A CONTRIBUTION TO STAINING METHODS.

I. A DIFFERENTIAL STAIN FOR CONNECTIVE-TISSUE FIBRILLÆ AND RETICULUM.
II. CHLORIDE OF IRON HÆMATOXYLIN FOR NUCLEI AND FIBRIN.
III. PHOSPHOTUNGSTIC ACID HÆMATOXYLIN FOR NEUROGLIA FIBRES.

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I. A Differential Stain for Connective-Tissue Fibrillæ and Reticulum.

The following method for staining connective tissue is simple and is believed to be better than any yet proposed for that purpose. It is not absolutely differential because, besides connective-tissue fibrillæ and reticulum, it also stains certain hyaline substances, but these latter usually are so different morphologically that confusion cannot arise. The method is also useful for the study of fibrin, smooth and striated muscle fibres, and amyloid. The manner of staining is in brief as follows:

1. Fix in corrosive sublimate solution or in Zenker's fluid.
2. Embed in celloidin or paraffin.
3. Stain sections in a $\frac{1}{20}$ to $\frac{1}{10}$ of a one per cent aqueous solution of acid fuchsin 1 to 3 minutes.
4. Wash in water.
5. Place in a 1 per cent aqueous solution of phosphomolybdic acid for 1 minute or longer (use platinum or glass needle).
6. Wash in two changes of water.
7. Stain in the following solution for 2 to 20 minutes or longer:

Aniline blue soluble in water (Grübler),	0.5
Orange G (Grübler)	2.0
Oxalic acid	2.0
Water 100.0	

8. Wash in water.
9. Dehydrate in 95 per cent alcohol.

10. Blot on the slide and clear in xylol, or clear in oleum origani cretici.

11. Xylol balsam.

The fibrillæ and reticulum of connective tissue, amyloid, mucus, and certain other hyaline substances stain blue; nuclei, protoplasm, elastic fibres, axis cylinders, neuroglia fibres, and fibrin, red; red-blood globules and myelin sheaths, yellow. The various structures do not stain with equal intensity, so that certain ones are brought out with great sharpness. This is particularly true of the fibrillæ and reticulum of connective tissue, also of fibrin and smooth and striated muscle fibres.

If it is desired to bring out the connective tissue as sharply as possible, omit the staining with acid fuchsin. Then the nuclei and protoplasm stain yellow and the blue fibrillæ and reticulum stand out more prominently.

The method is satisfactory only after hardening in corrosive sublimate solution or in Zenker's fluid, although fair results can be obtained after fixation by the method I have recommended for neuroglia fibres,[1] but the latter procedure is best suited to the central nervous system, under which subject it will be mentioned later. With alcohol and other fixatives everything stains blue.

The oxalic acid makes the aniline blue stain more intensely and quickly than it otherwise would. The orange G tends to limit the blue to connective tissue. The phosphomolybdic acid has two functions: it intensifies and fixes the acid fuchsin in certain histological elements while removing it from the connective tissue; it slows the action of the aniline blue and prevents it from gradually staining everything else in addition to the connective tissue.

The time of staining varies somewhat according to the structures it is desired to render prominent, the character of the tissue, and the thickness of the sections. Paraffin sections will stand longer staining than thick celloidin sections.

Elastic fibres stain pale pink or yellow. Sometimes the elastic laminæ of arteries seem to stain blue, but this is because the connec-

[1] *Journal of Experimental Medicine*, 1897, ii, p. 532.

tive tissue closely applied to the plates of elastic tissue stains deeply and conceals them.

This method is also useful in the study of fibrin, and of smooth and striated muscle fibres because they are brought out in sharp contrast to the connective tissue, especially if the staining with acid fuchsin is prolonged a little or if the sections are washed out for a few minutes in alcohol, which removes aniline blue rather quickly but does not affect the acid fuchsin.

The stain brings out clearly the deposit of amyloid in the tissues, especially the liver, because amyloid stains a light blue and stands out with the greatest sharpness from the red protoplasm of the liver cells. In places, where they have not degenerated, the connective-tissue fibrillæ can be seen running through or along side of the amyloid material.

The mucus in epithelial cells stains blue, as do also renal casts and certain other hyaline substances.

The method can be used also for the study of the central nervous system. It may be used after fixation in corrosive sublimate solution or in Zenker's fluid as already directed, or better still with slight modifications after the method I have recommended for the fixation of neuroglia fibres, namely:

Fixation.

1. Fixation of thin pieces of nervous tissue (2 to 5 mm. thick) in a 4 per cent aqueous solution of formaldehyde (10 per cent formol) for at least 4 days.
2. Saturated aqueous solution of picric acid 4 days or more.
3. 5 per cent aqueous solution of bichromate of ammonium 4 days in the incubator (use an abundance of the solution and change at the end of 24 hours so as to avoid any chance of a precipitate).
4. Alcohol. Embed in celloidin.

Staining Method.

1. 1 per cent aqueous solution of acid fuchsin, 2 to 5 minutes.
2. Wash quickly in water.
3. 1 per cent aqueous solution of phosphomolybdic acid, 1 to 2 minutes.
4. Wash in two changes of water.

5. 1 to 3 minutes in the aniline blue and orange G solution.
6. Wash in water.
7. Dehydrate in 95 per cent alcohol.
8. Blot and clear with xylol, or use oleum origani cretici.
9. Xylol balsam.

Connective tissue blue; neuroglia fibres deep red; axis cylinders and ganglion cells a lighter red.

II. Chloride of Iron Hæmatoxylin for Nuclei and Fibrin.

The results which may be obtained by this method are equally quick and satisfactory after all of the usual fixing reagents, except, perhaps, formaldehyde.

Celloidin or paraffin can be employed for embedding.

1. Stain sections on the slide for 3 to 5 minutes in a 10 per cent aqueous solution of ferric chloride.
2. Drain and blot the sections; then pour over them a few drops of a freshly prepared 1 per cent aqueous solution of hæmatoxylin. If all of the hæmatoxylin is precipitated by the excess of the ferric chloride, pour off the solution and add a fresh supply. In 3 to 5 minutes the sections will be colored a dark bluish black.
3. Wash in water.
4. Decolorize and differentiate in a $\frac{1}{4}$ per cent aqueous solution of ferric chloride. The sections should be kept constantly moving in the solution. The differentiation will be complete in a few seconds to one or more minutes.
5. Wash in water.
6. Dehydrate in alcohol.
7. Clear in oleum origani cretici.
8. Xylol balsam.

In the above directions definite strengths have been assigned to the solutions, but they may vary greatly without affecting the result. The important point is to get the sections stained deeply, and then to decolorize slowly. The differentiation can be stopped at any moment by transferring the sections to water. Sometimes it is advisable to examine the sections under the microscope to see if enough color has been extracted.

The strength of the hæmatoxylin solution is unimportant; it is

simply necessary to have enough hæmatoxylin to combine with all of the iron in and on the section. The simplest way is to dissolve by the aid of heat a pinch of the crystals in a few cubic centimetres of water in a test-tube. A little experience will determine about how much is needed. If a solution of hæmatoxylin more than one or two days old is used, the color obtained is grayish-blue and not so bright.

This method gives a sharp, permanent, dark blue stain to nuclei; it also stains fibrin of a grayish to dark blue color; if the decolorization is not carried too far, the contractile elements of striated muscle are brought out very sharply. In Zenker preparations the red globules appear of a greenish-gray color. Connective tissue is tinted a pale yellow. The nucleus of Amœba coli stains sharply by this method.

III. Phosphotungstic Acid Hæmatoxylin for Neuroglia Fibres.

This method was published originally in the Journal of Experimental Medicine (1898, III, p. 611). At that time the phosphotungstic acid manufactured by Merck was not pure. It contained a trace of phosphomolybdic acid and also some oxidizing agent which ripened hæmatoxylin at once. The staining solution made up according to the directions then given but with the pure phosphotungstic acid now manufactured gives negative results because the hæmatoxylin will not ripen spontaneously even after standing for months. The solution can be ripened, however, at once by the methods advised for alum hæmatoxylin; the best results are obtained by using peroxide of hydrogen.

To render the stain somewhat sharper, and to prevent the myelin sheaths from taking a bluish tint when the time of staining is prolonged, it has been found advisable to treat the sections with permanganate of potassium followed by oxalic acid.

The tissue should be fixed by the formaldehyde, picric acid, and bichromate of ammonium method described under the connective-tissue stain.

Staining Method.

1. Place the sections in a ½ per cent aqueous solution of permanganate of potassium for 15 to 30 minutes.

2. Wash in water.
3. 1 per cent aqueous solution of oxalic acid 15 to 30 minutes.
4. Wash in 2 or 3 changes of water.
5. Stain in the following solution 12 to 24 hours or longer.

Hæmatoxylin	0.1
Water	80.0
10 per cent aqueous solution of phosphotungstic acid (Merck)	20.0
Peroxide of hydrogen (U. S. P.)	0.2

Dissolve the hæmatoxylin in a little water by the aid of heat and add it, after cooling, to the rest of the water and the acid. Then add the peroxide of hydrogen. The solution keeps perfectly and can be used repeatedly.

6. Wash quickly in water.
7. Dehydrate quickly in 95 per cent alcohol.
8. Oleum origani cretici.
9. Xylol balsam.

Nuclei, neuroglia fibres, and fibrin, stain blue; axis cylinders and ganglion cells, pale pink; connective tissue, deep pink.

The blue color is a little sensitive to strong light, and on prolonged exposure will fade to pink.

If a permanent, isolated stain of the neuroglia fibres is desired, place the sections, after staining as directed in the phosphotungstic acid hæmatoxylin and washing in water, in a 30 per cent alcoholic solution of ferric chloride for 5 to 20 minutes (rarely longer); then wash in water and dehydrate as before. The nuclei, neuroglia fibres, and fibrin stand out sharply of a clear blue color; everything else is decolorized or appears of a pale yellowish or gray tint. The results obtained by this last step are practically identical with those obtained by either of the modified fibrin stains, and the method has the decided advantage of being applicable to any number of sections at once. The method will be found to bring out rather sharply in the nerve fibres outside of the cord the funnel-shaped markings in the myelin sheaths.

Phosphotungstic acid hæmatoxylin will be found occasionally useful as a stain for ordinary tissues hardened in various fixatives. Two to twenty-hours usually are required for staining. Nuclei, fibrin, elastic fibres, and the contractile elements of striated muscle stain blue; the other tissue elements stain pink to red.