On Elastic Fibers Stain with Hematein and its Staining Mechanism

By

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Introduction

One of the most popular methods for staining elastic fibers is Verhoeff’s iron hematoxylin method (1908).

Lillie et al. (1960, 1965) have first demonstrated that rodent elastic tissues are stained blue with a 0.01% hematoxylin in 0.01 M phosphate buffer at pH 7.0. However, Brissie et al. (1974) have reported that a characteristic protein elastin (a specific component of elastic tissue) increases its electron opacity, but does not stain, with 1% hematoxylin in 50% ethanol (pH 1.3). Since a hematoxylin solution saturated with urea (a powerful hydrogen bonding agent) blocks the ultrastructural staining, they have suggested that a staining of elastin depends on the hydrogen bonding.

On the other hand, an active dye component in staining solution containing hematoxylin appears to be hematein, an oxidation product from hematoxylin. This may be supported by the findings that elastic fibers can be stained redish yellow with a staining solution prepared by diluting ripened 1% hematoxylin in an ethanol-water mixture (1:9 v/v) with an equal volume of water (Seki, 1933).

Little is known, however, of the staining properties of hematein for elastic fibers.

The present work was undertaken to prove this mechanism, using the hematein purified from a commercial source as dye and rabbit aorta as organ to be stained.

Materials and Methods

1. Preparation of hematein

Hematein was purified from commercial source by the method of Arshid et al. (1954). Hematein content in the preparation was spectro-
metrically assayed according to the procedure of Marshall et al. (1974).

2. Preparation of staining solutions

Original staining solutions were prepared by dissolving 50 mg of the purified hematein in each 50 ml of the following buffer solutions: Walpole acetate-HCl buffer (pH 1.0, 2.0, or 3.0), 0.1 M acetate buffer (pH 4.0 or 5.0), 0.1 M phosphate buffer (pH 6.0, 7.0 or 8.0), and 0.05 M carbonate-bicarbonate buffer (pH 9.0 or 10.0). Staining solutions to be used for testing mordant-effects on hematein staining consisted of 50 mg of each mordant in 50 ml of the above hematein-buffer solution.

The mordants used here are as follows: aluminum ammonium sulfate, ferric ammonium sulfate, phosphotungstic acid, and ammonium molybdate.

3. Staining procedures

Rabbit aorta was fixed in 10% formol, Zenker-formol or Bouin's fluid and was dehydrated in ethanol. Sections were cut in paraffin at 5 μ and successively stained by the following procedures:

1) Deparaffinize and remove mercury precipitates with iodine-sodium thiosulfate.
2) Bring sections to water in the usual manner.
3) Stain in the dye solution for 30–60 minutes or more.
4) Wash in tap water for 5 minutes.
5) Dehydrate in ethanol, clear in xylene and mount in balsam.

To investigate whether the binding mechanism of hematein with elastic fibers depends on hydrogen or ionic bonds, urea (a hydrogen bond-agent) and NaCl (an ionic bond-agent) were added to the original hematein solution, respectively, and their effects were examined. The concentrations of these chemicals were, in both cases, at 0.5 M, 1.0 M, 2.0 M, 4.0 M and at their saturated concentration.

Enzymatic digestion method (Ross and Bornstein, 1969) was also applied to ensure the elastin staining with hematein as follows. A 0.1% solution of elastase (P-L biochemical inc.) in 0.2 M Tris buffer (pH 8.8) was used for digestion of elastic fiber preparation for either 2, 4, 6 or 8 hours at 37°C. After treatment of elastase, sections were stained in each staining solution. Section which was treated with a buffer solution without enzyme, was used as control.

Results

1. Buffer solution containing hematein alone

With the staining solutions at pH 6.0–8.0, elastic fibers were stained light blue for 60 minutes, strong blue for 3–4 hours (Fig. 1). However, they were not stained beyond the above pH range, regardless to the
length of staining time.

2. Buffer solution containing hematein and mordant

The elastic fibers were stained red purple to purple for 30-60 minutes after their immersion in the hematein-ammonium molybdate solutions at pH 1.0-3.0. Under these conditions, in particular the internal elastic membranes were intensively stained red purple. When pH of the dye-mordant solution was kept between 4.0 and 8.0, the elastic fibers were stained blue or dark blue for 30-60 minutes (Fig. 2). However, when the staining was conducted at pH 9.0-10.0 for 30-60 minutes, not only elastic fibers but also other tissue components were stained orange faintly.

As was done with ammonium molybdate, other compounds such as phosphotungstic acid, aluminum ammonium sulfate and ferric ammonium sulfate, were found to be good mordants for staining elastic fibers with hematein (Figs. 3, 4 and 5). The colors and their intensities stained with hematein solution containing one of three mordants (during the times cited at various pHs) were essentially the same as those obtained with ammonium molybdate as mordant.

3. Pretreatment of elastic fibers with elastase

Pretreatment of elastic fibers with elastase for 4 hours had no significant effect on the hematein-staining of their fibers. When the periods of the pretreatment were prolonged up to 6 and 8 hours, the staining of elastic fibers was strikingly inhibited or abolished completely (Fig. 6).

4. Effect of sodium chloride or urea on hematein-staining

NaCl or urea, when added to the hematein in 0.1 M phosphate buffer (pH 6.0-8.0) or the hematein-mordant in 0.1 M phosphate buffer (pH 6.0-8.0), inhibited the hematein-staining of elastic fibers (Fig. 7). The degree of the inhibition increased with increasing amounts of NaCl or urea. NaCl at saturated concentration abolished the hematein-staining of the elastic membranes (tunica media) completely.

Discussion

In contrast to the report by Lillie who used hematoxylin solution, our hematein method, if it is adopted, requires only one hour for staining elastic fibers. Hence it is obvious that hematein has a strong affinity to elastic fibers compared with hematoxylin. Hematoxylin, when dissolved in methanol, is known to be stable at least for 25 minutes and then gradually converted to its oxidized form, hematein (Marshall et al., 1974). Therefore aging of the hematoxylin solution
should gain the hematein content thereby facilitating the staining of elastic fibers. In the present work, the addition of mordants to the hematein solutions augmented, in all cases, the staining ability of hematein to elastic fibers. This may be explained by following reasons:

1) Mordant combines with hematein to form hematein-lake which possesses high affinity to elastic fibers.

2) Hematein-lake is more recognizable under light microscope than hematein, because the former is manifested as more coarse particles (Ohkura, 1952).

As was done with the conventional method, the pretreatment of elastic fibers with elastase for 8 hours abolished the staining of elastic fibers completely. This indicates that hematein is specially combined with elastin moiety in the fibers.

From the results obtained with urea (a hydrogen bond-agent) and NaCl (an ionic bond-agent), it seems likely that both hydrogen bond and ionic bond in elastin participate in the staining of elastic fibers with hematein.

Summary

1. Hematein shows a strong affinity to elastic fibers at pH 6.0-8.0.
2. Affinity of hematein to elastic fibers can be augmented following addition of the mordants such as aluminum ammonium sulfate, ferric ammonium sulfate, phosphotungstic acid and ammonium molybdate.
3. Both hydrogen and ionic bonds in elastin participate in the staining of elastic fibers with hematein.

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References

5) Marshall, P.N. and Horobin, R.W.: A simple assay procedure for mixture of


Explanation of Plate I

All specimens were prepared from Bouin's fluid fixed rabbit aorta.

Fig. 1. Elastic tissue was stained blue with a solution containing 50 mg of hematein dissolved in 50 ml of 0.1 M phosphate buffer at pH 7.0. ×100

Figs. 2-5. Elastic tissue was stained more intensely than in Fig. 1 by addition of mordants such as ammonium molybdate (Fig. 2), phosphotungstic acid (Fig. 3), aluminum ammonium sulfate (Fig. 4) and ferric ammonium sulfate (Fig. 5), respectively 50 mg, in the same solution as in Fig. 1. ×100

Fig. 6. Section was incubated at 37°C for 8 hours in 0.2 M Tris buffer (pH 8.8) containing elastase at a concentration of 0.01% prior to similar staining as in Fig. 2. The staining reaction of the elastic tissue was almost completely abolished (cf. Fig. 2). Only collagenous fibers in the tunica adventitia and tunica media were stained grayish purple. ×100

Fig. 7. Section was stained with a solution containing 2.0 M NaCl in the same solution as in Fig. 2. The staining reaction of the elastic tissues, especially in the tunica media, was weakened by addition of NaCl (cf. Fig. 2). ×100