AN ACRIFLAVINE ALCIAN BLUE TECHNIQUE FOR DUAL STAINING OF CARTILAGE AND MAST CELLS IN PARAFFIN SECTIONS

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Dewaxed and hydrated sections were treated with a 0.5% acriflavine hydrochloric acid solution of pH 1.5 for 30 min and then washed with distilled water followed by a rinse in descending concentration of ethanol and again in water. The sections were next treated with a 0.1% alcian blue acetic acid solution of pH 2.5 for 2-20 min, rinsed in water, dehydrated with ethanol and then cleared and mounted. Cartilage matrix appears bluish yellow and lacunar borders and chondrocyte cytoplasm yellowish blue. Mast cell cytoplasmic constituents appear bluish orange in contrast to the pale yellow of the nucleus.

For the differentiation of tissue acid mucopolysaccharides a series of combined stainings have proved to be useful, such as the colloidal iron-periodic acid-Schiff(PAS) (18), alcian blue-PAS(12), alcian blue-azure A(21), aldehyde fuchsin-alcian blue (22), alcian blue-safranin(21, 28), alcian blue-alcian yellow(17, 25) and high iron diamine-alcian blue(2, 23, 24) methods. Although differential staining of sulfated mucopolysaccharides has been relatively satisfactorily done by means of some of these methods, there are only a few dual staining techniques in which a dye with specific affinity towards sulfate groups of the substances is utilized.

Since Dodgson, Rose and Spencer (1) and Egami(3, 4) recorded the property of acriflavine to precipitate a variety of organic sulfate esters *in vitro*, this acridine dye has histologically been employed either as a fixative(7) or as a staining reagent(6, 13, 27) for demonstrating sulfated mucopolysaccharides in tissues. This specific staining property of acriflavine was combined with the alcian blue method of Spicer (21) in order to establish a dual staining method suitable for the characterization of sulfated mucopolysaccharides in cartilage and mast cells.

MATERIALS AND METHODS

Preparation of Tissue Sections

Tissue blocks containing tracheal, bronchial and ear cartilage and those of stomach and intestines were obtained from adult Wistar strain rats and C57BL/6J and ddN strain mice of both sexes sacrificed by ether anesthesia. The tissues were fixed in chilled 10% formalin in 95% ethanol(10) or in chilled 2% calcium acetate in 10% formalin(8) before embedding in paraffin. Sections were cut at a thickness of 6 to 8 μ and subjected to the following dual staining.

Staining Procedures

The dual staining with acriflavine(2, 8-diamino-10-methylacridinium chloride) (Katayama Chemical Works, Japan) and alcian blue 8GS(Schmid & Co., Germany) or 8GX(ICI, Inc., England) in this sequence was carried out according to the

following procedures.

1. Sections are dewaxed in xylene, hydrated through descending grades of ethanol and washed in distilled water.
2. The slides are immersed in a 0.5% acriflavine hydrochloric acid solution of pH 1.5 at room temperature for 30 min.
3. After briefly rinsing in distilled water, the slides are placed in 95% ethanol to remove excess dye and are rehydrated through descending grades of ethanol.
4. Sections are then stained in a 0.1% alcian blue acetic acid solution of pH 2.5 at room temperature for 2–20 min. This staining is to be stopped, before displacement of acriflavine by alcian blue begins to appear; an appropriate time of staining is about 2 and 15 min for cartilage and mast cells respectively.
5. Sections are washed in distilled water and, if necessary, counterstained lightly with Mayer's haemalum for nuclei and rewashed in water.
6. Sections are dehydrated through ascending grades of ethanol, cleared in two or three changes of xylene and mounted in balsam or an equivalent synthetic resin.

Histochemical Experiments.

Prior to the dual staining, a series of histochemical experiments were performed in order to gain insight into the significance of the staining. Among the experiments are methylation (incubation in 0.1 N HCl in methanol for 4 hr at 37°C or 60°C) (9), saponification (collodionization and subsequent immersion in 1% KOH in 70% ethanol for 20 min at 25°C) (21), sulfation (immersion in a mixture of 10 ml H₂SO₄ and 20 ml glacial acetic acid for 10 min at 4°C) (modification of 11) and methylation saponification sequence. As an additional experiment, further, more tissue sections were subjected to the reverse sequence of the dual staining, alcian blue acriflavine, both dyes being employed under the same histochemical conditions as those in the acriflavine alcian blue sequence.

Film Tests of Known Sulfated Mucopolysaccharides

Besides the experiments using tissue sections, the acriflavine alcian blue staining of two sulfated mucopolysaccharides of known chemical structure was tested. Films were prepared by placing a droplet of 1% casein (Merck) *M*/15 Na₂HPO₄ solutions containing chondroitin sulfate C or heparin sodium at a concentration of 1 : 50 and those devoid of any sulfated mucopolysaccharides on a slide. The films were dried, fixed in 10% formalin in ethanol for 1 hr, rinsed in distilled water and doubly stained according to the procedure described above.

RESULTS

Regardless of the rodent species from which tissues are prepared, the cartilage tissues are stained nearly overall bluish yellow or yellowish blue; the cartilage matrix appears bluish yellow and lacunar borders and chondrocyte cytoplasm yellowish blue (Figs. 1 and 2). Of the matrix, furthermore, the perilacunar areas of different sizes tend to be a little more alcian blue reactive than the remaining parts of the matrix (Figs. 1 and 2).

In mast cells of the gastrointestinal tracts of the both rodents the dual staining technique results in a bluish orange coloration of the cytoplasmic granules and

Fig. 1. Tracheal cartilage of a rat. The cartilage matrix is stained bluish yellow or yellowish blue. Acriflavine-alcian blue. $\times 280$.

Fig. 2. Tracheal cartilage of a mouse. The cartilage matrix is colored bluish yellow or yellowish blue. Acriflavine-alcian blue. $\times 280$.

Fig. 3. A mast cell in the stomach of a rat. The cytoplasmic constituents are tinged bluish orange. Acriflavine-alcian blue. $\times 980$.

Fig. 4. A mast cell in the small intestine of a mouse. The cytoplasmic components are stained bluish orange. Acriflavine-alcian blue. $\times 1,000$.

intergranular cytoplasmic matrix (Figs. 3 and 4). However, this coloration is at times variable in shade and appears to fluctuate between bluish orange and orange blue shades. The cell nucleus is stained pale yellow.

Prior methylation abolishes or decreases conspicuously the dual stainability of the cartilage tissues and mast cell cytoplasmic components, but saponification tends to diminish only slightly the dual stainability of the either structures. Prior sulfation induces an overall intense yellow coloration of the cartilage tissues with a faint blue tinge, and likewise increases appreciably the orange staining intensity of the mast cell cytoplasmic components. Saponification following methylation gives rise to moderate or weak blue coloration of the cartilage tissues, while it yields either moderate or weak staining with a yellowish blue shade in mast cell cytoplasmic constituents.

In both the cartilage tissues and mast cells the reverse sequence of the dual staining, alcian blue acriflavine results in yellow or orange coloration of the tissue components with little or no blue shade.

When stained by the acriflavine alcian blue sequence of the dual staining, the casein films containing chondroitin sulfate C or heparin sodium are colored bluish yellow or bluish orange in shade, and those devoid of either sulfated mucopolysaccharides remain unstained.

DISCUSSION

On the basis of a series of biochemical data on the reaction of acriflavine with a variety of organic sulfate esters (1, 3, 4, 26), Takeuchi (27) has previously suggested that the capability of this dye to stain tissues is due to its formation *in situ* of insoluble salts with sulfate esters of mucopolysaccharides. On the other hand, the affinity of alcian blue towards carboxyl groups of mucopolysaccharides has been advocated by Mowry (12) and largely accepted by Quintarelli and Scott (16), Spicer (21), Spicer and Meyer (22) and Yamada (29). In the present technique acriflavine and alcian blue have been reacted at pH 1.5 and 2.5 respectively and at the respective values of pH reactive sulfate and carboxyl groups of mucopolysaccharides appear to be in the state of dissociation. According to Egami (3, 4) the bond between acriflavine and sulfate esters is supposed to be ionic in nature, whereas that between alcian blue and carboxyl and other acidic groups has recently been concluded to be similarly ionic (14). In view of these facts the present dual stainability of the cartilage tissues and mast cells with acriflavine and alcian blue is provocative of the idea that reactive sulfate and carboxyl groups of mucopolysaccharides involved in the structures are significantly responsible for the acriflavine and alcian blue reactions respectively. It appears, however, impertinent to conceive that the present dual staining technique is a method which enables one to differentiate, with thorough precision, the both acidic groups from each other; in addition to physical factors such as size of dye particles and density of substrates (5), numerous chemical facets like possible capability of basic proteins (16) and sulfates (21) to obstruct the access of alcian blue to carboxyl groups and masking of polysaccharides by proteins (15) are reported to influence the staining reactions of acid mucopolysaccharides. In the present technique the time of immersion of sections in the alcian blue solution of a low concentration (0.1%) has been limited to a minimum (2–20 min) in order to avoid the possible displacement of acriflavine

by the phthalocyanine dye such as that reported in the alcian blue alcian yellow sequence (19, 25). Moreover, the effects of prior methylation and methylation saponification sequence upon the dual reaction of the tissue structures, above all upon that of the cartilage tissues indicate that the above idea is, to an appreciable extent, likely. The methylation is known to hydrolyze sulfate esters and methyl-esterify carboxyl groups in polysaccharides and saponification following methylation to hydrolyze the methylesters and to recover the originally reactive carboxyl groups (20, 21). The effect of sulfation upon the dual staining of the either tissue structures confirms the property of acriflavine to combine with sulfate esters in mucopolysaccharides and endorses the likelihood of the above idea. Likewise, the acriflavine alcian blue stainability of chondroitin sulfate C and heparin sodium is taken to be in favor of the idea.

The reverse sequence of the dual staining, alcian blue acriflavine is inadequate for use as a histochemical technique, because the carboxyl groups of mucopolysaccharides, though appearing to be stained first by alcian blue at pH 2.5, are subsequently rendered undissociated by the acriflavine solution of pH 1.5 to release the former dye and to be decolorized.

From what has been observed here, the present acriflavine alcian blue technique is postulated to be useful for the characterization of some acid mucopolysaccharides with reactive sulfate and carboxyl groups.

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