationic dye-metal complex with a binding capacity for nucleic acids was a generally

accepted concept at that time. Later, Lillie et al (1976a, b) demonstrated by acid nucleic acid extraction and DNase digestion procedures that alum hematoxylin did not bind to nucleic acid, but mainly to basic nucleoproteins. On the other hand, its binding to elastic fibers remains uncertain.

Using tissue sections and polyamino acid model tissues, we investigated the staining properties of hematoxylin for elastic fibers, and compared the results with conventional staining methods for elastic fibers.

Materials and Methods

Specimens from rabbit thyroid gland, hypophysis, pancreas, aorta, stomach, small intestine, sublingual gland, and skin were fixed in 10% formalin, embedded in paraffin and sectioned at a thickness of 5 μ m.

Preparation of staining solutions

Orcein, resorcin-fuchsin, iron-hematoxylin and aldehyde-fuchsin were prepared according to the procedures of Lillie and Fullmer (1976). Modified alum hematoxylin solutions for the staining of elastic fibers were prepared as follows: hematoxylin (100 mg) was dissolved in 100 ml of 70% ethanol and added to 20 mg of sodium iodate, 300 mg of potassium alum, 5 g of chloral hydrate, and 100 mg of citric acid. Alternatively, Mayer's hemalum (30 ml) was added to 70 ml of absolute ethanol and mixed well for 15–20 min. The solutions were filtered and just prior to use were adjusted to about pH 8 using saturated aqueous lithium carbonate.

Deparaffinized sections were stained with one of these staining solutions for the stated periods.

Preparation of model tissues

Model tissues were prepared according to the specifications of Cooper (1971), using the following synthetic polyamino acids (Sigma): poly-L-alanine (approx. mol. wt. 30,000), poly-L-arginine approx. mol. wt. 60,000), poly-S-benzyl-L-cysteine (approx. mol. wt. 7,700), poly-S-carbobenzyl-L-cysteine (approx. mol. wt. 10,400), poly-glycine (approx. mol. wt. 6,000), poly-L-histidine (approx. mol. wt. 80,000), poly-L-leucine (approx. mol. wt. 15,000), poly-L-methionine (approx. mol. wt. 30,000), poly-L-phenylalanine (approx. mol. wt. 9,000), poly-L-serine (approx. mol. wt. 5,000), poly-L-tryptophan (approx. mol. wt. 5,000), poly-L-tyrosine (approx. mol. wt. 130,000), and

poly-L-valine (approx. mol. wt. 9,000).

Blocking reactions

To examine the effects of various blocking reactions on the staining properties of the present elastic fiber stains, we performed aldehyde blocking (Pearse, 1968), benzoylation (Lillie and Fullmer, 1976), benzyl arginine blocking (Lillie et al., 1971), deamination (Pearse, 1968), methylation (Fisher and Lillie, 1954), nitration (Lillie and Donaldson, 1972), permanganate oxidation, and performic acid oxidation (Pearse, 1968).

Results

1. Tissue sections

The staining results obtained with the different elastic fiber stains are summarized in Table 1. Elastic fibers and thyroid colloid were stained with all the tested elastic fiber stains. Mucous cells of the sublingual gland and tracheal cartilage were strongly stained with all the stains except modified alum hematoxylin (Figs. 1–10). On the other hand, surface epithelial mucous cells of the stomach were most strongly stained with modified alum hematoxylin.

2. Model tissue sections

Among the synthetic polyamino acids used, tyrosine was distinctly stained with all the stains except modified alum hematoxylin. The latter stain showed an affinity for arginine and histidine (Figs. 11–17).

3. Blocking reactions

The effects of the blocking reactions on the staining of elastic fibers are shown in Table 2. The affinity of modified alum hematoxylin for elastic fibers was remarkably decreased by permanganate oxidation and deamination (Figs. 18–20), while these blocking reactions did not

Table 1. Reactions of various tissue components upon staining for elastic fibers.

	Orcein	Resorcin-fuchsin	Iron-hematoxylin	Aldehyde-fuchsin	Modified alum hematoxylin (pH 8.0)
Aorta					
Elastic fibers	4	4	4[4]	1(4)	4
Thyroid gland					
Colloid	2	2	3[2]	2(3)	2
Trachea					
Cartilage capsules	4	4	4[0~±]	4(4)	0~±
Sublingual gland					
mucous cells	3	3	4[±]	4(4)	±
Hypophysis					
Neurosecretory					
substances	1~2	1~2	4[2]	2(3)	1~2
Small intestine					
Goblet cells	3	3	±~1[0~±]	4(4)	0~±
Pancreas					
B-cells	2	2	3[1]	2(3)	±~1
Stomach					
Surface mucous					
cells	1	1	3[±~1]	1(4)	4
Skin					
Horny layer	1	1	4[4]	±(2)	2
Hair cortex	1	±	±[±]	2(4)	0

()=5-min oxidation with 0.3% $\text{KMnO}_4 \cdot \text{H}_2\text{SO}_4$, []=2-min differentiation with 1% FeCl_3 , 0=negative reaction, ±=feeble or equivocal reaction, 1~n=the higher the number, the greater the intensity of the reaction.

negatively affect the other staining methods. Methylation inhibited the staining of tracheal cartilage, sublingual mucous cells and goblet cell mucin by resorcin-fuchsin, orcein, aldehyde-fuchsin and iron-hematoxylin.

Discussion

Conventional elastic fiber stains exhibit an affinity for some tissue components other than elastic fibers. Resorcin-fuchsin stains the B-cells of the hypophyseal anterior lobe (Romeis, 1940), neurosecretory substances (Hiraoka and Imoto 1955; McGuire and Opel, 1969), cartilage, and some mucins (Puchtler et al., 1961), cartilage

capsules, interstitial matrix, and mast cell granules (Goldstein, 1962). Cartilage and mast cells (Unna, 1894), mucus (Zimmermann, 1898), nuclei, cartilage, mast cell granules, epithelium, collagen (Engle and Dempsey, 1954), and neurosecretory substances (Hiraoka and Imoto, 1955) are stained with orcein. According to Scott and Clayton (1953), aldehyde-fuchsin exhibits an affinity for hyaline cartilage and mast cells (strong reaction), goblet cells (moderate reaction) and B-cell granules of the islets of Langerhans, thyroid colloid, argentaffine cell granules, keratin and acrosomes of spermatozoa (weak reaction). Further, Verhoeff's (1908) iron-hematoxylin stains myelin sheaths as well as nuclei

Table 2. Effects of blocking reactions on the staining of elastic fibers.

	Orcein	Resorcin-fuchsin	Iron-hematoxylin	Aldehyde-fuchsin	Modified alum hematoxylin (pH 8.0)
Direct control	3	3	4	2	3
Aldehyde blocking	3	4	4	1~2	2
Benzoylation	4	4	4	3	2
Benzyl arginine blocking	4	4	4	2	3
Deamination	3	4	4	2~3	1
Dinitrophenylation	4	3	4	3	3
Methylation					
60°C, 4 hours	4	4	4	1~2	2
60°C, 8 hours	4	4	3~4	±~1	1~2
Nitration	4	4	4	4	1
Oxidation					
KMnO ₄ •H ₂ SO ₄	3	2	4	4	±
Performic acid	4	4	4	3~4	4

0=negative reaction, ±=feeble or equivocal reaction, 1~n=the higher the number, the greater the intensity of the reaction.

(Puchtler and Waldrop, 1979).

Modified alum hematoxylin did not show an affinity for the so-called acid mucopolysaccharide-rich tissue sites such as cartilage and mucous cells of the sublingual gland, which are stained with conventional elastic fiber stains. Fullmer and Lillie (1956) noted that orcein, which contains anionic, cationic and non-ionic compounds (Pearse, 1968), has some of the characteristics of a basic dye; it combines with nuclei (this reaction is prevented by brief methylation), and it also combines with the products of cysteine acid cleavage (this reaction is blocked by extended methylation). Scott and Clayton (1953) reported that in the absence of oxidation, aldehyde-fuchsin exhibits an affinity for strong sulfur acid. According to Bangle (1954), the violet or purple color seen upon aldehyde-fuchsin staining, may be ascribable to the formation of azomethines or Schiff's base between acetaldehyde and the open amino groups of the basic fuchsin dyes. Such a reaction introduces into the basic fuchsin molecules the indamine

group, —N=, which is a known basic chromophore (Bangle, 1954). Sumner (1965) suggested that post-oxidation, the staining of cystine-rich tissue components, such as neurosecretory substances, with aldehyde-fuchsin may be due to ionic links with the acidic products of cystine oxidation and that acidic mucins are probably stained by ionic linkage to sulfate groups. Concerning iron-hematoxylin, hematein and Fe⁺⁺⁺ form a variety of cationic, anionic and non-ionic chelates; and the ratio of these compounds changes with time (Puchtler and Waldrop, 1979). The staining compounds of resorcin-fuchsin remain to be fully elucidated. Since strongly basophilic materials such as cartilage and mast cell granules are stained with resorcin-fuchsin, it appears that conventional elastic fiber stains possess basic compounds or basic dye characteristics. On the other hand, modified alum hematoxylin does not stain the basophilic materials and does not show the staining pattern of basic dyes (Akita, 1981) after oxidation with permanganate or performic acid, suggest-

ing that it does not possess basic dye characteristics.

Fullmer and Lillie (1956) noted that orcein combines with elastin in some types of electrostatic bonding, probably not of the ionic type. Furthermore, based on methylation experiments, they concluded that elastic fiber stains such as orcein and resorcin-fuchsin probably react with an acid in elastin (Fullmer and Lillie, 1957). Puchtler et al. (1961) suggested that the binding of resorcin-fuchsin is due to non-ionic bonds, and Goldstein (1962) stated that the staining of elastic fibers with orcein, resorcin-fuchsin and aldehyde-fuchsin depends on a non-ionic link, probably a hydrogen bond, since the staining is inhibited by urea, a powerful hydrogen bonding agent. According to Puchtler and Waldrop (1979), the binding of Verhoeff's iron-hematoxylin apparently occurs mainly via van der Waals forces and hydrogen bonds.

Mowry (1978) suggested that aldehyde-fuchsin colors cystine-containing peptides and proteins such as elastic fibers. However, Puchtler et al. (1979) pointed out that since aldehyde-fuchsin does not color renal basement membranes, in which Böck (1978) demonstrated cystine, cystine does not play a role in the binding of this dye under the condition tested. In our poly-amino acid experiments, orcein, resorcin-fuchsin, aldehyde-fuchsin and iron-hematoxylin did show an affinity for tyrosine, whereas modified alum hematoxylin did not. However, in the present study, we did not determine whether the conventional stains reacted with tyrosine in the tissue sections, although we suspect that they do not always stain tyrosine-rich tissue components.

While the staining mechanism of modified alum hematoxylin remains unclear, it apparently differs from that of conventional stains. Further studies using suitable models with characteristic amino acids

in elastin, e.g. desmosine and isodesmosine, are needed to clarify the staining mechanism and the staining materials in elastic fibers.

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Explanation of Figures

Plate I

Figs. 1-10. Tracheal cartilage and sublingual mucous cells are strongly stained with orcein (Figs. 1 and 2), resorcin-fuchsin (Figs. 3 and 4), iron-hematoxylin (Figs. 5 and 6) and aldehyde-fuchsin (Figs. 7 and 8), but not with modified alum hematoxylin (Figs. 9 and 10).
×300.

Plate II

- Figs. 11-17. Orcein (Fig. 11), resorcin-fuchsin (Fig. 12), iron-hematoxylin (Fig. 13) and aldehyde-fuchsin (Fig. 14) show an affinity for the poly-L-tyrosine floccules in gelatin, but modified alum hematoxylin does not (Fig. 15). Poly-L-arginine (Fig. 16) and poly-L-histidine (Fig. 17) are stained with modified alum hematoxylin. The arrows indicate the floccules of polyamino acids. $\times 300$.
- Figs. 18-20. Elastic membranes and fine fibers in the aorta are clearly stained with modified alum hematoxylin (Fig. 18). The staining reactivity of elastic fibers is decreased by permanganate oxidation (Fig. 19) and deamination (Fig. 20). $\times 300$.