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Origins of...

Tinctorial methods in histology

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Tinctorial methods, by definition, are those that employ dyes to demonstrate tissues and their constituents. The following account will not be concerned with techniques where a colour compound is formed in situ. This means that silver techniques, and what is traditionally termed histochemical techniques, will not be considered. Equally, there will be no attempt to consider each and every method in what is a highly impressive repertoire. The choice of subject will be restricted to methods that were either influential in their day, or have stood the test of time and remain a feature of the current histological scene.

The early dyes

Over the years a wide range of dyes has been used for histological staining methods. Most of these have been adapted from those in use in the textile dyeing industry, and it is only in recent decades that a clearer appreciation has been reached of the underlying mechanisms that result in the binding of dyes to tissue.

The dye with probably the greatest claim to antiquity is indigo. It is extracted from the indigofera plant, and there is evidence of its use to dye cloth by the ancient Egyptians some 3000 years ago. Coincidentally, the Arabic for indigo is annil from which is derived the word aniline, the substance extracted from indigo in the early 19th century.[1] The aniline group of dyes would become the dominant force in the growth of the textile dyeing industry and histological stain technology. Examples of other early dyes are woad from the plant *Isatis tinctoria* much favoured by the ancient Britons, and, moving into the 13th century, the use by Robin Hood *et al* of Lincoln Green, which comes from the plant *Genista tinctoria*.

Turning to rather more contemporary times, the pioneer microscopists employed a variety of naturally occurring dyes to colour their somewhat rudimentary specimens. These included Tyrian purple from a type of Mediterranean shell fish, alizarin from the madder plant, saffron from the stamens of the crocus, and carmine, much favoured by the early microscopists and first used by Leeuwenhoek in the 18th century.[2] This last dye is extracted from cochineal, which is obtained from the female beetle of that name. Iodine was also quite popular for colouring specimens, particularly among French workers. Raspail used iodine in 1825 to demonstrate starch in plant cells, and Claude Bernard, the famous French physiologist, used it to stain a substance in liver[3] that he would later (1849) identify and call glycogen.

Early staining procedures

It should be borne in mind that early microscopy was performed either on plant material or simple tissue preparations, and it was only when fixation and sectioning techniques improved in the latter half of the 19th century that histological staining, as we know it today, became a reality.

As indicated previously, cochineal was one of the earlier, commonly used dyes often in simple alcoholic solution. The dominant figures of 17th century microscopy Leeuwenhoek and Hooke used cochineal[4] as did Sir John Hill, a noted English microscopist who in 1770 stained wood fibres by this means.[5] In later years a number of eminent German microscopists would advance the knowledge of tissue structure, again using carmine staining. They included Ehrenberg in 1838, Goppert and Cohn in 1849, Corti in 1851,[6] and von Gerlach in 1858.[7]

Picric acid was used for dyeing silk in the 1840s, but seems not to have been taken up by histologists until Roberts, an English microscopist, used it as a general stain for tissue protein in 1863. A significant event took place in 1856 in terms of dye formation, when a young English chemist named Henry Perkin (fig 1), synthesised the first aniline dye that he named aniline purple,[8] and became better known as Perkin's mauve. This opened up new horizons for the dyeing of textiles and, ultimately, the staining of tissue in the histology laboratory. Perkin's fortunes were assured when, in future years, his purple dye came to be used to colour some of the early postage stamps and a favourite ballgown belonging to Queen Victoria. He was knighted in 1906. Other new aniline dyes for textiles soon appeared and include some of the now familiar names. Basic fuchsin was synthesised in 1858, aniline blue in 1862, eosin in 1871, and methylene blue in 1876.

Although the synthesis of aniline dyes was to usher in a new era of tissue staining it is important to remember that there is one natural dye, in particular, which came into histological use within a few years of Perkin's discovery, and has remained a constant feature ever since. This refers, of course, to haematoxylin, which is extracted from a tropical logwood, largely obtainable from Central America. It seems to have been first used non-commercially by

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Figure 1 A young Henry Perkin.

Figure 2 A young Paul Ehrlich.

Reichel in the 18th century[6] and in a commercial sense in the 1840s.[9] The active stain ingredient is created by oxidation, and it was Mayer in 1891 who demonstrated that the compound so formed was haematein.[10] Waldeyer is credited with the introduction of haematoxylin in 1863 as a tissue stain,[11] although this is not without dispute.

Bohmer in 1865 combined alum with haematoxylin to form a distinctive albeit imprecise stain for cell nuclei.[12] More selective nuclear staining was achieved when in 1886 Ehrlich (fig 2) added acetic acid to the solution.[13] A number of the earlier alum haematoxylin solutions are still very much in use today and include those of Ehrlich of 1886, Harris 1900, and Mayer's haemalum 1903. Harris was an American born in 1875 about whom little is known. Paul Ehrlich was one of the giants of German medical science with significant contributions to bacteriology, haematology, and immunology as well as histology. He was cousin to Carl Weigert (fig 3), another famous figure. Much of Ehrlich's creative work was carried out in Berlin and Frankfurt, where he was to die in 1915 at the age of 61. Paul Mayer, who died in 1923 aged 78, was of German-Italian extraction and spent much of his working life as a histologist at the Zoological Station in Naples, Italy. As well as his haematoxylin solution he is also remembered for the mucicarmine and mucihaematein techniques for mucin, both published in 1896. The use of haematoxylin is not, of course, restricted to the demonstration of cell nuclei. Other uses have appeared and include staining for myelin in 1889, muscle in 1900, elastin in 1908, and, in more modern times for neuroendocrine cells (1969), etc.

Figure 3 Carl Weigert.

Before concluding this section on the historical development of histological dyes mention must be made of their commercial procurement. As has already been indicated, commercial dye production was primarily aimed at the textile industry rather than the laboratory and, initially, little or no attempt was made to classify and standardise dyes in a scientific sense. An important step forward was when a Dr Grubler of Leipzig formed in 1880 a company for the express purpose of marketing dyes for microscopy. Later in 1897 another similar company was formed by a Dr Hollborn and for many years these two German compa-

nies successfully marketed much of the world's histological dyes.

The development of histological techniques

Synthetic dyes began to be more widely used in the early 1860s but the actual methods were relatively crude. They invariably consisted of one-step staining, the excess stain being washed off in water or alcohol before the slide was mounted. In 1867 Schwartz introduced two-dye sequential staining interspersed with a simple washing stage.[14] The technique was further refined when in 1869 Bottcher incorporated an alcohol differentiation step[15]; however, some simple staining regimens remained popular. An example is the metachromatic staining of cartilage which dates from 1875 and is attributed to the celebrated French histologist Louis Ranvier who used the dye cyanine.[16]

It was at this time that the haematoxylin and eosin method was born. The H&E has survived the passage of time to become the standard morphological staining method for just about every histological laboratory in the world. As we have seen, alum haematoxylin had emerged as a nuclear stain and one of the early counterstains was aniline blue—hardly an ideal choice. Eosin had been reported as a general stain for tissues by workers such as Dreschfeld and Fischer in the 1870s. The actual combination of the two dyes to form a single method has been variously attributed to Wissowzky in 1875, Reynaud in 1876, and Busch in 1876–78.[17] Eosin is also a fluorochrome and, a century later, this property would be utilised by the increased fluorescence shown by eosin stained myocardial fibres when undergoing early necrotic change.[18]

The 1880s saw the inception of three tinctorial techniques that are in popular use today. What is popularly known as the ZN technique seems to have had a particularly complex genesis. The original stain was devised by Robert Koch in 1882 who used an alkaline methylene blue solution to stain the tubercle bacilli, counterstaining with Bismarck brown. In the same year Ehrlich modified this by changing the primary stain to a mixture of aniline and methyl violet, and the counterstain to methylene blue. Later that year Friedrich Ziehl replaced the aniline with phenol—that is, the primary stain is now a carbol (from carbolic acid) methyl violet. Finally, Friedrich Neelsen in 1883 replaced the methyl violet with basic fuchsin. Thus was realised the now-familiar carbol fuchsin stain.[19] Interestingly, given the context of this staining method, both Ehrlich and Neelsen contracted tuberculosis during their lifetime.[8] The former survived his infection, but Neelsen succumbed to his at the comparatively early age of 40. The second of the three techniques is the Gram stain for organisms. Hans Gram, who died at the age of 85 in 1938, was a Professor of Medicine in Copenhagen although his famous technique was devised in 1884 while working in Berlin. The method was based on an earlier one devised by the ubiquitous Paul Ehrlich, and a key feature was the Lugol's iodine step following the initial staining by gentian violet. The iodine was used almost by accident as Gram had intended it as a counterstain rather than as a dye trapping agent for what came to be known as the Gram positive bacteria.[20] It is of interest that Jean Lugol was a Paris physician who in 1830 developed the solution that carries his name as a treatment for scrofula (cervical gland tuberculosis). The third of these three techniques is the van Gieson for collagen. Ira van Gieson was a New York neuropathologist of Dutch-Jewish parentage who died in 1913, having published his technique in 1889.[21]

The closing years of the 19th century brought a number of significant tinctorial methods to the fore, one of which, although primarily a haematological stain, has enjoyed considerable use by histologists (and cytologists). This is the stain formulated by Dimitri Romanowsky a Russian protozoologist working in St Petersburg, where he died aged 60 in 1921. The dye compound is actually based on an earlier one devised by Ehrlich who is also credited with having coined the term neutral dye.[22] Romanowsky described his now famous staining technique in the course of his 1892 MD thesis on malaria infected blood cells. A number of important modifications to his method have appeared over the years, and include those of Leishman in 1901 and Giemsa in 1902. William Leishman, who died in 1926 aged 61, was of English stock and served in the Royal Army Medical Corps where he rose to become Lieutenant General. Gustav Giemsa, on the other hand, was a German bacteriologist and chemist at Hamburg University. He died aged 81 in 1948.

The demonstration of lipids in tissue received a boost in 1896 when an Italian named Daddi—about whom little is known—introduced the Sudan dyes for that purpose.[23] Another important landmark in the history of histological staining methodology was the advent of the so called trichrome techniques in 1900. These, together with the phosphotungstic acid–haematoxylin method of 1897, are designed to show in contrasting colours a wide range of tissue constituents—particularly muscle and the connective tissues. The early histopathologist, whose name is most often associated with this group of tinctorial methods, is Frank Mallory who was Professor of Pathology at the Boston City Hospital.[8] He died in 1941 having only three years earlier published an authoritative text on histological techniques when aged 76. Another classic trichrome method later appeared in 1929 and is that of Pierre Masson (fig 4) a French pathologist who settled in Montreal, Canada where he was to die aged 79 in 1959.

At the end of the 19th century a number of now familiar methods for elastic fibres made their appearance. The first of significance was Unna's orcein technique of 1890, Paul Unna being a Professor of Dermatology in Hamburg, Germany. As a stain for elastin orcein has diminished in popularity over the years but, as modified by Shikata in 1974, has taken on a new lease of life in the demonstration of viral hepatitis antigen. Unna is also credited with

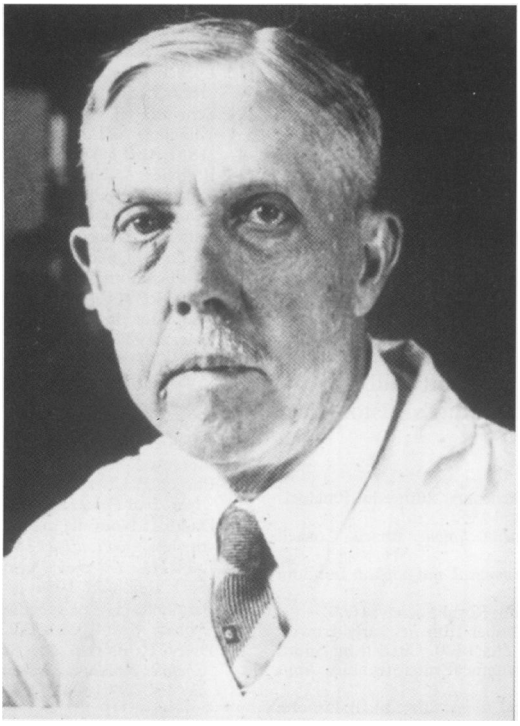

Figure 4 Pierre Masson.

having discovered the existence of plasma cells,[8] and his name is linked with the original histological technique for these cells namely the Unna-Pappenheim. This was published in 1899 by Pappenheim (a student of Unna's) and was based on an original technique by the ubiquitous Paul Ehrlich. Unna's modification of the solution followed in 1902. The outstanding contribution to elastic fibre staining, however, was undoubtedly that of Carl Weigert working in Breslau and Leipzig, who gave us his classic elastin stain in 1898. The iron haematoxylin solution for cell nuclei followed in 1904, the year of his death at the relatively early age of 59. Another still popular elastin stain is the 1908 method of the American pathologist and ophthalmologist Frederick Verhoeff, who was based in Massachusetts.

Staining techniques: the later years

It was inevitable that, as time passed, the momentum of tinctorial method innovation would slacken as other forms of histological demonstration technique took shape; immunocytochemistry is the most obvious example. Indeed, from the 1920s onwards comparatively few significant staining techniques emerged.

Congo red is a cotton dye introduced to the textile industry in 1884, and takes its name from the Congo Free State which was formed in that year.[24] Griesbach seems to have used Congo red in 1886 as a stain for axons but it was another German worker, Bennhold, who described in 1922 its all important use for identifying amyloid in tissue sections.[25] The diagnostic value of this was considerably heightened when in 1927 two French workers, Divry and Florkin, demonstrated the green birefringence effect of amyloid deposits stained with Congo red.[26] In the same year Southgate, an English pathologist at the Croydon General Hospital, published his variant of Mayer's mucicarmine solution. This remained for many years the standard method in Britain for mucins. Curiously, Southgate's mucicarmine has waned in popularity in this country, while in America Mayer's format continues to flourish.

The year 1950 saw the appearance of several tinctorial methods of note. Harold Steedman, a research scientist at Glasgow University, published the alcian blue technique for acid mucins,[27] and George Gomori the aldehyde fuchsin technique for elastic fibres and mast cells.[28] The latter worker also published, in that year, his one-step trichrome technique for which there are adherents today. Gomori, who died at the relatively young age of 53 in 1957, was an American pathologist of Hungarian extraction, at the University of Chicago.[29] A remarkable researcher, he is chiefly remembered for his original contributions to the fields of silver demonstration techniques and enzyme histochemistry. Steedman, who was also well known for his publications on ester wax and microtomy, died in 1991 at the age of 84.

In this period a new staining technique made its appearance for the demonstration of normal myelin; the luxol fast blue of Kluver and Barrera in 1953.[30] This dye is closely related to alcian blue, being of the copper phthalocyanin group. Its affinity for myelin, however, remains poorly understood.

Tinctorial methods and the modern laboratory

The more traditional staining methods will probably continue to play a part in histology either, as in the case of the H&E, to afford a diagnosis per se, or as special stains to assist in arriving at a diagnosis. As special stains they can be used alone, or in a complementary role to the more sophisticated technology. The advent, and now wide employment, of immunocytochemistry plus the growing involvement of molecular biology in diagnosis has, inevitably, signalled a decreasing role for the tinctorial method. However, certain of these stains are still very much in evidence—for example, in the demonstration of lipids and carbohydrates. In the field of infective agents, particularly those associated with the immunocompromised disorders, there has been something of a resurgence in the use of tinctorial methods such as the ZN. Other, newly identified, pathogens have created a demand for the more traditional staining methods, a good example being gastritis and the need to demonstrate the associated helicobacter organisms. As discussed, the main challenge to the use of tinctorial methods in histology has come from immunocytochemistry as a routine tool. Consequently, the more subjective, colourful staining techniques such as those for the constituent cells of the anterior pituitary gland and the pancreas are rarely called for. It is probably also true to say that the trichrome methods are less often used. However, an important role for staining techniques is that they continue to complement the information furnished by an antigen–antibody reaction. To give but one example: by means of immunocytochemistry it is now possible to

characterise the precise type of abnormal protein present in certain amyloid deposits. This might well be the A4 protein that is associated with Alzheimer's disease and found in blood vessel walls and plaques of affected brain tissue. Lacking the availability of a suitable pan-amyloid antiserum in the immunocytochemical armamentarium, however, there is still a well defined need for an initial indicator for the presence of amyloid per se. Until such a general amyloid antiserum becomes available it is likely that the Congo red staining method will continue to flourish. It would seem, therefore, that the tinctorial method still has an important part to play in today's histological environment.[31]

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