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Review

Optical properties of amyloid stained by Congo red: History and mechanisms

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ABSTRACT

Amyloid stained by Congo red has striking optical properties that generally have been poorly described and inadequately explained, although they can be understood from principles of physical optics. Molecules of Congo red are orientated on amyloid fibrils, and so the dye becomes dichroic and birefringent. The birefringence varies with wavelength in accordance with a fundamental property of all light-transmitting materials called anomalous dispersion of the refractive index around an absorption peak. The combination of this and absorption of light, with modification by any additional birefringence in the optical system, explains the various colours that can be seen in Congo red-stained amyloid between crossed polariser and analyser, and also when the polariser and analyser are progressively uncrossed. These are called anomalous colours.

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1. Introduction: amyloid and Congo red

In 1953 Missmahl and Hartwig wrote: 'Die Entdeckung der Doppelbrechung in der Amyloidsubstanz hat eine längere und sich mehrfach wiederholende Geschichte'. (The discovery of the birefringence of amyloid has a long and many times repeated history.) Because many misquotations, misunderstandings and misattributions of historical matters related to amyloid have been passed from paper to paper, extracts from the most relevant papers are copied from the original, with translations of German and French extracts by DBB, to allow readers to check the meaning for themselves. The spelling of the German in quotations and titles of papers in the list of references is copied exactly from the original and so may not follow precisely the current reformed German spelling.

The quotation from Missmahl and Hartwig (1953) can be used as an example of confusion about optical properties of amyloid, because most of the practical and theoretical study has not been on

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birefringence of amyloid alone, but amyloid stained by Congo red. Indeed, the more important factor in the properties is Congo red.

Congo red is a synthetic compound given a variety of formal chemical names, but each molecule can be considered to consist of two molecules of naphthionic acid (1-naphthylamine-4-sulfonic acid or 4-aminonaphthalene-1-sulfonic acid) coupled to one molecule of benzidine (diaminophenyl or 4-(4-aminophenyl)aniline) by azo groups, –N=N–. This is now given the Colour Index number 22120 and name direct red 28 (Horobin and Kiernan, 2002). The chemical was supposedly first made in 1883 by a method patented in 1884 by a German chemist, Paul Böttiger, who had been looking for a pH indicator (Steensma, 2001). Böttiger may not have been the first to make Congo red (Armstrong, 1935).

Congo red was found to be a direct dye for cotton, which meant that there was no need for a mordant, or a chemical required to fix the colour in materials. Cotton is cellulose from plant cell walls. The linear Congo red molecules align themselves along the linear molecules of cellulose, with hydrogen bonds between the two substances (Wälchli, 1945). Congo red was the first direct dye, and had great commercial success. Congo Red was originally a trademark, and other early trade names for this included Congo, various types of Cotton Red and Direct Red, Cosmos Red, and Chokusetsu Red (Rowe, 1924). Chokusetsu means direct in Japanese.

The dye is too easily washed out of fabrics to be useful these days for most colouring purposes, and there are other drawbacks such as the toxicity of benzidine used in its manufacture, but Bennhold (1922) in a short technical paper described the specific staining of amyloid with Congo red, and this is now one of its major uses.

Bennhold was not the first to use Congo red as a stain for microscopy. Griesbach had done this in 1886 in a study of many azo dyes, but he did not mention amyloid (Griesbach, 1886). Bennhold is credited as the first to report its value in detection of amyloid. He discovered this in a study of intravenous injections of Congo red, which others had used to determine blood volume (Bennhold, 1923). Bennhold was a physician in Hamburg at that time, and investigated the distribution and elimination of Congo red in 21 normal people and 82 patients with various conditions.

He found that in patients with what would now be called the nephrotic syndrome there was more rapid clearance of Congo red from the blood than in controls and most other conditions, with appearance of the dye in the urine in nephrotic patients, which suggests that Congo red was bound to albumin or other plasma proteins. In 10 patients said to have amyloidosis complicating pulmonary tuberculosis, most had even more rapid clearance, including when no Congo red appeared in the urine. Necropsy on a patient with amyloidosis who died 20 h after injection gave the explanation, because the liver and spleen appeared red macroscopically, and unstained frozen sections of liver, spleen and kidney had red areas on microscopy, which showed that Congo red specifically bound to amyloid.

Intravenous injection of Congo red was used in clinical practice for about 40 years in the diagnosis of amyloid. For example, this was still in a guide called Today's Tests (1968). Biopsy of an affected organ with staining of sections by Congo red is now the usual way to make a diagnosis of amyloid.

Amyloid, meaning starch-like, was first used by Vogel and Schleiden (1839) as a name for a material in cell walls of the seeds of some plants: 'Das Amyloid kommt gebildet in der Natur vor'. (Amyloid occurs already formed in nature.) This had a property of starch, namely reaction with iodine to give a blue colour, but did not have all the properties of starch. The name amyloid was applied later in several different senses, including to cellulose after treatment with sulphuric acid. This gave cellulose a property that unmodified cellulose did not have, which was reaction with iodine to give blue (Puchtler and Sweat, 1966).

Application of the term amyloid to human disease was initially inconsistent and confused (Puchtler and Sweat, 1966; Schwartz, 1970). Virchow, who is usually said to have introduced the term into pathology, wrote a few papers which are difficult to understand, such as Virchow (1854). He used amyloid first as a morphological description of various concentric structures resembling starch granules, including corpora amylacea in the brain, previously named by Purkinje. Later he used amyloid to mean an abnormal material detected by chemical tests, which he thought resembled cellulose rather than starch. This use of the term was eventually to lead to the modern meaning of amyloid as extracellular, insoluble fibrils derived from various misfolded proteins.

2. Optical properties of Congo red-stained amyloid and orientated Congo red: the background

Amyloid stained by Congo red has striking optical properties that have mostly been badly described and inadequately explained. These properties are largely due to the fact that the dye molecules have an orientated arrangement on amyloid fibrils, as they do on cellulose molecules, with hydrogen bonding between amino and other groups on the dye and hydroxyl groups on amyloid (Puchtler et al., 1962). Solutions of Congo red, in which the molecules are randomly orientated, generally do not show these properties.

Congo red molecules can also be orientated in ways that have been used in optical investigations. These include crystallisation and use of flowing solutions, which are technically difficult (Wälchli, 1945); polishing or stroking of dried drops of a solution, which do not give particularly well orientated preparations (Missmahl, 1957; Taylor et al., 1974); and smearing of drying drops with a single stroke in one direction on a glass slide, which is the simplest way to obtain clean, well-orientated material that can be used repeatedly in investigations of optical properties (Zocher, 1925; Missmahl, 1957). In these preparations the asymmetric molecules become orientated with their long axis in the long axis of needle-shaped crystals, in the direction of flow, and in the direction of polishing or smearing.

When stained by Congo red, amyloid and other materials, such as cellulose paper and cotton, appear various shades of red in unpolarised light, and so do solutions of Congo red and preparations of orientated Congo red. This is because the dye, as usually used, has its maximal absorption of wavelengths in the blue/green part of the visible spectrum. When white light is altered by most absorption of these wavelengths, the appearance is red.

Congo red has to be in alkaline or weakly acid solutions for red to appear, when it is the disodium salt. In strongly acid conditions, as the free acid, the maximal absorption moves to longer wavelengths in the yellow and orange, giving a violet or blue colour. This explains how Congo red can be used as a pH indicator, changing colour in the pH range 3–5 (Horobin and Kiernan, 2002). The possibility of change of colour is another reason why Congo red is rarely used to dye clothes now. The peak absorption may also move to longer wavelengths when there is increased binding to a substrate, which is called the bathochromic shift (Amelin and Tret'yakov, 2003). The effects of pH and binding may explain some of the reported differences in optical properties of Congo red-stained amyloid and orientated Congo red, for instance in the wavelengths at which a property is at its maximum.