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Original Articles

Differential Staining of Collagen Types in Paraffin Sections: A Color Change in Degraded Forms

R.D. Lillie*, R.E. Tracy, P. Pizzolato, P.T. Donaldson, and C. Reynolds

Department of Pathology, Louisiana State University Medical Center, New Orleans, Louisiana, USA

Summary. The Herovici combination of picro methyl blue or aniline blue and picro acid fuchsin in proper proportion colors mature dense collagen red while reticulum and newly formed collagen are blue. Fetal collagen, wound healing collagen, subepidermal finely fibrillar collagen, deteriorating collagen in and over arterio-sclerotic lesions tend to take the blue stain. Arterial adventitial, deep dermal gastric submucosal collagens are red.

Denaturation of collagen with 2 N–3 N acetic acid or with neutral or slightly acid water (pH 4) at 95° C fragments coarse red collagen and turns its staining reaction toward blue. Renal and gastric mucosal reticulum and subepidermal collagen resist these treatments better than coarse collagen, the fine medial collagen of large arteries tends to retain morphologic integrity but alters its color to light blue. *Clostridum histolyticum* collagenase destroys the blue staining of renal, gastric mucosal and pulmonary parenchymal reticulum in 10–15 min, while it often takes an hour or more to destroy the red staining coarse collagens. Fibrous plaque collagen succumbs gradually to collagenase, while it appears to resist hot water denaturation better than the adventitial collagen of the same artery or that of deep dermis of the same patient. Unlike the allochrome and the periodic acid Schiff methods the blue Herovici reaction does not stain glomerular capillary and pulmonary capillary basement membranes.

The findings indicate the existence of tinctorially and chemically distinct collagens. The NK-19 lot of aniline blue was effective at a 1:19 aniline blue:acid fuchsin ratio. Herovici used a 1:2 ratio, other samples require intermediate ratios.

Key words: Arteriosclerosis – Fibrous plague – Aging – Wound healing.

Offprint requests to: Dr. R.E. Tracy, Department of Pathology, L.S.U. Medical Center, 1542 Tulane Avenue, New Orleans, Louisiana 70112, USA

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Introduction

Apart from fairly numerous methods combining elastin and collagen stains and combinations of Bielschowsky (1904) – Maresch (1905) silver methods for reticulum with Van Gieson stain, procedures demonstrating differing types of fibrillar connective tissue in differing color have been few.

Lillie's (1951, 1952) allochrome procedure combined the positive periodic acid Schiff (PAS) reaction (McManus, 1947; Lillie, 1947) of certain vascular and epithelial basement membranes (red) with a picro methyl blue (Curtis, 1905) connective tissue stain to give contrasting blue collagen and red membranes. Sweat, Puchtler and Woo have published a variant of this method (1964). Böck (1978a, b) indicates that the thiosulfation method of Castino and Bussolati (1974) by showing high sulfur collagens is demonstrating the same basement membrane structure as the PAS reaction. But this has not thus far been combined in a trichrome collagen method.

Ladeweicig (1938) reported a phosphotungstic acid sequence to a one solution aniline blue, orange G, acid fuchsin stain in which part of the connective tissue fibers stained red, part blue. Fibrin was also red, mucin and amyloid were light blue.

Our recent (1979) phosphomolybdic acid Victoria blue method gave dark blue dense collagen and light green areolar fibrils.

Clark included a naphthol yellow – HCl (flavianic acid), acid fuchsin, trypan blue method in the 1973 edition of Staining Procedures. This gave some red, some blue collagen. Herovici's (1963a) picric acid, methyl blue, acid fuchsin stain was reported to stain precollagen blue and collagen red. He used it later (1963b, 1964) on cervical cancer classification by its stromal nature.

Leather industry studies (Gustafson, 1956) indicate that some days exposure of hides to 3 N acetic acid occasions an irreversible 50% swelling of collagen, more pronounced than with formic, propionic or hydrochloric acids at the same pH level. This was thought to be accompanied by hydrogen bond cleavage and partial protein chain separation. We thought that such a procedure might be applied histochemically to afford some measure of collagen depolymerization.

Hot water hydrolysis was used by Mall (1891, 1896) to help distinguish reticulum and collagen. This test also seemed suitable for experimental exploration to see whether collagen could be altered toward "precollagen" as shown by the Herovici method.

The effect of clostridial collagenase on collagen and reticulum also merited experimental exploration.

Material and Methods

Material comprised skin, normal and arteriosclerotic arteries, kidney and lung to represent vascular and epithelial basement membranes, trachea for cartilage and collagens, and various other normal and pathological tissues taken from human autopsies on individuals ranging from stillborn to old age. The human material was supplemented with certain experimental animal material not readily available from human sources.

All material was studied as paraffin sections of tissue fixed, often in parallel, in neutral buffered formol and in Carnoy's ethanol:chloroform:acetic acid 6:3:1 mixture.

The dyes used were aniline blue NK-19 and acid fuchsin CR-28. Herovici mixed 50 cc 0.1% aqueous methyl blue (50 mg) with 50 cc 1.2% picric acid containing 100 mg (0.2%) acid fuchsin. To the mixture he added 10 cc glycerol and 0.2 cc 1.3% lithium carbonate (saturated) and stained 2 min. Our procedures are modifications of this mixture.

Since optimal concentrations of acid fuchsin, methyl blue and aniline blue in saturated picric acid have generally been 50–100 mg/100 cc picric solution, picro methyl blue (PMB), picro aniline blue (PAB) and picro acid fuchsin (PAF) were made at 0.1% (100 mg/100 cc saturated aqueous picric acid). After a successful preliminary trial with an old methyl blue sample, aniline blue was substituted when it was found that methyl blue was not available in our usual American biological stain catalogs.

A 15:30 PAB:PAF ratio duplicated the results of the Herovici mixture. Optimal red blue differentiation was obtained at 5:95 with our dye lots. Substitution of 0.25% HCl for Li_2CO_3 and glycerol gave some improvement of results. Thus a 40 cc batch is 2 PAB:38 PAF:0.1 HCl. This mixture was used in all studies reported here.

For some of the hot water denaturation tests a purified reagent was prepared: A liter of deionized distilled water was alkalinized by addition of 2 cc 28% NH_3 and boiled down to about 600 cc to drive off NH_3 and any amino chloramines which may have come from the local chlorination procedure for Mississippi River water. At the end of this treatment the pH had returned to about 4.5. $CaCO_3$, 2 g was then added and shaken thoroughly; 18 h later the water was redistilled in glass and a neutral distilled water was obtained. Exposure of sections to water at approximately 95° C was done by immersing the testing chamber in a bath of boiling water, thus avoiding the disruptive effect of bubble formation on the surface of the sections. The house supply of deionized distilled water (pH 4.0) was used. Repetition of these experiments with purified neutral water gave essentially similar staining results. Intervals were set at 5, 10, 15, and 20 min. Collodionizing of the sections was needed to avoid section losses at intervals of 30, 45, 60, and 120 min.

A series of Carnoy and formol fixed sections were subjected to clostridial collagenase digestion (Lillie and Fullmer, 1976, p. 302). Nutritional Biochemicals Lot 2659 at 10 mg/33 cc pH 7.5 0.05 M Tris HCl buffer was used first for 2 h, then at 1 h, 30 min and 15 min on arteries, skins, kidney, lung with artery and bronchiole, and antropyloric mucosa. Collodionization was necessary to prevent loss of sections.

Staining Results

Kidney showed sharp dark blue staining of the reticulum surrounding the renal tubules and the glomerular capsules, no staining of capillary tufts occurred. Fibers in the sheaths of small arteries and of the renal fibrous capsule were bright red. The reticulum of pelvic fatty tissue was blue. Tubular epithelium was faintly yellow, nuclei refractile and slightly stronger yellow. Erythrocytes were bright yellow or even orange yellow. Arteries showed deep red staining of adventitial collagen and blue staining of perivascular and adipose tissue areolar fibers. Media and intima normally presented mingling of light red to pink collagen, yellow muscle and wavy refractile light orange elastic fibers. In young infants light blue staining was seen in the inner layers of the intima. Fibrotically thickened intima of mature arteries was generally blue. In fibrous arteriosclerotic plaques the dense fibrous tissue was red; looser collagen in vacuolated and degenerating areas was blue. Skin of adults normally presented a narrow subepidermal zone where a few blue fibers were seen, representing the epidermal basement membrane and associated collagen. Most of the dense bundles of dermal collagen were brilliant red. Dark blue basement membranes surrounded sweat gland tubules and less constantly other skin glands. In infants the amount of blue subepidermal tissue was greater than in adults, and in stillborn infants collagen of dermis was almost exclusively blue. Lung presented scantly

blue fibrils in the parenchyma, not outlining capillaries as the PAS and allochrome methods do. Red stained collagen occurs around arteries and bronchioles, and also was found encapsulating light pink cartilage. Epithelia were light yellowish with deeper sometimes pink or orange nuclei. Cartilage nuclei were often pink. Striated muscle was yellow with fine blue reticulum, in places condensing to coarser red fibers. Gastric mucosa presented light yellow glands in a delicate blue reticulum, thickening basally in areas to red fibers. Submucosal collagen when dense was deep red and blue when loose and fine. Healing wounds of the skin in human and of the muscle in rats had exclusively blue staining of newly formed collagen.

Collagen Denaturing by Acetic Acid

Formol and Carnoy fixed paraffin sections were stained directly with the Herovici variant given above and after exposure to 3 N acetic acid: 1) 5 days at 23° C 2) 1 day at 50° C 3) $1^1/_2$ days at 50° C and 4) 1 day at 50° C in 3 N HAc containing 10% NaCl. Section losses were frequent, especially in group 4. Generally the formol fixed material in group 1 resembled the controls. In group 2 red stained collagen was reduced in amount, and in some sections converted to blue. In group 3 there was insufficient section survival for adequate appraisal. With Carnoy fixed material, some tests in which tissue survived showed collagen, normally red, replaced by purple or blue. In groups 2 and 3, normally blue renal reticulum was extracted and lost in Carnoy but not formol fixed material.

Exposure for 3 and 18 h at 50° C to 1 N and 2 N HAc, and at 50° C to 2 N HAc including 10% NaCl were tried. Using 1 N HAc no convincing alterations were produced. With 18 h 50° C in 2 N HAc partial alteration of normally red staining dermal and arterial adventitial collagen to reddish purple, to bluish purple and to blue occurred.

Hot Water Hydrolysis

After exposure of Carnoy fixed material to 95° C water the normally few blue fibers beneath the epidermis expanded in 5 min to a fairly broad blue zone, the moderately dense red dermal collagen shrank to dense red fragments in a pale bluish background. In 10–15 min, the amount of dense red dermal material decreased, the background faded to gray and then pink and except for a few blue fibrils in papillae the subepidermal zone faded to gray. In collodionized sections exposed for 30, 45, and 60 min to hot water, little further change was noted. At 120 min, dermal collagen became purple to blue. The subcutaneous reticulum colored blue throughout. Formol fixed tissue showed red dermis throughout a full hour, with little fiber shrinkage.

Arterial adventitia in Carnoy fixed material paralleled the behavior of dermal collagen. In formol fixed tissue some blue fibers appeared in adventitia with longer exposure. A fibrous plaque remained partly red, but showed a blue gray intimal area at 1 h.

In Carnoy fixed kidney, the blue staining of intertubular reticulum continued

deep blue in the medullary area, weakening in parts of the cortex, and focally losing all staining at 1 h. Periarterial stroma was altered from red to blue and renal capsule became blue. With formol fixation, blue reticulum was retained throughout. Some red was seen about arterioles. Arteries and capsule became largely blue in 1 h but retained some red staining.

In some stillborn and newborn infants formol fixation did not completely protect dermal and arterial adventitial collagen against 95° C water hydrolysis. In both of these locations partial collagen fragmentation appeared in as little as 10 min, and staining altered through purplish red to orange pink and light and deep blues in some fibers. The alteration was less than in Carnoy fixed tissues.

In hairless newborn mice perichondrial collagen stained red around faintly blue cartilage. In the dermis only a narrow deeply situated zone colored purplish red, being blue elsewhere. After 5 min 95° C hydrolysis all layers stained blue, both with formol and Carnoy fixations, and very little red remained in perichondrial collagen.

Clostridial Collagenase Digestion

At 1 h digestion dermal collagen was represented by scattered patches of amorphous blue to purple material. A fibrous plaque was pale pink and partly vacuolated. The adventitia was disorganized and replaced by fragmented red and bluish purple collagen. The gastric submucosal collagen, normally red, was fragmented and blue to purple. No blue reticulum was found in gastric mucosa, renal or pulmonary parenchyma at 2 h, 1 h, 30 or 15 min. The renal capsule was faintly bluish at 2 h, no arteries were stained.

In the shorter time the dermal collagen colored alternately red and pink at 15 min, paler at 30 min, and at 1 h was swollen, blurred bluish red alternating with clear areas. The arterial adventitia was first lighter and deeper red, at 30 min pale red and pink, at 60 min blue red and purple to pink. The dense areas of the fibrous plaque faded from red to pale red; the vacuolated degerating areas were yellow, deepening to orange at 60 min. The amount of blue staining developing in dermal and adventitial collagen varied in different sections, reaching its greatest development in 30–60 min. Reticulum of adipose tissue in periarterial and subcutaneous tissue stained blue or purple up to 1 h.

Discussion

Herovici (1962) stated that the blue staining by his mixture distinguished precollagen from red collagen. His precollagen is otherwise "young collagen" without statement as to how its age was established. Our experience with the chiefly blue stain of dermal collagen and the amount of blue fibrils in aortic intima of stillborn infants confirms that young collagen elects the blue dye. The replication of these findings in hairless newborn mice supports this view. The deposition of fine blue fibrils in the healing area of incised wounds of human skin and stomach and in experimental wounds in rat muscle further supports the view

that newly formed collagen selected the blue stain. All of these collagens stain normally red with the unmodified Van Gieson stain. The appearance of a finely fibrillar blue staining connective tissue immediately beneath the epidermis recalls Unna's (1902) observation of a subepidermal collagen which stained red by the Ehrlich-Biondi-Heidenhain acid fuchsin, orange G, methyl green method, while the rest of the dermis stained orange red.

Blue staining appears also in the degenerating arterial intima overlying fibrous arteriosclerotic plaques and in vacuolated areas of degeneration within them. (Overt atheromas were not represented in the material of this study.) This may be pointing to a depolymerizing of collagen which returns to the less organized form seen in the late embryonic stage.

Application of the acetic acid, the hot water and the collagenase tests also displaced Herovici staining of collagen from red toward blue, as well as obviously fragmenting the red staining coarse collagen.

The renal intertubular reticulum stained dark blue in the 2:38:0.1 PAB:PAF:HCl mixture. Capillary loops in glomeruli regularly remained unstained, as did also pulmonary capillaries. In this feature they apparently follow the behavior of the picromethyl blue component of Lillie's (1952) allochrome stain. The blue staining of renal reticulum, the subepidermal collagen and the gastric interglandular reticulum resisted both 2–3 N acetic acid and 95° C water much longer than did the red staining of the coarse dermal and arterial adventitial collagen. Collagenase took 1–2 h to hydrolyze the coarse dermal and arterial adventitial collagen but destroyed the staining capacity of renal reticulum in 5–10 minutes. These findings suggest that the red staining arises from a property of some collagens which is easily destroyed by gentle hydrolysis, converting them into the blue staining form. A depolarization should be considered.

While hot water hydrolysis largely or completely destroyed coarse dermal and arterial collagen in 1 h in Carnoy fixed tissue, with formol fixation these tissues resisted the changes in adult individuals. In neonatal tissue the formaldehyde protection was much less evident, though still present to a limited extent.

From the foregoing studies we recognize: 1) A coarse collagen typically stained red by the Herovici procedure which is rapidly altered by 2–3 N acetic acid, hydrolyzed by hot water and digested in an hour by *Clostridium histolyticum* collagenase. 2) A finely fibrillar subepidermal collagen staining blue by Herovici, which is present in all age groups, apparently scanty in young adults, and which is resistant to hot water and increased in demonstrable amount by hot water. 3) The blue staining renal intertubular reticulum which is relatively resistant to acetic acid and hot water hydrolysis but promptly destroyed by collagenase. 5) The finely fibrillar light red collagen of the arterial media which changes to light blue on 5–20 min 95° C hydrolysis, but does not alter morphologically. 5) The compact red staining collagen of the fibrous arteriosclerotic plaque which appears relatively resistant to hot water, both in lack of color change and obvious morphologic alteration. Collagenase gives gradual fading of the red. 6) The interglandular gastrointestinal reticulum which stains blue but apparently intergrades basally into slightly coarser red staining collagen. 7) The fine Van Gieson positive reticulum, stained blue by Herovici, which is seen in subcutaneous, periarterial and renal pelvic fatty tissue. This staining was little altered by acetic acid, hot water or collagenase.

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