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Demonstration of Spirochaetes

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Demonstration of spirochaetes in paraffin sections have been of great interest to me for several years. It is a known fact that spirochaetes are often very difficult to stain. I would like to share my experiences with other technicians on the staining of spirochaetes. The good staining results which I have enjoyed have been based on modifications of two well known methods for spirochaete demonstration. Following is my modification of Gabriel-Steiner's original technique, published in the *American Journal of Clinical Pathology*, May 1950.

A Rapid Technique for Demonstration of Treponema Pallidum (Syphilis)

Microtomy
Cut paraffin embedded sections at 4-6 micrometers.

Solutions

Gum Mastic Solution (Stock)

Gum mastic	50.0 gm
Alcohol, 100%	500.0 ml

Shake solution well and place in the dark for a week. Use the supernatant only when the color becomes clear yellow.

Uranium-Gum Mastic Solution
(Prepare Fresh)

Uranyl nitrate, 6 hydrat	2.0 gm
Alcohol, 100%	85.0 ml
Gum mastic stock solution	15.0 ml

Silver Nitrate Solution
(Place in 60° C oven for 30 minutes before using.)

Silver nitrate	1.0 gm
Distilled water	100.0 ml

Brenzcatechin Solution
(Prepare fresh and place in 60° C oven for 4 hours before using.)

Brenzcatechin or pyrocatechol	5.0 gm
Distilled water	100.0 ml

Alcohol Gum Mastic Solution
(Prepare fresh)

Gum mastic stock solution	25.0 ml
Alcohol, 100%	75.0 ml

Inter-Laboratory Routing

1. _____
2. _____
3. _____
4. _____

The inter-laboratory routing slip is provided for your convenience to ensure all histologists and pathologists within you facility have an opportunity to review Histo-Logic.

INSIDE THIS ISSUE

Staining Procedure

1. Deparaffinize slides in xylene for 15 minutes.
2. Place slides in 2 changes of 100% alcohol.
3. Place slides in uranium-gum mastic solution for 5 minutes.
4. Rinse in distilled water for 2 minutes.
5. Place in 1% silver nitrate solution for 3 hours in a 60° C oven.
6. Rinse in distilled water for 2 minutes.
7. Place in 95% alcohol for 2 minutes.
8. Place in 100% alcohol for 2 minutes.
9. Place in alcohol gum mastic solution for 5 minutes.
10. Place in 5% Brenzcatechin solution for 45 minutes, in a 60° C oven. (Shake solution before using.)
11. Rinse in distilled water for 2 minutes.
12. Dehydrate through alcohols 70%, 95%, 100% and acetone for 1 minute each.
13. Clear with xylene 2 times, 2 minutes each.
14. Mount coverslip with resinous media.

Results

Spirochaetes are black (Fig. 1). Other tissue elements are yellow to light brown.

Notes

1. All glassware used for gum mastic solution must be cleaned with 100% alcohol.
2. All glassware used for silver nitrate must be cleaned well and rinsed with distilled water.
3. A good control slide should be included.
4. One can use the same technique for 2 micron plastic sections. I recommend EFL 67 plastic resin, which contains:
   a) 100 ml hydroxyethylmethacrylate and butoxyethanol
   b) 10 ml PEG 400 and N.N.-Dimethylanalin. (Rudell, C.L.: Stain Tech., 43, pp. 253-255, 1967).

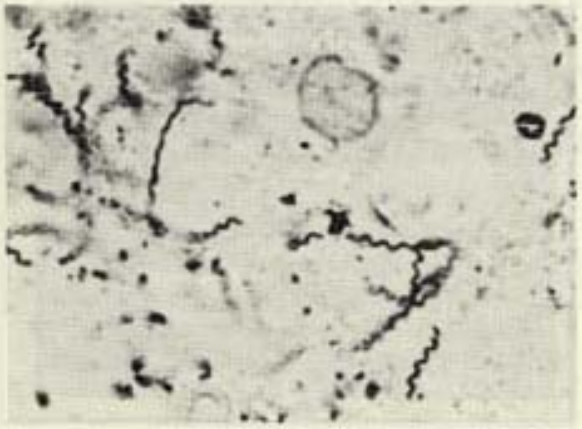

Figure 1: Cork screw shaped treponema pallidum is well demonstrated in this photograph. Note the absence of excessive silver reduction on the surrounding tissues. X2400

The Demonstration of Leptospira Icterohaemorrhagiae (Leptospirosis or Weil's Disease) in Paraffin Sections

Following is my modification of the Warthin Starry technique.

Microtomy

Cut paraffin embedded sections at 4-6 micrometers.

Solutions

Sodium acetate (1.6% in distilled water)	1.0 ml
Acetic acid (1% in distilled water)	25.0 ml
Distilled water	475.0 ml

Check pH before use.*

Silver Nitrate Solution

Silver nitrate	1.0 gm
Acetate buffer solution	100.0 ml

Developing Solution

Silver nitrate (2% in acetate buffer)	20.0 ml
Gelatin (5% in acetate buffer)	50.0 ml
Hydroquinone (0.15% in acetate buffer)	30.0 ml

(Mix immediately before use and warm to 55° C).

Staining Procedure

1. Deparaffinize slides in xylene and hydrate through graded alcohol to distilled water.
2. Place in acetate buffer solution for 10 minutes in a 60° C oven.
3. Place slides in silver nitrate solution for 1 hour in a 60° C oven.
4. Rinse in 60° C acetate buffer for 1 minute.
5. Develop sections in the developing solution for 2-6 minutes at room temperature. (Before using this solution, warm to 55° C).
   Develop 3 slides of the same paraffin block as follows:
   a) The first slide for about 1 minute (color should be very pale yellow).
   b) The second slide for about 2-4 minutes (until deep yellow).
   c) The third slide for about 3-6 minutes (until light brown to brown).
   Check the color of the slides after developing, since the color is more important then the time of development.
6. Place in acetate buffer for 10 minutes in a 60° C oven.
7. Rinse in distilled water for 1 minute.
8. Dehydrate through alcohol, 70%, 95% 100% and acetone for 1 minute each.
9. Clear with xylene twice, 2 minutes each.
10. Mount coverslip with resinous media.

Results
Spirochaetes are black (Fig. 2). Mast cell granules are yellow-brown to deep brown. Other tissue elements are yellow to brown.

***Notes**
If the pH of the acetate buffer is below 3.2 the spirochaetes will not develop properly. Better staining of the spirochaetes is achieved by using a pH of 3.8 to 4.0. If the pH is higher then 4.2 the background will stain too dark.

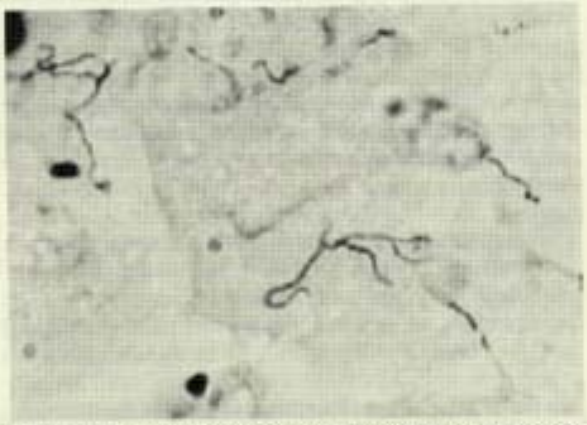

Figure 2: Leptospira Icterohaemorrhagiae are well demonstrated with the use of the modified method presented herein. X2400

A Combined Haematoxylin-Giemsa

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Editor's Note
Please note that the success of this staining procedure is based upon the postfixation of tissue in Heidenhain's solution as suggested below.

Postfixation and processing of Tissue With Heidenhain's* Solution

1. Lillie's 10% buffered neutral formalin ... 1 hour
2. Heidenhain's solution ... 1 hour
3. Heidenhain's solution ... 1 hour
4. Alcohol, 70% ... 1 hour
5. Alcohol, 100% ... 1 hour
6. Alcohol, 100% ... 4 hours
7. Alcohol, 100% ... 1 hour
8. Xylene ... 1 hour
9. Xylene ... 1 hour
10. Xylene with paraffin wax ... 1 hour
11. Paraffin wax ... 1 hour
12. Paraffin wax ... 1 hour

***Heidenhain's Solution (Fixative)**

Mercuric chloride	4.5 gm
Sodium chloride	0.5 gm
Distilled water	80.0 ml
Formaldehyde, 40%	20.0 ml

Tissue exposure to Heidenhain's should be limited to the times suggested since extended exposure may produce staining problems.

Staining with Haematoxylin-Giemsa

1. Xylene ... 3 minutes
2. Xylene ... ½ minute
3. Xylene ... ½ minute
4. 100% alcohol ... ½ minute
5. 100% alcohol ... ½ minute
6. 95% alcohol ... ½ minute
7. 70% alcohol ... ½ minute
8. Tap water ... ½ minute
9. 0.5% aqueous iodine, ... 2 minutes
   (To remove mercury precipitate, if present)
10. Tap water ... 1 minute
11. 5% sodium thiosulphate ... ½ minute
12. Tap water ... 1 minute
13. Hansen (or Harris) haematoxylin ... 5 minutes
14. Tap water ... ½ minute
15. Tap water ... ½ minute
16. Tap water ... 1 minute
17. Tap water ... 2 minutes
18. Giemsa stain solution diluted 1:9 ... 5 minutes
19. Distilled water ... ½ minute
20. 1% acetic acid ... ½ minute
21. 100% alcohol ... 1 minute
22. 100% alcohol ... 1½ minutes
23. Xylene ... 1 minute
24. Xylene ... 2 minutes
25. Mount coverslip with resinous media.

Staining Results
Nuclei slate blue chromatine, pink or turquoise nucleoles. Cytoplasm blue or slate blue in mesenchymal cells, blue in most epithelial cells. Mucinous substances in glands and connective tissue light blue to dark blue, in goblet cells with greenish tinge. Squamous epithelial cells blue in basal and parabasal layers, pink in intermediate and superficial layers. Keratin varying from red to green, hair

matrix light green. Cytoplasm of plasma cells, deep blue with juxtanuclear halo. Russell bodies green or turquoise. Mast cells and Nissl substance blue. Macrophages in some locations, i.e., degenerating thyroid follicles, with light green granules. Melanin and lipofuchsin dark blue-green, while haemosiderin remains unstained. Collagen and amyloid pink, unstriped muscles purple. Cartilage deep blue. Thyroid colloid blue, blue-green, purple or red, apparently according to functional state. Granules in eosinophil leucocytes red, in neutrophils pink, in Paneth cells brownish-red, in Kulchitzky cells brownish-orange, in serous glands of pancreas, gastric and salivary glands deep blue. Gastric parietal cells and onchocytes pink. Cells of adenohypophysis well differentiated. Vegetable cells green (e.g. in bronchioles, gastric ulcers and in foreign body granulomas in hands or feet).

Comments

In routine examination of biopsies and of surgical material, some of these properties are of special value. Nuclear staining is very sharp with distinct chromatin and mitotic figures. The cell membranes are well demonstrated, often slightly basophil even in cells otherwise eosinophil. Differentiation between cells and intercellular material, notably connective tissue, is correspondingly well marked. Melanin is easily recognized and not confused with haemosiderin. In cervical biopsies, reserve cell hyperplasia and incomplete squamous metaplasia is usually found to carry, on the surface, blue glandular epithelium. Plasma cells are easily recognized, a feature of special importance in bone marrow biopsies. One advantage must be mentioned separately. Comparison between cytological (fine needle) biopsies stained with May-Grunwald-Giemsa and histological material stained with haematoxylin-Giemsa is greatly facilitated, due to the cytoplasmatic staining properties of the latter. Likewise, hemopoietic cells in bone marrow sections are well differentiated.

Professional Attitudes

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Positive professional attitudes do not seem as prevelant in our sector of the health care field as they once were. Dedication to a technical profession such as histotechnology was an admirable characteristic frequently encouraged and sometimes taught as part of the school curriculum. Although this characteristic remains just as desirable, it is much less often emphasized to students, trainees and employees under our supervision.

A person with a positive professional attitude advocates high-quality patient care and places achievement quality results in his/her work over anticipation of days off, holidays and payday. Unfortunately, we sometimes see evidence of a lack of devotion to the patient. Although remote from the patients, we in histopathology are no less responsible for the patient's overall welfare than the clinician and nurse who minister directly to them. A lack of dedication is frequently coupled with symptoms of low self-esteem and lack of motivation. Such negative attitudes do not exert a good influence on less experienced coworkers who through a more positive emotional and professional atmosphere might develop an attitude of dedication to our profession.

Self-esteem, pride in oneself, leads to esteem for those around us. Motivation is improved immeasurably when we feel good about ourselves and our professional behavior.

Expressions that reflect a lack of self-esteem and indicate lower standards of performance and lack of professionalism are: "I am not going to do that, it isn't my job"; "I am not going to stay late and do that frozen section—I got stuck doing that last week"; "Why should I devote so much time working for the society—'they' don't appreciate me"; "Why should I write an article for the Journal—they won't publish it anyway." To know we did a task exceedingly well when it demanded our best and to be a cooperating part of the team joining other team members to accomplish sometimes undesirable tasks, is to enhance our image of ourselves.

In many instances, we are not well motivated because we have no professional goals or direction. We as experienced leaders need to be well-motivated and cultivate high self-esteem in our subordinates rather than feel threatened or intimidated by co-workers who appear to be more capable than we are. When we feel jealous or are threatened by another person's skill and knowledge, we are deprived of being all that we could be professionally. We may even compromise our integrity. Integrity, or trust-worthiness, should always be present in those who have committed themselves to serving patients who depend on our skills and integrity for accurate diagnoses without thich proper treatment cannot be effected.

We must never forget that the specimens we deal with represent humans whose welfare, although indirectly, still very much depend on us.

As leaders we should leave our profession a legacy of pride in our work, the example of high self-esteem, and the ability to empathize with the patients.

A Useful Haematoxylin Without Toxic Chemicals

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Haematoxylin is one of the oldest and most valuable histological stains. Haematein, the active coloring agent, is formed by chemical oxidation or a nonchemical process requiring a number of weeks.

In alum haematoxylin, the selective staining power is due to the combination of haematein with alum. Harris demonstrated that it is possible to oxidize and ripen alum haematoxylins by adding *mercuric oxide* and Mayer recommends adding *chloral hydrate* as a preservative. *Mercuric oxide* and *chloral hydrate* are *toxic chemicals*, therefore it is best not to use them, if possible.

After more than a year and many experiments, I have successfully prepared a very useful haematoxylin without toxic chemicals.

Advantage of this Haematoxylin:
- contains no toxic chemicals
- filtering is not necessary
- shelf life is months to years
- it is easy to prepare
- low price (cost)
- can be used for: routine histology; plastic embedded sections; frozen sections; cytology

Preparation and Materials
1. Take a one liter flask with 815.0 ml hot tap water and dissolve 25.0 gm aluminum ammonium sulphate, $AlNH_4(SO_4)_2.12\ H_20$. Allow solution to cool to room temperature.
2. Add 25.0 ml ripened 10% haematoxylin in absolute alcohol to above solution and stir well.
3. Dissolve 0.2 gm sodium iodate, $NaI0_3$, in 50.0 ml cold tap water and add to above solution and stir well.
4. Add 100 ml of diethylene glycol, $C_4H_{10}O_3$ and stir well.
5. Add 10 ml glacial acetic acid and stir.

This haematoxylin solution, with a pH of 3.10 to 3.30, is ready for immediate use.

References

1. Culling, C.F.A.: Handbook of Histopathological and Histochemical Techniques. Butterworth & Co., London, 1974.
2. Lillie, R.D. & Fullmer, H.M.: Histopathologic Technic and Practical Histochemistry. McGraw-Hill, New York, 1976.
3. Bancroft, J.D. & Stevens, A.: Theory and Practice of Histological Techniques. Churchill Livingstone, London, 1977.

Drying and Staining Slides with Microwaves

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There is no telling what will happen when four histotechs put their heads together and decide to do something. Histotechs are innovative and often come up with new and better ways to do something in the histology laboratory.

We bought a microwave oven in February 1987 and since that time have developed and tested a container for drying tissue slides in the microwave. The container is used numerous times daily. Slides are dried in a 750 watt oven at full power for 2 minutes and 15 seconds, with absolutely no harm to the tissue, no matter how delicate it may be. We cut 2 hours off of our morning schedule, which enables the pathologist to receive the slides much sooner.

The container holds 20 slides, has a divider down the middle with an attached handle. The divider was added so that the container may be used for staining also. We use an automatic slide stainer and have experienced no problem with sections falling off. With the use of this container, you don't have to cram slides (as you would in a coplin jar) for staining. Additionally, you can dry slides in 2 minutes instead of 20. For additional information, please contact the authors.

Frozen Section Technique

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This alternative freezing method is time saving and also reduces potential hazards to technician and tissue specimens.

Fresh tissue sample is positioned on the object holder with a few drops of "Tissue-Tek" O.C.T. compound. Using clamp forceps the object holder is lowered into an insulated container of liquid nitrogen. Immerse in the liquid nitrogen, 10 seconds, until O.C.T. appears opaque. Time will vary slightly, depending on tissue type; fatty specimens require longer exposure to liquid nitrogen. Your regular cryostat procedure may be followed from this point.

This method reduces potential health hazards to technicians by eliminating the use of freon sprays. Rapid freezing reduces ice crystallization and subsequent artifacts to tissue. Noticeable improvements in cellular detail has been recognized by the pathologist.

Did you Know

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Did You Know:

— that 10% formalin penetrates 2 mm of most tissues in the first 4 hours of exposure. Continued tissue exposure results in the fixation penetration of 10 mm in 24 hours.

— that studies have shown that 10% formalin penetrates 5 mm of tissue in 1 hour with the use of the "Vacuum Infiltrator Processor" (VIP) sold by Miles, Diagnostic Division, Elkhart, Indiana.

— that formaldehyde forms methylene compounds with amino, amide and hydroxy groups and thus offsets the solubility and reactivity of proteins.

— that tissue bound formaldehyde cannot be removed by water washing prior to processing. Only adsorbed and/or loosely bound formaldehyde is displaced by washing in running water.

— that coagulant fixatives include alcohol, picric acid, mercuric chloride and chromium trioxide.

— that noncoagulant fixatives include formalin, potassium dichromate and osmium tetroxide.

Continued on page 267

Alteration in Tissue Structure Due to Heat, Fixation and Processing

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The photographs of tissue alterations due to several facets of histologic technique are presented to remind us that detailed care and attention is essential when handling tissue specimens. They are intended as a guide which should provide some insight into morphologic tissue changes if one does not observe accepted and proven steps in technology. The liver specimens were obtained from an autopsy and therefore some autolytic changes had occurred prior to the performance of the designated tests. Photograph A (top) was taken as close to the middle of the section as possible. Photograph B (bottom) was taken at the periphery (1 mm from edge) of the specimen. Comments regarding results are not necessary since the photographs are self explanatory. *Note:* The only variables in handling these tissues are those indicated in the captions. All other steps were performed identically in a conventional manner.

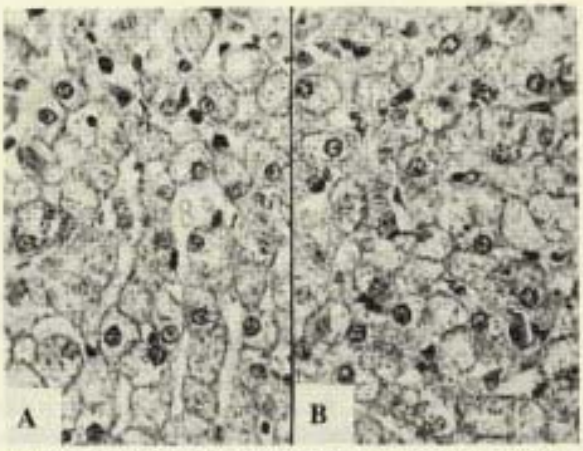

Figure 1, a & b: 48 hours in buffered neutral formalin and 5 hours in 60° C paraffin wax, following routine dehydration and clearing. H&E X300

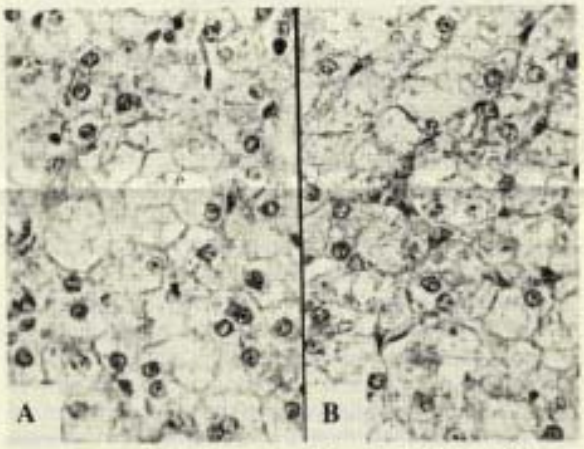

Figure 2, a & b: 48 hours in buffered neutral formalin and 5 hours in 75° C paraffin wax, following routine dehydration and clearing. H&E X300

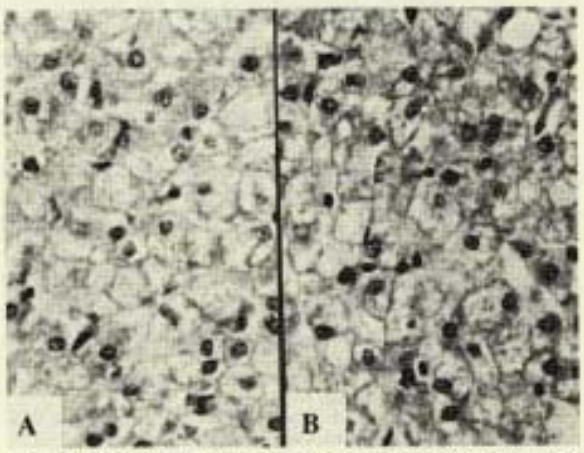

Figure 3, a & b: 48 hours in buffered neutral formalin and 5 hours in 80° C paraffin wax, following routine dehydration and clearing. H&E X300

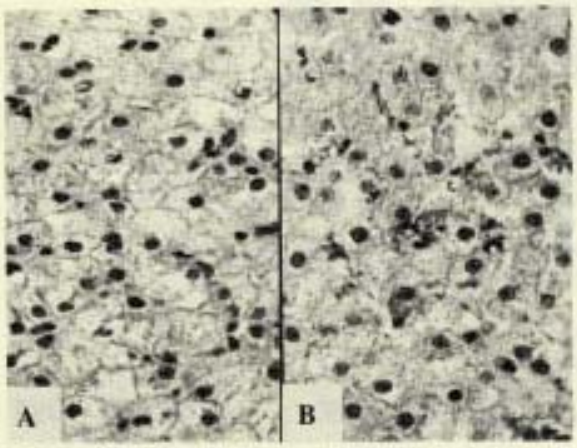

Figure 4, a & b: 48 hours in buffered neutral formalin, room temperature air dried overnight (16 hours) and processed in a routine manner. H&E X300

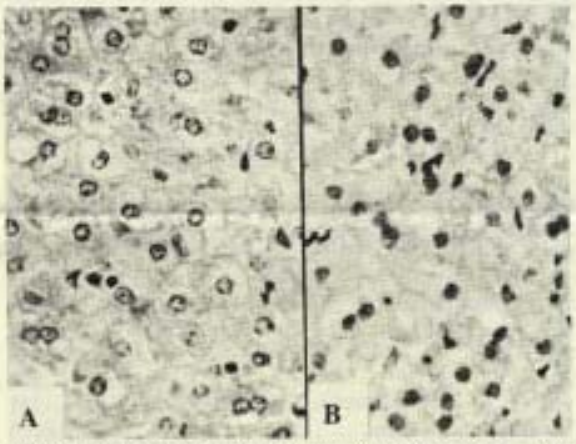

Figure 5, a & b: Specimen was placed in normal saline overnight (16 hours) and fixed in buffered neutral formalin for 48 hours. Processed in a routine manner. H&E X300

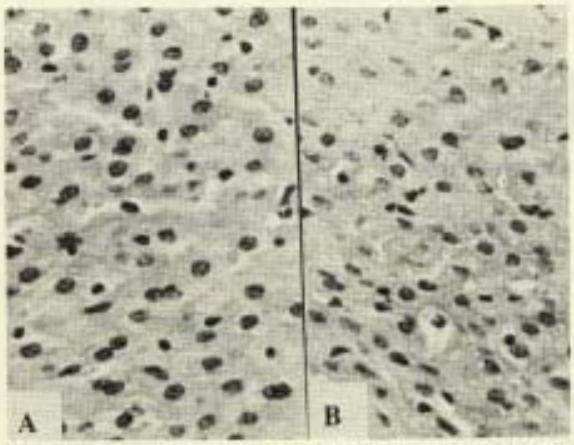

Figure 6, a & b: Specimen was allowed to set unfixed at room temperature overnight (16 hours). Fixed in buffered neutral formalin for 48 hours. Processed in a routine manner. H&E X300

Smith's Method for Juxtaglomerular Granules (A Correction)

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We encountered certain difficulties when applying for the first time, the method for juxtaglomerular apparatus as given in the AFIP Staining Manual[1].

After oxidization with potassium permanganate, there follows decolourization with sodium thiosulphate (hypo). The decolourization did not occur and sections remained brown. On completion of the staining procedure on the undercolourized section, it was noted that the permanganate brown masked the staining reaction of the Biebrich scarlet/ethyl violet complex. Decolourization with oxalic acid was then performed resulting in a marked improvement, but not completely satisfactory results.

Reference to the original article as published by Carlton L. Smith[2] showed the decolourizing agent to be 1% $Na_2S_2O_5$ sodium metabisulphite, not sodium thiosulphate as published by the AFIP Manual. Satisfactory results on human tissue were obtained using the correct method.

Another noteworthy observation was in the recognition of the neutralization end point when preparing the Biebrich scarlet/ethyl violet complex. No abrupt colour change from red to violet occurred as described by Smith. A good guide signalling the end point was the formation of the precipitate on the side of the flask upon swirling.

References

1. Luna, L.G.: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd ed., McGraw-Hill Book, Co., NY, 1968.
2. Smith, C.L.: Rapid Demonstration of Juxtaglomerular Granules in Mammals and Birds. Stain Tech., Vol. 41, No. 5, pp. 291-294, 1966.

Continued from page 266

— that Carnoy's fixation is invaluable for studies of early myocardial and vascular lesions. Myocardial infarction alterations of myofibril, 2 hours old, are demonstrated well with Masson's trichrome stain.

— that Zenker's fluid, which contains no reducing agent, does not precipitate, change color, or undergo any significant pH change in a 48 hour period.

— that Zenker-formal, which causes a great deal of shrinkage, is widely used because of its excellent cytoplasmic preservation.

— that Flemming's fixative without acetic acid is the best fixative for muscle from the shrinkage stand point.

Subject Index ..Vol. XVII, 1987

Author Index...Vol. XVII, 1987

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