

KIDNEY: BASEMENT MEMBRANE, PAS

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## A MOST BODACIOUS STAIN

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Tech Points

*Quick – name one stain that will demonstrate a type of microorganism, several types of simple polysaccharides, 2 main types of mucopolysaccharides, a couple of types of connective tissue, DNA but not RNA, the colloid of thyroid, some lipids, and is usually used with a blue or green counterstain. That incredible, versatile stain is, ta dah ...*

*the PAS, also known as the Periodic Acid-Schiff.*

**H**ow can one stain demonstrate so many seemingly different tissue components? We'll get in to the chemistry of it in a moment, but the basis is that the Schiff dye molecule will combine with aldehydes to make a pink color. Any sugars or polysaccharides present in the tissue can be oxidized into aldehydes that will bind with the Schiff reagent dye.

Tissue components that stain with the PAS are:

Simple polysaccharides, which are sugars, starch, cellulose, and chitin.

- Glycogen, a type of sugar, can be found in abundance in the cytoplasm of some cells (such as liver hepatocytes, striated muscle fibers, and ectocervical cells), on fungus (but not on bacteria to any large extent), and on several connective tissues (such as reticulin and basement membrane and some lipids (glycolipids)). Glycogen can be differentiated from other simple polysaccharides or neutral mucins that also stain PAS positive by using enzyme digestion with diastase or amylase, applied before the PAS stain, would remove positive staining from the glycogen, while the other carbohydrates would continue to stain PAS positive.
- Starch and cellulose are found in plants, which can sometimes be seen as small bits of food in the sections of the digestive system, or which have been aspirated into the lungs.
- Chitin is found on fungus walls, as well as insect bodies and parasite hooklets, the latter two of which can incidentally end up in human bodies.
- The colloid of thyroid is a glycoprotein – a protein compound with glycogen.

Mucopolysaccharides are divided in neutral and acid mucins. The neutral mucins are found in the mucous cells of the stomach, the goblet cells of the small and large intestines, the mucin cells of lung and endocervix, and in cartilage. Because of the neutral mucins, all of these tissue components will stain PAS positive. On the other hand, acid mucins, found also in the goblet/mucin cells of the GI and lung, but not the stomach, tend to NOT stain with the PAS procedures. DNA has a sugar known as deoxyribose. When it is hydrolyzed with an acid, the DNA sugar creates an aldehyde which will combine with the Schiff,

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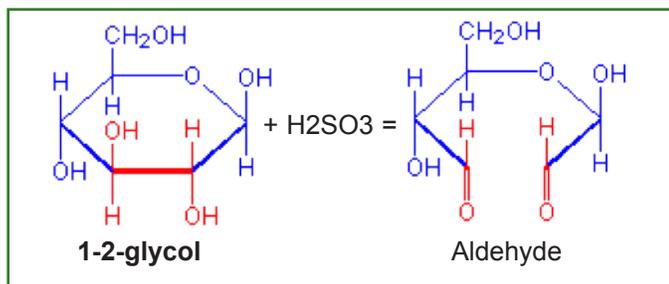
staining the nuclei. RNA contains ribose sugar, which will not create an aldehyde when hydrolyzed, and thus will not stain with Schiff.

So, while all of the tissue components in the opening paragraph all seem different, they all have a type of carbohydrate that can be converted to an aldehyde, which will stain magenta with the Schiff dye molecule.

### OXIDATION

The most common oxidizer used to demonstrate these carbohydrates is usually periodic acid. The formula for periodic acid is  $\text{HIO}_4$ ; it is an acid (H) with one or more iodines (I). But there are more oxygens (O) than iodines (in this case, 4 oxygens to 1 iodine).

The slides are placed in 0.5% – 1.0% periodic acid for anywhere from 5 to 30 minutes, and washed in water to remove the periodic acid. Only those carbohydrates that contain a 1-2-glycol (Carbon-Hydroxyl (–C–OH) group on position 1 and 2 of the carbohydrate) will be converted to two aldehydes (Carbon double-bonded to an Oxygen (–C=O)). If there are a lot of these types of 1-2-glycol carbohydrates all in one area (like in goblet cells, basement membrane, fungus, etc.), then there will be a lot of carbohydrates created for the Schiff molecule to bind to and later to be seen as magenta. There are amino acids with –OH groups, or that are aldehydes =O, but because they are randomly spaced, there will not be enough to demonstrate (stain) the amino acid proteins in general.



Chromic acid could also be used as an oxidizer. Whereas periodic acid stops oxidation at the aldehyde stage, chromic acid will oxidize the 1-2-glycol carbohydrates to aldehydes, and then continue to oxidize the aldehyde to carboxyl ions, which will not stain with Schiff. It is sometimes used to demonstrate fungus. With the PAS, fungus will stain magenta, but so will a lot of the background, such as reticulin, basement membrane, keratin, mucin cells and goblet cells, glycogen, etc. Therefore, it can sometimes be a little difficult finding magenta colored small fungi or sparse fungi against a background that has a lot of magenta staining. Since fungi have chitin, and most human and animal tissue does not, the chromic acid, if used in the correct percent and time, will overoxidize the background carbohydrates to the carboxyl ions so the background will not stain with Schiff. Chitin will continue to have some aldehydes, and thus will stain magenta with Schiff. The Chromic Acid-Schiff stain is also known as the Bauer-Feulgen procedure.

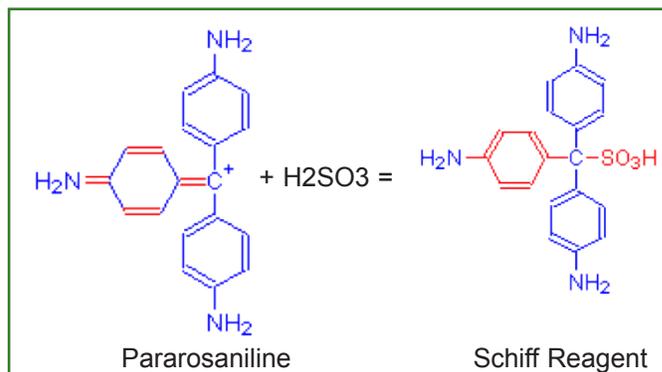
### Did you know?

*The proper pronunciation for the chemical Periodic Acid is per-i-o-dik (purr-eye-oh-dik). The word that is pronounced peer-e-odd-ick means reoccurring at different time intervals. That certainly does NOT apply to this chemical! So remember, it is ALWAYS purr-i-oh-dik, if it's the chemical...we should never (not even peer-i-odd-ically) refer to it as something else! Just a little pet peeve of mine, which I had to get out of the way! Peggy*

### SCHIFF REAGENT

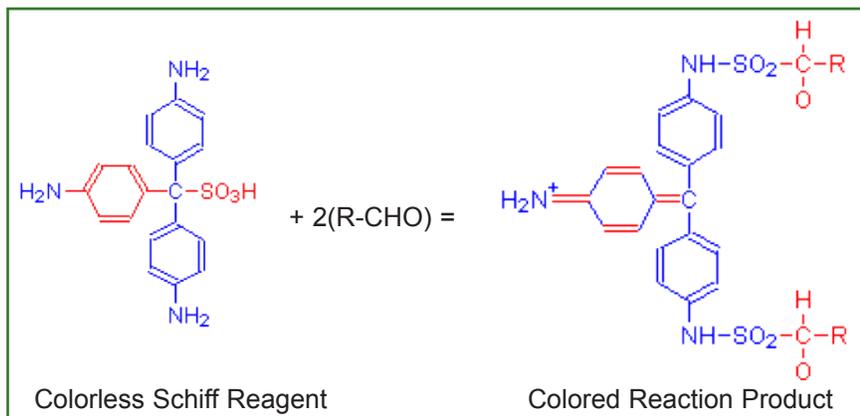
The dye that is used in the Schiff reagent could be basic fuchsin, new fuchsin, or pararosaniline. This dye is dissolved in water, and sodium or potassium metabisulfite is added, along with hydrochloric acid. The solution is allowed to set in the dark overnight. During this time, the sulfites (–SO<sub>3</sub>H) bind to the dye, changing some of the double bonds on the dye molecules to single bonds, and moving some double bonds around.

After setting overnight, the Schiff reagent should be a pale yellow to a red-brown color. These colors are due to impurities in the basic fuchsin dye. These impurities can be removed by adding some activated charcoal to the solution and then filtering. The Schiff reagent should be colorless at this point.

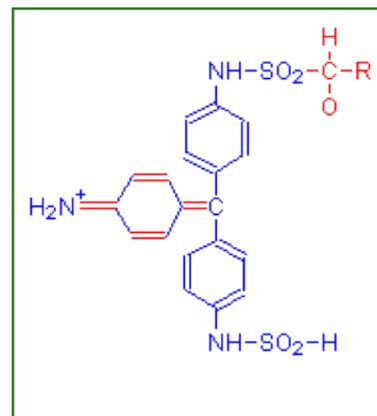


PARAROSANILINES QUINOID RING IS LOST WITH THE ADDITION OF -SO<sub>3</sub>H AND DOUBLE BONDS CHANGE TO SINGLE BONDS IN THE FORMATION OF SCHIFF'S REAGENT.

The slides are placed in the Schiff solution for 10-30 minutes. In the traditional view, the 2 amino groups (–NH<sub>2</sub>) on the altered dye molecule will combine with the 2 aldehydes that have been formed on the oxidized carbohydrates in the tissues. This creates a new dye molecule. There is a newer alternative theory that the Schiff reagent only needs to bind to one of the aldehydes, on only one of the amino groups.



SCHIFF'S SINGLE BONDS REFORM INTO A QUINOID RING WITH THE ADDITION OF ALDEHYDE.



A POSSIBLE ALTERNATIVE PRODUCT.

## WATER RINSES

The next step involves washing the slides in running tap water. This removes the hydrochloric acid in the Schiff reagent, raising the pH, allowing the sulfonic acid group to become detached, rearranging the double bonds, and the red magenta color appears. Washing in cold water takes at least 5-10 minutes, using warm water shortens the time.

## COUNTERSTAIN

A common counterstain is hematoxylin, usually either Gill, Mayer or Harris. Since this is a counterstain which needs to be pale, only a short amount of staining time is needed. Nuclear staining of blue helps the pathologist determine which part of the tissue is involved (for example, are the glomeruli or tubules involved in the kidney, or did the fungus invade the blood vessel?).

Some labs use a light green counterstain when looking for fungus with the PAS. The background is stained a pale green, which highlights the magenta of the fungus. A light counterstain must be used, as too dark of the green could mask the magenta fungus.

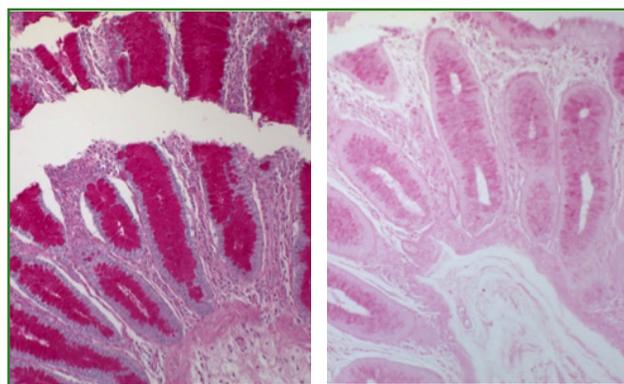
## PAS FALSE POSITIVE OR INCREASED BACKGROUND

- Fixation in Gluteraldehyde: This di-aldehyde fixative could bind both aldehydes to tissue amino acids. However, some gluteraldehydes may bind only one aldehyde to the tissue proteins, leaving the second fixative aldehyde available to bind to a Schiff molecule.
- Frozen sections: Some glycolipids that are normally removed during processing, will now be available for staining with PAS technique. Though this isn't a true false-positive (since glycolipids would be expected to stain), since they are normally not seen in processed tissue, they might be interpreted as increased background.

## PAS FALSE NEGATIVE OR DECREASED POSITIVE STAINING

- Under-oxidation: Not enough aldehydes are created, so not enough Schiff molecules bind to the tissue. Due to either too low a percent of oxidizer, or too short of a time in the oxidizer.

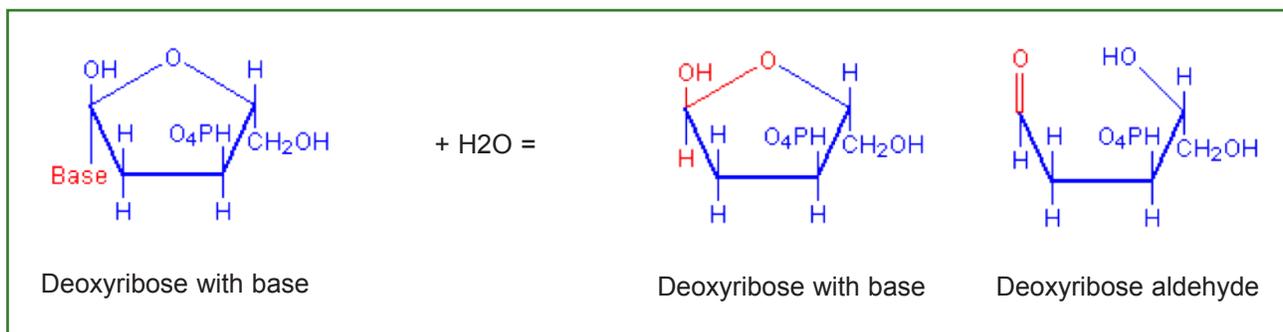
- Over-oxidation: Too long of a time in a strong oxidizer, such as chromic acid, that can over-oxidize the carbohydrate past the aldehyde stage, to the carboxyl ion stage, which will not stain with the Schiff reagent.
- Under-staining: Too short of time in Schiff reagent. Not enough Schiff molecules bind to the aldehydes in the tissue.
- Weak Schiff reagent: Either made incorrectly, or used over and over again, until there are too few dye molecules to properly stain the tissue section.
- Under-washing: Too short of time in the water rinse after the Schiff reagent. Not enough Schiff molecules are converted from the colorless form to the magenta form.
- Over-counterstaining: Too dark of a green counterstain can mask the magenta staining.
- Too thin of tissue: Sections that are typically cut at 1-2  $\mu\text{m}$ , such as kidney biopsies, have fewer carbohydrates than a 4-5  $\mu\text{m}$  section. To make certain as many carbohydrates are changed to aldehydes and later stained with Schiff, increase the time in both the periodic acid and the Schiff reagent.



LARGE INTESTINE: NEUTRAL MUCINS  
GOOD STAINING (RIGHT); POOR STAINING (LEFT)

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## FEULGEN

Even though we use the H&E to demonstrate nuclei, the hematoxylin doesn't stain just the DNA in the nucleus. It is staining other proteins in the nucleus also. If we need to demonstrate just DNA, the Feulgen stain must be done.

DNA is made up of nitrogenous bases (purines and pyrimidines), sugar (deoxyribose), and phosphate groups (phosphoric acid). When placed in hydrochloric acid (HCl) at 60° C for a specific amount of time, RNA is broken down so that it cannot be demonstrated. In DNA, the nitrogenous bases are broken down, while the phosphate groups remain but are not involved in the Feulgen reaction. The deoxyribose sugar is broken open (hydrolyzed) by the HCl, creating an aldehyde. This aldehyde can then bind with the Schiff reagent, as discussed above, staining only the nuclei a magenta color.

Since HCl is a strong acid, any carbohydrates in the tissue background (glycogen, mucin, etc.) are all overoxidized, so no staining in Schiff will occur.

The amount of time in the HCl is dependent upon the type of fixative used. Over- or under-hydrolysis in HCl can result in an understained to false-negative reaction, by either not enough aldehydes being made (under-hydrolysis) or the aldehydes being further broken down to carboxyl ions (over-hydrolysis).

Use of a fixative with acid in it, such as Bouin (acetic acid and picric acid), and tissue that has been decalcified in acid, will result in a false-negative stain. The acids will over-hydrolyze the DNA during fixation, and cause the deoxyribose sugar to go past the aldehyde stage, and will no longer stain with the Schiff dye molecule.

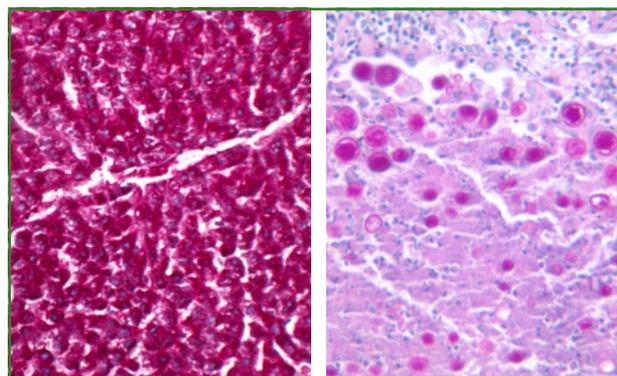
Counterstain is optional, but if light green is used, a very pale counterstain must be used. Too dark of a counterstain could either cause the nuclei to appear more blue (magenta + green), or the dark green background may overwhelm the nuclear staining.

## CONCLUSION

This staining procedure seems somewhat simple – put slides in 2 reagents (periodic acid and Schiff), rinse, and then counterstain. But there is some elegant chemistry going on.

The PAS stain can be used to demonstrate:

- Basement membrane disorders, particularly of the kidney
- Glycogen storage disease
- Adenocarcinoma (neutral mucins)
- Fungal diseases
- Food particles in lung aspirates



PAS STAINING GLYCOGEN (LEFT) AND FUNGUS/YEAST (RIGHT)

Let's thank Joseph McManus, MD, for inventing this stain in 1946. He was a Canadian pathologist who spent the majority of his career at the University of Alabama.

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