THE INNOVATOR

from ANATECH LTD. *"the leader in innovative histology chemicals"*

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THREE DYES, THREE DILEMMAS

Alcian blue

Perhaps the most critical dye in our story is Alcian blue (CI 74240). Developed in the 1940's by Imperial Chemical Industries (ICI) of the UK, it somehow found its way into the biological stain field in the 1950's. At first the dye proved to be frustrating to the biologists using it, because of lot-to-lot variability in intensity and specificity. ICI had been having trouble with the dye's solubility under textile dyeing conditions, and various process changes in manufacturing were made during the 1950's and 1960's. Each different batch was given a code, and today it is generally recognized that only Alcian blue 8GS and 8GX were reliable for biological staining.

To make matters worse, safety and environmental problems plagued ICI, and they ceased manufacturing the dye, probably by the mid 1970's. Poor acceptance by the textile industry undoubtedly played a decisive role in abandoning the dye. Alcian blue (and Alcian yellow) are in a rather unusual class called ingrain dyes, which require a dyeing procedure very different from other textile dyes. The colorant is introduced to the fabric or yarn in a water soluble form, then the ionizing groups are removed by treatment with strong alkali, leaving an insoluble pigment trapped within the fibers. Staining as we know it apparently was not involved, although any histotechnologist knows that Alcian blue certainly stains lab coats permanently without the alkaline treatment! The process never gained popularity with the textile mills.

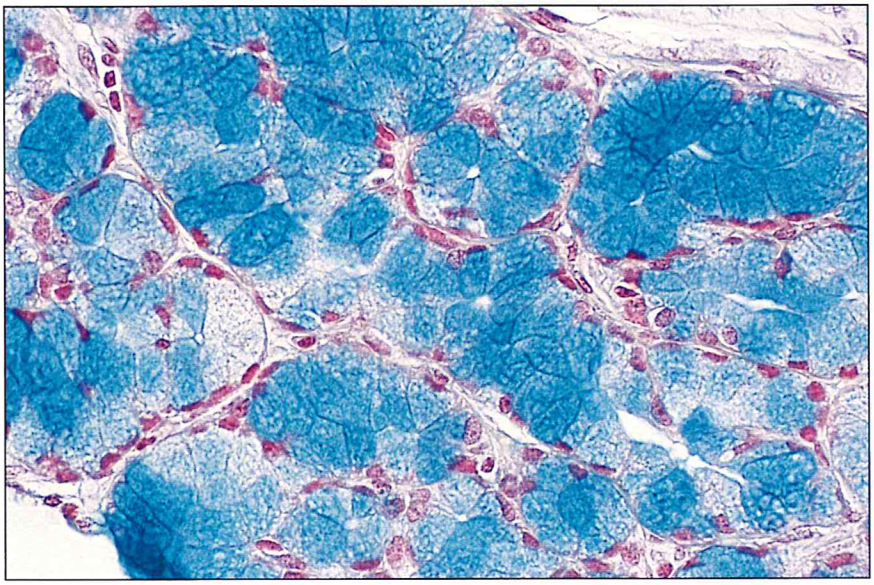

Figure 1.

Pig duodenum fixed with NBF, stained with Alcian blue at pH 2.5 and counter stained with Brazilliant!. Mucus in Brünner's glands stains blue, but it is not possible with this slide alone to determine if that mucus is carboxylated or sulfonated (see Figure 2). 40x

Welcome...

Nearly all of the dyes used in histology come from the textile industry, and thus are subject to the market pressures of that sector. Biological demand is so insignificant it rarely plays a role in supply or quality issues. In short, we are at the whim of forces completely beyond our control.

Recently, several dyes have presented some unique challenges. Worldwide supplies of Alcian blue were exhausted, with no apparent hope of replenishment. Nuclear fast red continued to present supply and quality problems. Congo red, along with other benzidine-based dyes, was declared an OSHA carcinogen. Others are in similar trouble.

We understand that histotechnologists, pathologists, researchers and, indirectly, patients, all depend upon dyes such as these, many of which act in unique ways. In this issue of The Innovator, we tell the story of three of these dyes and how we have succeeded in overcoming their obstacles.

Our first step in tackling any problem is to understand the underlying chemical mechanism. What really makes each of these so unique? Second, does any other dye have that same property, and if so, can we substitute one for the other? Finally, if not, is there another way around the problem? This Innovator will show how we approached each situation. We hope you enjoy the solutions!

After ICI ceased production, small lots of Alcian blue from other sources appeared on the biological stain market. All seemed to have been made in countries whose disregard for health and safety is well known in the dye industry. Purportedly, the risks proved too great even for these suppliers, however, and today few, if any, are willing to continue manufacturing Alcian blue using the ICI process.

We began our research by turning to computer-generated molecular modeling studies to discover why Alcian blue works as it does. We learned that Alcian blue's ionic groups are effectively isolated from the rest of the molecule, concentrating the charge in a very small volume (picture a tiny cloud of positivity). Nearly every other basic dye has its positive charge spread across the entire molecule (a very large cloud of positivity whose magnitude of charge is the same as the small cloud). Chemists call these hard and soft cations, respectively. Tissue anions have similar variability in their charge sites. The carboxylic and sulfonic acids of mucopolysaccharides are hard anions. When a hard cation (Alcian blue) meets a hard anion (mucopolysaccharides), a very stable salt forms that cannot be destained by usual staining procedures. Other basic dyes with soft ionic clouds are attracted to the hard negative clouds in the tissue, but are so poorly matched in geometry they cannot bind tightly enough to withstand subsequent staining procedures and dehydration. Thus, Alcian blue is highly selective for the tissue substances (given the proper solution pH), and forms insoluble complexes that withstand harsh subsequent treatment (like PAS) without destaining. That is what makes this dye so important.

Do any other dyes have this attribute? Yes, two others to be exact, out of thousands listed in the *Colour Index* and *Conn's Biological Stains*. Alcian yellow is the only one familiar to histologists; basic red 18 is the other, and is unknown in our field. The former is yellow, the latter is deep orange. Do they work in all Alcian blue procedures? Yes! Are they practical substitutes for Alcian blue? No, because they are not blue, and they too are unavailable. After all that we felt we were back to square one.

We next looked at alternative synthetic pathways for making the dye, and this proved fruitful. We now have a new Alcian blue that is made responsibly and dependably here in the US. We do not call it Alcian blue 8GX because that designates a different manufacturing process, but our dye functions indistinguishably from the original. Dye content is notably higher (60–65%) than recent material which scarcely met the minimum for certification (50%). Our Alcian blue works in all procedures that we could find in the literature. To top it off, the Biological Stain Commission has certified it.

It is our intent to resupply the world with Alcian blue. You may now buy it from a variety of vendors here and abroad, as dye powder or as a stain solution. It is ours, and you are welcome to purchase it from them or from us directly, whether you are an end-user or another vendor.

Nuclear fast red

This dye is an obsolete pigment for paint and ink, so there is little incentive to produce it. Not surprisingly, therefore, availability is undependable and quality is variable. Recent batches are reported to leave a white precipitate on slides. We became intrigued with nuclear fast red years ago, and began a quest for a red nuclear stain that did not have these problems, yet shared that dye's specificity as a nuclear stain.

Many red dyes exist, but few stain nuclei selectively and remain in the section during dehydration. Basic fuchsin and safranin do a fair job of staining nuclei, but are readily extracted by alcohol during final dehydration. We wanted something as crisp, specific and color-fast as hematoxylin, only red, so we turned to hematoxylin's close cousin, Brazilin. As hematoxylin is extracted and purified from the logwood tree, so is Brazilin purified from Brazilwood, another tropical tree. Sufficiently pure Brazilin is not generally available, so has been used rarely in histology. Fiber artists do use it to dye natural fibers a dull red. It has little

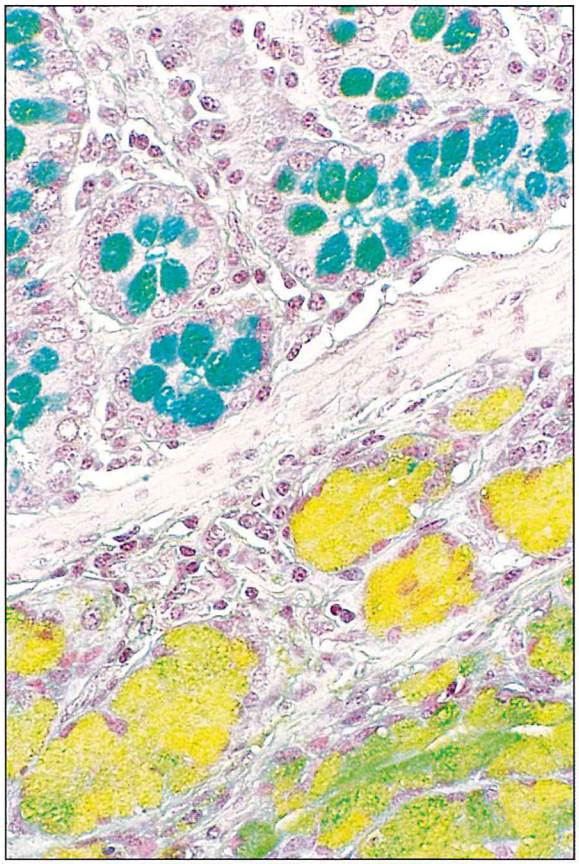

Figure 2.

Same tissue as in Figure 1 stained to differentiate carboxylated mucus in Brünner's glands (yellow) from sulfonated mucus in goblet cells (blue-green). Alcian blue pH 1.0 + periodic acid + sodium metabisulfite + Alcian yellow + Brazilliant!.

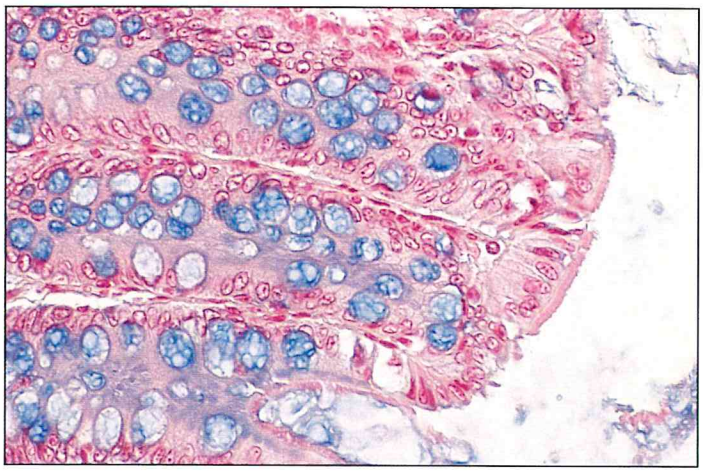

Figure 3.

Opossum small intestine fixed in Prefer. Alcian blue pH 2.5 + Brazilliant!. 40x

other use today. The problem for us was obtaining good quality dye and learning how to make superior stains with it.

We now offer both the dye, Brazilin (CI 75280), and a Harris-type formulation of that called Brazilliant!. For those who like to make their own stains, our Brazilin can replace hematoxylin in most formulations, but you should double the amount of dye. You must first oxidize Brazilin to Brazilein with sodium iodate (or let it ripen naturally over several months), then complex it with aluminum. Harris solutions will give the most specific staining of nuclei, while Gill and Delafield's will impart a light pink background color to the cytoplasm, just as in the hematoxylin counterparts.

Brazilliant! is a nuclear stain of unsurpassed clarity and contrast, as you can see from Figures 1–3. The solution is stable for well over a year, so you can enjoy the convenience of a ready-to-use product without worrying about it expiring before you use it up. Brazilliant! does not develop a surface film like Harris hematoxylin sometimes does, and rarely needs to be filtered except to remove cellular debris.

Either way, your solution or ours can be used as a counterstain for any special stain or chromagen that is not compatible with blue nuclei, such as Perl's iron and blue alkaline phosphatase chromagens. We love it with our Alcian blue. Always place the red solution last in your procedure. We stain for 6 minutes, rinse in deionized or distilled water, then dehydrate starting in 70% alcohol. No "bluing" is needed; in fact, alkaline tap water or bluing agents will lighten the stain.

Please remember that no stain will make poorly fixed nuclei look good. If your tissue is properly fixed and stains well with a quality Harris hematoxylin, Brazilliant! will duplicate that result in red. Also note that the color red is not as dark as blue. Stains that impart a slight hue to nuclei might shift the pure red of Brazilin to an orange-red, brown-red or violet red.

Congo red

We have never been fond of Congo red (CI 22120), and judging from the number of published procedures for using it, many others have the same complaints. It is typically weak, and many pathologists resort to fluorescence or polarization microscopy to satisfy themselves that amyloid is really present. Solutions have questionable stability if made up completely, or require the addition of some key ingredient just before use. Neither condition is satisfactory for a stain that is used with decreasing regularity in a busy clinical setting.

In 2000, all dyes based upon benzidine and its methoxy and ethoxy derivatives were declared known human carcinogens and placed on OSHA's list. Congo red is one such dye. The change will have two effects on histology. Immediately, any lab using Congo red must incorporate new information into its Chemical Hygiene Plan and safety training programs. Work areas must be posted to notify personnel that an OSHA carcinogen is in use. Bottles must be specially labeled. If you deal with formalin now, these changes are minor (but don't neglect them!).

We are more concerned with a greater problem which may not appear for a few years. Once again, the textile industry may determine how we stain for amyloid. Benzidine-based textile dyes were very popular and comprised most of the so-called direct cotton dyes. They were an improvement over older colorants, especially for cotton where fading was so common, but they posed a significant health risk to workers exposed to industrial quantities of dye powder. Dye manufacturers long ago began developing substitutes, and voluntarily have been phasing out benzidine-based dyestuffs ever since. For example, in 1971 there were 890 direct dyes in commercial production. By 1982, 107 new direct dyes had been brought to market (nearly all of which were not benzidine-based), while production of 320 others was abandoned. Most of the latter were made with benzidine or its derivatives. As is so often the case, the safer dyes also worked better.

There is a trend here that cannot be ignored. We believe that the prospects for Congo red are dim: if history is any indication, it won't be long before it too is phased out. It won't be tomorrow, but it will happen. Spurred by the disappearance last year of Alcian blue, Alcian yellow and Celestine blue, we decided to introduce a substitute for Congo red.

We had been studying the mechanism of Congo red staining several years ago, again with our molecular modeling software. Amyloid is a heterogeneous group of macromolecules that all have accordion pleats. Within the pleats are hydrogen-bonding sites, much like docking ports on a space station. To stain amyloid selectively, a dye must be able to fit within the pleat, and

have paired docking probes just the right distance apart (12–16 Å). Only long, flat dyes would fit, and few of these have proper docking probes (hydrogen-bonding sites). The majority are benzidine dyes. Fortunately, we did find a few that were in a new class, based on ureylene instead of benzidine. Sirius red F3B (CI 35780) is one of them, but it has solubility problems that we felt were significant.

We chose instead a ureylene relative, which we call Amyloid Red (CI 29200). It has the same wonderful selectivity for amyloid that Congo red possesses. It differs in being more stable and easier to use. The color is slightly darker, less pink. It is fluorescent and forms a birefringent complex with amyloid. We think you will rarely need to resort to fluorescence or polarization microscopy because of the superior color of the dye. If there is doubt after staining with Amyloid Red, use immunohistochemistry to be really sure.

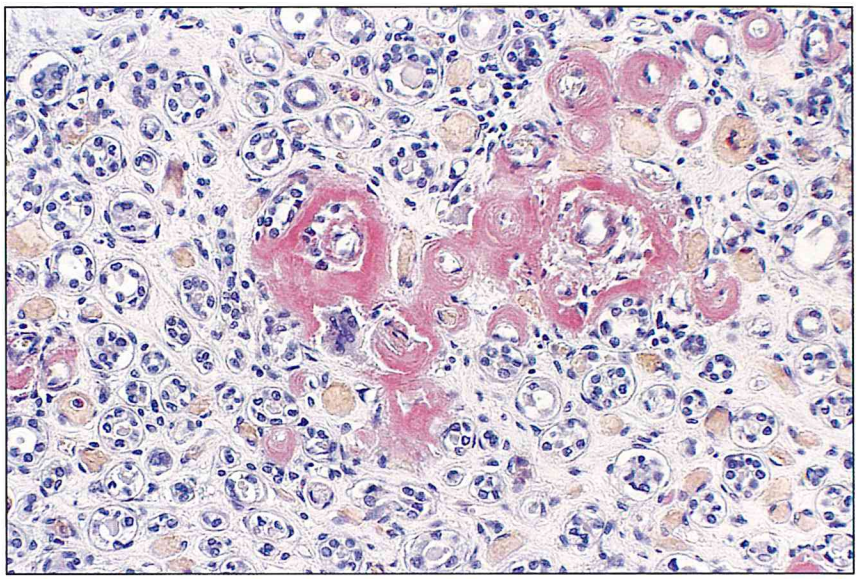

Figure 4.

Human kidney fixed in NBF and stained with Amyloid Red and Harris Hematoxylin. Amyloid stands out clearly against a colorless background; erythrocytes are pale orange. 20x

Keep in mind that amyloid stains best when it is freshly formed, and gradually loses its ability to bind direct dyes as it ages. We're talking here about its age while still in the patient, not the time wet tissue or unstained slides are stored. Whether you use Congo red, Sirius red F3B or Amyloid Red, old deposits will stain lightly; Congo red will be lightest of the three dyes.

Summary of New Products

We hope that you have enjoyed reading about our research, and will try one or more of these products when you need to reorder. All come with complete instructions, including suggested staining protocols. The dyes are accompanied by recommended formulations for stains.

Cat. #	Product	Unit	Price (2001)
862	Alcian Blue, Certified (dye)	25 g bottle	$87.50
863	Amyloid Red (dye)	50 g bottle	$75.00
861	Brazilliant! (stain)	quart	$40.00
860	Brazilin (dye)	50 g bottle	$50.00

ANATECH LTD. 1020 Harts Lake Road, Battle Creek, MI 49015 ❧ 616-964-6450 ❧ 800-262-8324
fax: 616-964-8084 ❧ e-mail: email@anatechltdusa.com ❧ website: anatechltdusa.com