STAINING METHODS FOR OSMIUM-METHACRYLATE SECTIONS*

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In the study of tissues by electron microscopy, it is desirable to have contiguous sections available for examination by light microscopy for purposes of orientation and selection of appropriate fields. Tissues fixed in osmium tetroxide and embedded in methacrylate often provide suboptimal results when stained by the usual methods for light microscopy.[1] The purpose of this investigation was to develop methods of staining these tissues and to determine which histochemical techniques could be applied without modification.

The procedures were used primarily on renal tissues obtained by needle biopsy. Excellent results were obtained with a number of different staining methods; several are illustrated in a color plate recently published.[2] Some of the techniques have been found to be applicable to other tissues, such as eyes, arteries, and developing teeth. Two of the methods yielded good results with the pituitary gland of the rat.

MATERIAL AND METHODS

Of the many groups of dyes tested, the tri-phenyl methane derivatives were the most satisfactory. These dyes included malachite green,‡ light green SF yellowish, fast green FCF, basic fuchsin, acid fuchsin, methyl violet,‡ crystal violet,‡ ethyl green, and aniline blue. Other satisfactory groups were: (a) nitro and azo dyes: picric acid, orange G, ponceau 2R, Biebrich scarlet, Bismarck brown R, Congo red; (b) quinone-imines: thionine, azure A, methylene blue, toluidine blue O,§ gallocyanin, Celestin blue B, safranin O,‡ and azocarmines B and G; (c) xanthenes: pyronin B, eosin Y, ethyl eosin, erythrosin, and phloxine B; (d) phthalocyanin: Alcian blue 8GS; and (e) natural dyes: hematoxylin and cochineal (carmine).

A new staining method was devised employing basic fuchsin, alum, and hematoxylin (FAH). Staining procedures which were modified were the hematoxylin and eosin, Mallory-Heidenhain "azan," Mallory connective tissue, Masson trichrome, colloidal iron stains, and the

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‡ Aqueous mount.

§ Acetone dehydrated.

methyl violet stain for amyloid. Staining methods applied without change were the periodic acid-Schiff, Feulgen, methenamine silver, von Kóssa, gallocyanin-chromalum, Alcian blue, and the following elastic tissue stains[3]: Gomori's aldehyde fuchsin, Weigert's resorcin fuchsin, Unna's orcein, and Verhoeff's iodine-iron-hematoxylin.

Preparation of Tissues

Renal tissue was obtained by needle biopsy[4] and other tissues by open biopsy or at necropsy. Tissues were cut into 1 mm. cubes and fixed for 1½ to 2 hours in 1 per cent osmium tetroxide buffered at pH 7.4 with veronal acetate.[5] Sucrose was added to the solution of osmium tetroxide according to the method of Caulfield.[6] The tissue was dehydrated in graded alcohols, embedded in n-butyl methacrylate containing 3 per cent paste catalyst (1,2-dichlorobenzoyl peroxide), and incubated at 45° C. for 18 to 24 hours. The details of the fixation and embedding procedure may be found elsewhere.[7]

Sections were cut with a Porter-Blum microtome at 1 to 6 μ, depending upon the stain to be employed (see individual staining procedures). Sections were floated onto a water bath containing 20 per cent acetone, mounted on albuminized slides, and air dried. Before staining, sections were passed through xylene to remove methacrylate and then through graded alcohols to water.

Staining Procedures

Fuchsin-Alum-Hematoxylin (FAH). Goodpasture's aniline carbol fuchsin solution, MacCallum's variant[8]:

30% alcohol	100 ml.
Basic fuchsin	0.59 gm.
Aniline	1 ml.
Phenol	1 gm.

Note: This solution is stable and may be reused for several months.

Method: (Sections cut at 1 to 2 μ.)

1. Stain in Goodpasture's solution for 1 minute.
2. Rinse quickly in distilled water.
3. Treat with 5% aqueous ferric ammonium sulfate for 5 minutes.
4. Rinse in distilled water.
5. Stain in Harris' hematoxylin[3] for 15 minutes.
6. Rinse in distilled water.
7. Rinse in tap water, 1 to 2 minutes.
8. Dehydrate quickly in 95% and 100% alcohols, clear in xylene, and mount.

Results: Nuclei, pink or blue-gray; endothelial cytoplasm, grayish rose; basement membrane, rose to purple; connective tissue fibers, blue-gray; hyalin of diabetes (early), bright rose; (late), purple; amyloid, bright rose; calcium, dark blue to black; pituitary acidophil granules, rose.

Hematoxylin and Eosin Variant.

Method: (Sections cut at 1 to 2 μ.)

1. Stain in Harris' hematoxylin for 1 to 2 minutes.

2. Wash in running tap water for 2 minutes.
3. Counterstain in 1% eosin Y in 0.1% aqueous solution of calcium chloride for 5 minutes.
4. Dehydrate quickly in 95% and 100% alcohols, clear in xylene, and mount.

Results: Nuclei, blue; cytoplasm, pink.

Note: 1% phloxine B in 0.1% $CaCl_2$ or 1% erythrosin in 0.01% $CaCl_2$ may be substituted for eosin Y.

Mallory-Heidenhain "Azan" Variant.

Mallory III solution[3]:

Aniline blue	0.5 gm.
Orange G	2 gm.
Acetic acid	8 ml.
Distilled water	100 ml.

Method: (Sections cut at 4 to 6 μ.)

1. Stain in 1% azocarmine B in 1% acetic acid for 4 hours at 50 to 55° C.
2. Rinse in distilled water.
3. Counterstain in Mallory III for 5 minutes (aniline blue in aqueous or oxalic acid solutions, and light green or fast green in acetic acid solution may be substituted.)
4. Rinse in distilled water.
5. Dehydrate in 95% and 100% alcohols, clear in xylene, and mount.

Note: A Mallory connective tissue variant may be employed by substituting azocarmine with 0.5% cold aqueous acid fuchsin for 30 minutes.

Results: Nuclei, red; basement membrane, blue; collagen, blue; glomerular fibrinoid, orange-red.

Masson Trichrome Variant.

Method: (Sections cut at 4 to 6 μ.)

1. Stain in Weigert's acid iron chloride hematoxylin (Lillie's variant[3]) for 10 minutes.
2. Rinse in distilled water.
3. Rinse in tap water for 3 minutes.
4. Stain in ponceau-acid fuchsin[3] for 30 minutes.
5. Rinse quickly in distilled water.
6. Stain in 2% light green SF yellowish in 2% acetic acid for 5 to 10 minutes. (1% fast green FCF in 1% acetic acid for 3 to 5 minutes may be substituted.)
7. Rinse quickly in distilled water.
8. Dehydrate in 95% alcohol (check degree of light green staining), 100% alcohol, clear in xylene, and mount.

Note: Because red tones were diminished by use of phosphomolybdic and phosphotungstic acid mordants (customarily applied in trichrome staining), these were omitted.

Results: Nuclei, dark gray; basement membranes and hyalin of diabetic glomerulosclerosis, green; collagen, green; arteriolar hyalin and fibrinoid, red.

Colloidal Iron Variants.

Method: (Section pituitary at 4 to 6 μ; other tissues at 1 to 2 μ.) Technique of Rinehart and Abul-Haj,[9] with the following modifications after the colloidal iron-ferrocyanide steps:

1. Rinse in distilled water.
2. Treat with 0.5% aqueous periodic acid or sulfurous acid (6 ml. of 10% sodium meta-bisulfite, 5 ml. of 1 N HCl, 100 ml. distilled water) for 5 minutes.
3. Wash in running tap water, 5 minutes.

4. Stain in Grenacher's alum carmine[3] for 40 minutes. (Omit for sections of pituitary.)
5. Rinse in distilled water.
6. Counterstain in 0.5% aqueous acid fuchsin for 15 minutes.
7. Rinse in distilled water.
8. Dehydrate in 95% and 100% alcohols, clear in xylene, and mount in polystyrene resin.[3]

Note: This technique may be applied to formol-paraffin sections if the staining time in acid fuchsin is reduced to ½ minute or less and followed by differentiation in tap water.

Results: Nuclei, blue or rose; cytoplasm of glomerular epithelium, light blue; cytoplasm of glomerular endothelium, pink; connective tissue ground substance, blue; collagen, pink; glomerular hyalin (diabetic), pink; basement membrane, pink; pituitary gland: acidophils, pink; basophils, blue; Golgi apparatus, pink.

Another variant of the colloidal iron technique is the application of the periodic acid-Schiff (PAS) reaction following the colloidal iron-ferrocyanide steps.[10] (Alcohol rinses are replaced with distilled water and the thiosulfate reducing rinse with a 5 minute tap water wash.) After the final sulfurous acid rinse nuclei may be stained with Harris' hematoxylin. The results given above will then be changed to light gray or blue nuclei and dark rose collagen, basement membranes, endothelial cytoplasm, and glomerular hyalin of diabetes.

Periodic Acid-Schiff.

Method: (Sections cut at 4 to 6 μ.) Method of McManus.[11] Counterstain with Harris' hematoxylin and fast green if nuclear and connective tissue staining is desired. Leucofuchsin (Schiff) reagents: cold aqueous preparation of Longley,[12] boiled aqueous preparation of Coleman,[13] or the following alcoholic preparation:

1. Mix together:
   100% alcohol ..... 70 ml.
   Basic fuchsin ..... 0.5 gm.
2. Mix, dissolve, and add to the above solution:
   Hydrochloric acid (conc.) ..... 3 ml.
   Distilled water ..... 30 ml.
   Sodium meta-bisulfite ..... 1 gm.
3. Store in tightly stoppered bottle in dark overnight until solution is amber. Shake with 0.3 gm. activated charcoal for 2 minutes and filter through Whatman #2 paper. Store in dark at 0 to 5° C. When the solution has further decolorized to pale buff, it is ready for use.

Note: 1. Two alternate oxidants for the Schiff reaction (1% potassium permanganate and 4% chromic acid) were tested, but the staining was less intense.
2. The aqueous leucofuchsin reagents listed above can be used for the Feulgen reaction[14] after hydrolyzing tissues for 30 minutes in 1 N HCl at 60° C.

Results: Positive PAS reaction in basement membrane, hyalin, "fibrinoid," and glycogen.

Methyl Violet Variant for Amyloid.

Method: (Sections cut at 4 to 6 μ.)

1. Stain in 1% aqueous methyl violet 2B (color index #680) for 5 minutes.
2. Rinse in distilled water (to redden amyloid).
3. Decolorize connective tissue quickly (to light blue) with 80% alcohol (see note below).
4. Stop decolorization with distilled water rinse.
5. Drain and mount in glycerol gelatin (Kaiser-Mallory).[8]

Note: Because the customary acetic acid differentiating rinse (Mallory, Lillie)[8] decolorized amyloid, it was replaced by alcohol.
Results: Amyloid, dark rose; other glomerular structures, light rose to light blue.

Discussion

Standard histologic staining methods were altered for application to osmium-methacrylate tissues by increasing staining times, omitting or varying some mordanting and differentiating rinses, and substituting dyes where the original did not stain. Tissue sections were cut at 1 to 6 μ, depending upon the staining procedure to be used.

Some sections mounted in Permount® (Fisher Scientific Company, New York) faded rapidly. Since Permount is excellent for preserving most tissue stains, fading is apparently peculiar to osmium-fixed, methacrylate-embedded tissues. An acrylic resin, Krylon ("Crystal Clear" #1303, Krylon, Inc., Norristown, Pennsylvania) was tried as a mounting medium without the use of cover slips,[15] and there was less fading. The refractive index of acrylic resins (1.49) was not high enough to give a sharp image under high dry lens; however, with immersion oil and the 97x lens, definition was far greater than in sections mounted in Permount.

The fuchsin-alum-hematoxylin (FAH) method was simple and rapid and gave constant results. Aniline carbol fuchsin, which demonstrated hyalin, amyloid, and pituitary acidophils in this technique, was selected because it could be counterstained and dehydrated without being decolorized.

The connective tissue stains which were adapted were Mallory-Heidenhain "azan," Masson trichrome, and Mallory connective tissue. Elimination of decolorizing and mordanting acids and careful control of staining times minimized the diffuse and variable staining reactions experienced by Houck and Dempsey.[1]

McManus' periodic acid-Schiff and Gomori's methenamine silver techniques were applied without variation. Both methods demonstrated hyalin, fibrinoid, basement membrane of renal glomeruli and tubules, and Bruch's membrane of the eye. In accordance with the experience of others,[16,17] methenamine silver provided greater contrast than PAS so that thinner sections (1 to 2 μ) could be examined; these provided better cytologic detail. The use of periodic acid as an oxidant in the methenamine silver method gave greater basement membrane contrast, whereas chromic acid oxidation provided nuclear detail. It has also been possible to show glycogen with the PAS reaction and reticulum fibers by using Wilder's ammoniacal silver nitrate in osmium-methacrylate tissues.[1]

A variant of the hematoxylin and eosin technique was developed by the addition of 0.01 to 0.1 per cent calcium chloride to the fluorans (eosins). This procedure intensified the staining reaction of the fluorans[3,18] and prevented their decolorization in dehydrating alcohols.

Nuclear chromatin was stained by the Feulgen and gallocyanin-chromalum[19] methods. A modification of the latter technique, using phloxine B as a counterstain, was developed by Runge, Vernier and Hartmann[20] for use on osmium-methacrylate tissues. However, this method has the disadvantage of requiring a staining time of 24 to 48 hours.

Nuclei were easily stained by methyl green-pyronin methods, but results were too capricious to make them valuable for histochemical determinations. A rapid nuclear and cytoplasmic stain was possible with chloroform extracted methyl green[18] counterstained with 0.5 per cent aqueous acid fuchsin; however, results varied with slight changes in staining times and rapidity of alcoholic dehydration.

Colloidal iron and Alcian blue[21] stains produced intense colors but did not appear specific for acid mucopolysaccharides. The colloidal iron variant could be used for staining of both osmium-methacrylate and formalin-paraffin tissues. Up to the present this has been the only procedure which has provided differential staining of the cells of the anterior pituitary following osmium-methacrylate treatment.

With the methyl violet and FAH stains amyloid was demonstrated and its presence confirmed by examining paraffin sections from the same specimens stained by standard techniques for amyloid.

Summary

A number of staining procedures were successfully applied to osmium-fixed, methacrylate-embedded tissues. A new technique employing fuchsin-alum-hematoxylin (FAH) was devised. Several of the histochemical reactions could be utilized without modification. It was necessary to vary a number of standard procedures because of the difficulty in counterstaining osmium-methacrylate tissues. By changes in mordants and decolorizers and by the substitution of dyes, variants of standard techniques gave results which reproduced those obtained with formalin-fixed, paraffin-embedded sections.

By the use of these techniques it was possible to obtain excellent material for examination by light microscopy. Moreover, contiguous sections were available for investigation by both electron and light microscopy.

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