

Origins of ...

Mucin staining

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Introduction

This paper will survey the origin and development of mucin staining. There is insufficient space to include lectin histochemistry and immunohistochemistry in depth, though reference will be made to two important interfaces between mucin staining and immunohistochemistry, converging on sialic acid and the protein backbone of the mucin molecule, respectively.

Natural dyes

Having gone to the trouble of inventing the light microscope, it must have been in a mood of frustration that Anton van Leeuwenhoek (1632-1723) attempted to discern structure in transparent sections. By applying saffron (*Crocus*) solution, Leeuwenhoek was able to elucidate the structure of muscle preparations. Like all histological staining, mucin staining began with the use of natural dyes. Carmine is derived from cochineal, extracted from dried female insects. The Greeks and Romans extracted cochineal from the insect *Dactylopius coccus* and the Aztecs from *Coccus cacti*. Earlier still, no higher authority than the Divinity had exhorted Moses to prepare offerings of rams' skins dyed red (almost certainly with cochineal) (Exodus 25:5). More female than male insect offspring are produced (300:1), an economic boon as only the females produce the dye. To harvest cochineal, egg laden and almost spherical female insects are scraped from the plants on which they live, killed and dried. Carmine is derived from cochineal by boiling the dye with alum to precipitate out a water soluble, partially purified product. Paul Mayer (1892) published several tests to determine the purity of the products.¹ Carmine was used initially as a general histological dye, particularly as a nuclear stain. Rawitz (1899) described "mucicarminic acid" for staining mucin based on the formula: carminic acid 0.5 g, AlCl₃ 1 g, 50% alcohol 100 ml.² A further modification through Southgate³ enjoyed several decades of use, but has now been largely supplanted by periodic acid Schiff (PAS) and alcian blue. The disappearance of mucicarmine from the histological repertoire heralded the virtual death knell of carmine itself, easily the most highly prized dye in the histological investigations of the late nineteenth century.⁴

Before turning to PAS and alcian blue, it should be recalled that another natural dye, derived from 'logwood', also came to be modi-

fied (by Paul Ehrlich) and used as a stain for mucin.⁵ Haematoxylin is extracted from the tree *Haematoxylon campechianum*, so named because it originated in the Mexican state of Campeche (though now grows in the West Indies).⁶ Ehrlich's haematoxylin stains acid mucins (for example, of intestine, salivary glands, oesophageal glands and bronchial glands) but not neutral mucin (for example, of stomach), as well as being an excellent nuclear stain. Ehrlich's haematoxylin is an alum haematoxylin, ripening naturally over a period of about two months.

Alcian blue—a synthetic dye

We turn from the almost mystical world of natural dyes to the hard science of histochemistry. In fact, the dividing line between the empirical staining by dyes used in the textile industry and the more theoretical approach based upon an understanding of chemical (organic, enzymic and immunological) reactions is not clear cut. The physical factors determining reagent-tissue reactivities cut across traditional histochemical classifications; these include van der Waals forces, hydrogen bonding (mucicarmine), covalent bonding (PAS) and electrostatic or coulombic attractions (alcian blue and high iron diamine). Alcian blue is a synthetic basic dye, a metal complex of copper with phthalocyanin substituted by quaternary ammonium groups. It was the first (1948) of a family of alcian dyes to be introduced by an ICI chemist named Haddock.⁶ Alcian blue was derived from an earlier, water insoluble dye, monastral fast blue, used for dyeing cotton. It was described initially as a mucin stain by Steedman in 1950.⁷ The dye binds through electrostatic forces generated by the carboxyl group of sialic acid or sugars with sulphate substitution. The more highly acidic sulphated mucins can be demonstrated selectively by lowering the pH, as shown by Mowry in 1958.⁸

Periodic acid Schiff

Periodic acid (HIO₄) is an oxidising agent, used initially by Jackson and Hudson (1937) for the chemical estimation of polysaccharides.⁹ McManus (1946) was the first to apply periodic acid to the histological demonstration of mucin,¹⁰ whereas Hotchkiss (1948) emphasised the legitimacy of periodic acid as a specific histochemical reagent.¹¹ Pearse reinforces the fact that the whole of the modern

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histochemistry of the mucopolysaccharides is bound up with the PAS reaction.¹² Periodic acid breaks the C–C bond in 1:2 glycols of monosaccharides, converting the glycol groups into dialdehydes. Because the aldehydes are not oxidised further, they can be localised with Schiff's reagent (used earlier in Feulgen staining) to give a substituted dye that is magenta in colour. The intensity of the colour reaction is directly proportional to the number of reactive glycol structures. However, the batch and quality of the Schiff's reagent (a basic fuchsin solution: F(SO₂H)₂) will also affect staining intensity.

Apart from the currently popular combined alcian blue–diastase PAS sequence,⁸ many modifications of the PAS technique have been described. Most of these modifications relate to the fact that sialic acid exists in several variant forms. The presence of O-acetyl groups at C₄ and/or the C_{7–9} side chain means that the 1:2 glycol groups are no longer available for conversion to dialdehydes. Colonic sialic acid—for example, is heavily O-acetylated, making it relatively non-reactive with PAS and also resistant to neuraminidase digestion. Culling and coworkers developed the periodate borohydride/potassium hydroxide/PAS (PB/KOH/PAS) technique to demonstrate O-acetyl sialic acid. Following periodate oxidation, dialdehyde reactivity is blocked by sodium borohydride. KOH then removes (saponifies) the O-acetyl groups, unmasking PAS reactive 1:2 glycol groups. A positive (magenta) reaction confirms the presence of O-acetyl sialic acid.¹³ The same team developed this approach further to allow sialic acid without O-acetyl substituents (and any other PAS positive sugars) to be stained blue and O-acetyl sialic acid to be stained magenta. This was achieved with the sequence periodic acid/thionin Schiff/saponification/PAS (PAT/KOH/PAS).¹⁴ Spicer had shown earlier that the interposition of phenylhydrazine between periodic acid and Schiff blocked the staining of neutral sugars but not sialic acid.¹⁵ The final and most complex development of the PAS stain involved not only phenylhydrazine (P) but also borohydride (Bh) interposition (to improve specificity)—that is, PAPT/KOH/Bh/PAS.¹⁶ These methods were developed with the hope that they would allow distinction to be made between colorectal and other adenocarcinomas. Unfortunately, sialomucin in colorectal neoplasms is changed from the O-acetylated to the non-O-acetylated form.¹⁷ Attention was then focused on the nature and diagnostic significance of this switch. This was facilitated by the development of the mild PAS technique, specific for non-O-acetyl sialic acid, by Veh *et al.*¹⁸ This extremely simple method has been used to shed light on factors determining sialic variation in normal, precancerous and cancerous lesions of the large intestine. For example, it has been shown that there is genetic polymorphism of the O-acetyl transferase gene that accounts for the fact that 9% of caucasian colons fail to express O-acetyl sialic acid.^{19, 20}

Mucins have been shown to include blood group structures.²¹ Over the years that followed

the development of biotechnology to produce monoclonal antibodies, probes directed against particular blood group structures have been made available. Antibodies specific to the sialylated blood groups SLe^a (gastrointestinal cancer antigen or GICA), SLe^x (CD15s), STn, and other structures such as small intestinal mucin antigen (SIMA), have been heralded as cancer specific or cancer associated—for example, in relation to colorectal cancer.^{22–24} Much of this altered colorectal cancer mucin reactivity reflects merely the switch from O-acetyl to non-O-acetyl sialic acid. The presence or absence of O-acetyl groups profoundly affects the antigenicity of sialylated structures. The antibodies PR3A5,²⁵ 3NM,²⁶ and MMM17²⁷ react only with structures that include O-acetyl sialic. Conversely, TKH2²⁸, AM-3²⁹ and anti-SIMA³⁰ are reactive only in the presence of non-O-acetyl sialic acid. The tumour associated antigens SLe^a, SLe^x and STn are expressed constitutively by normal colorectal goblet cells, though rendered cryptic by the usual O-acetylation of sialic acid.^{28, 31, 32} This remarkably simple and unifying phenomenon, namely the switch from O-acetyl to non-O-acetyl sialic acid, calls for reappraisal of the immunohistochemical literature on colorectal mucins and signals the danger of applying immunohistochemistry in the absence of classic mucin histochemistry, founded essentially on the 50 year old PAS technique and its modifications.

The enzyme galactose oxidase selectively converts 1:2 glycol groups in galactose and N-acetyl galactosamine (GalNAc) to dialdehydes. The galactose oxidase-Schiff (GOS) technique demonstrates terminal galactose and GalNAc groups within—for example, gastric surface and crypt columnar cell mucus.³³ Mucin within gastric mucous neck cells and pyloric glands is GOS negative. This again illustrates how the principle of the broad spectrum PAS stain can be adapted for more specific purposes, thereby uncovering phenotypic variation of mucin within a single organ.

Staining of sulphated mucin

Basic aniline dyes show a high binding affinity for sulphate groups. The first aniline dye to be produced was orcein. Known originally as French purple (circa 1300 AD), this was produced by exposing a lichen extract (*Rocella*) to air oxidation in the presence of ammonia formed in fermented urine.⁴ Orcein is now used as an elastic stain, but the disulphide bonds must first be oxidised. Orcein and other aniline dyes, notably aldehyde fuchsin, will bind to sulphate groups in mucins without the requirement for prior oxidation. Gomori (1946) was the first to use aldehyde fuchsin as a histological stain for elastin.³⁴ Spicer and Meyer (1960) combined the technique with alcian blue to distinguish sulphated mucins (purple) from carboxylated mucins (blue).³⁵ The difficulty with this technique is that a single goblet cell, a single mucin molecule, or even a single oligosaccharide chain could include both sialic acid and sulphated sugars. Indeed, sialic acid itself could be sulphated. This

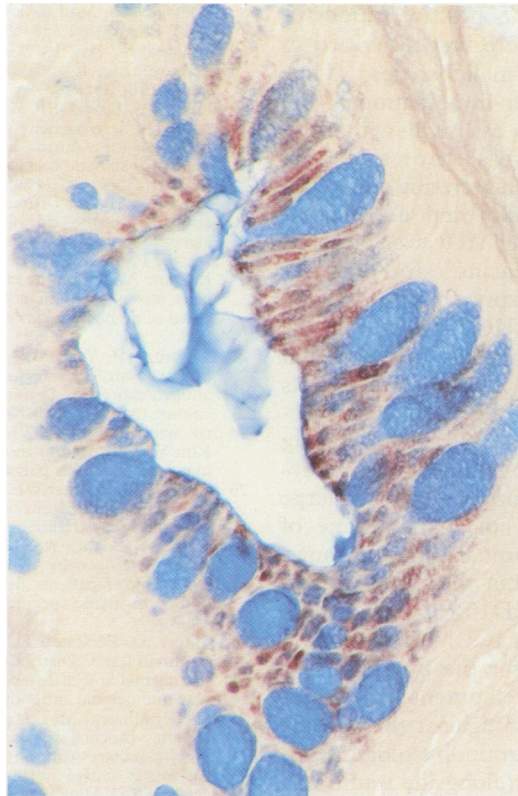


Figure 1 Incomplete intestinal metaplasia (type III) of gastric mucosa in which goblet cells secrete non-sulphated acid mucin (blue) and columnar mucous cells secrete sulphated acid mucin (brown/black) (HID/AB).

means that the result of staining is often an indecipherable mixture of purple and blue. The high iron diamine/alcian blue (HID/AB) sequence³⁶ provides an improved colour contrast, sulphated mucins staining brown and carboxylated mucins (sialomucins) staining blue (fig 1). This method has been used extensively in the study of disorders of the colorectum,³⁷ stomach³⁸ and Barrett's oesophagus.³⁹ The AB/PAS and HID/AB techniques facilitated recognition of complete and incomplete variants of intestinal metaplasia of gastric mucosa, incomplete sulphomucin secreting forms showing a selective association with gastric cancer.³⁸ However, interpretation of the HID/AB technique has provoked controversy.⁴⁰

Future of mucin staining

The main practical use of mucin histochemistry lies in the diagnosis of adenocarcinoma, particularly poorly differentiated adenocarcinoma. The stains in routine use are alcian blue and diastase PAS, either alone or in combination. Clearly this practice will continue long into the future. New developments will arise from an improved understanding of the nature of cancer mucin and this will certainly acquire diagnostic importance.

It is generally assumed that cancer 'mucin' is the counterpart (albeit differing qualitatively) of the normal secretions of mucinous cells of crypts, ducts or acini.⁴¹ Nevertheless, mucin secreting adenocarcinomas may arise in tissues or organs that do not normally secrete mucin, such as prostate, breast and ovary. Mucinous metaplasia is only a partial explanation for this phenomenon. Intraluminal PAS positive mate-

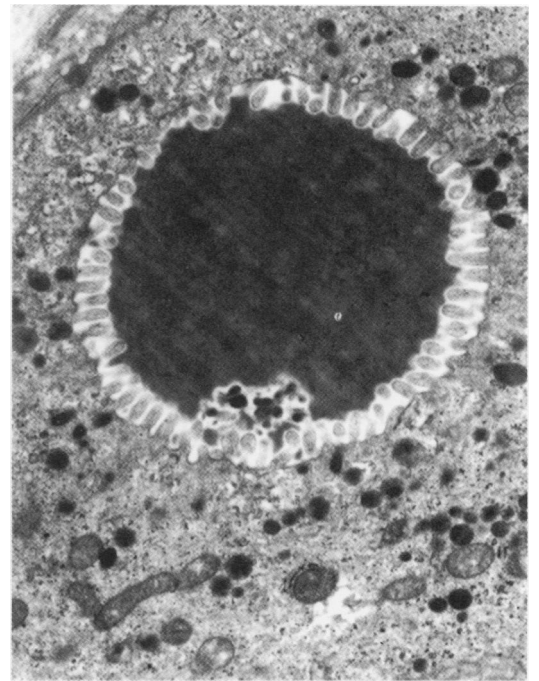


Figure 2 Intracytoplasmic lumen in a metastatic adenocarcinoma at ultrastructural level. Small electron-dense cytoplasmic secretory vacuoles are present in the surrounding cytoplasm (EM $\times 10\ 000$).

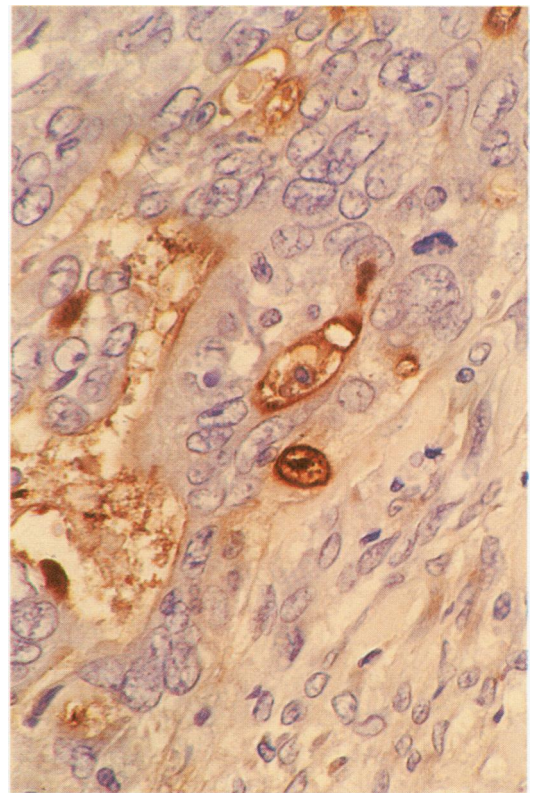


Figure 3 Intracytoplasmic lumen stained for MUC1⁴⁵ which highlights the glycocalyx ring and central droplet. Macrolumina also contain MUC1 positive material (immunoperoxidase).

rial may not be secretory mucin at all, but rather represent upregulated membrane associated glycoprotein (glycocalyx) that is normally elaborated by non-mucin secreting columnar or cuboidal cells.⁴² The related epitopes to which monoclonal antibodies have been raised include epithelial membrane antigen (EMA), human milk fat globules (HMFG1 and 2) and the underlying protein

backbone (MUC1).⁴² PAS positive intracytoplasmic lumina (ICL) were first described in non-mucinous epithelial malignancies.⁴³ They arise through intracellular invagination of the apical membrane, bearing microvilli with a glycocalyx coat (fig 2). ICL have also been described in typical, mucin secreting adenocarcinomas (fig 3).⁴⁴ In colorectal adenocarcinoma, ICL express mainly MUC1 as opposed to goblet cell MUC2 mucins.⁴⁵ Interestingly, macrolumina may also express predominantly MUC1 as opposed to MUC2 mucin, indicating that much of the PAS positive material that we loosely equate with secretory mucin is nothing of the sort.⁴⁵ In the normal colorectum, MUC1 can be demonstrated following periodic acid oxidation to remove the carbohydrate chains.⁴⁵ Its distribution in normal large bowel is limited to the apical membrane of immature crypt base columnar cells and shows exact co-localisation within the type 2 blood group substances Le^x (CD15) and Le^y (these are also expressed within ICL).⁴⁵ The cloning of mucin genes coding for the protein backbone (MUC1-7),⁴⁶ development of monoclonal antibodies directed against the specific tandem repeats⁴⁷ and immunohistological application of these probes (alongside traditional mucin staining) will transform our understanding of the nature of mucin and its role in health and disease.

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