A METHOD OF ELECTIVE STAINING OF ACID (SULFATED) MUCOPOLYSACCHARIDES WITH BASIC BROWN*

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The acid mucopolysaccharides are the object of the increased attention of both biochemists and morphologists. Nevertheless the existing methods of detection of acid mucopolysaccharides in histological preparations are insufficiently effective. For instance, metachromatic staining with the thiazine dyes, which plays a considerable part in the study of the acid mucopolysaccharides [13, 3], does not exhibit specificity, for it has recently been shown that metachromasia is given by high polymer phosphates and nucleic acids beside acid mucopolysaccharides [14, 8, 2].

Hale's method, which is so often used for detection of acid mucopolysaccharides, like its modifications, is not specific, for it reveals nucleic acids, lecithin, sphyngomyelin, fibrinogen and other substances in addition to acid mucopolysaccharides [3, 10, 7].

Abroad in recent years alcyan blue has been used widely as a stain for acid mucopolysaccharides [18], although it has not become popular in the Soviet Union because the dye is in extremely short supply. It must be pointed out, however, that the detection of acid mucopolysaccharides by alcyan blue is hardly a specific procedure, for when sections are stained with this dye elective staining of the acid mucopolysaccharides takes place only after exposure for 10-40 seconds, after which other structures not containing acid mucopolysaccharides very quickly begin to take up the stain [3]. This shows beyond doubt that alcyan blue combines not only with acid mucopolysaccharides but also with other substances.

Our method of elective staining of acid mucopolysaccharides is by means of the dye basic brown** (Bismarck Brown, vesuvine). Although the ability of basic brown to stain nuclei, cartilage and mucus has been known for some time [4], this stain has not been widely adopted in histological practice. We have previously described a special technique for the elective staining of mast cells by means of basic brown [6].*** Later we showed that when this method of staining is used, besides the granules of mast cells, other structures take up the stain. The common feature of all the histological elements staining by this method is that they contain acid mucopolysaccharides.

Any form of fixation which preserves acid mucopolysaccharides may be used (absolute alcohol, alcohol-formalin, etc.). Especially good results are given by Shabadash's No. 1 neutral fixing agent (copper nitrate 1.8 g, calcium nitrate 0.9 g, alcohol 100 ml, concentrated formalin 10 ml). The virtues of this fixing agent are explained by results [15] showing that acid mucopolysaccharides are rapidly precipitated by alcohol in the presence of ions of the heavy metals. Embedding in paraffin wax is carried out by the usual method, so that film preparations can be made.****

* The paper was presented at the Conference on the Histochemistry of the Polysaccharides in Moscow, 1960.

** Its nomenclative number in the Union Reagent Catalog is 1863.

*** Unfortunately the work of Hardy and Wesbrook [9], who used Bismarck Brown to stain most cells, was not known to the authors at the time when they developed the method.

**** The method which is being described was not tested in celloidin and frozen sections. We consider that it should be possible to stain acid mucopolysaccharides in frozen and celloidin sections.

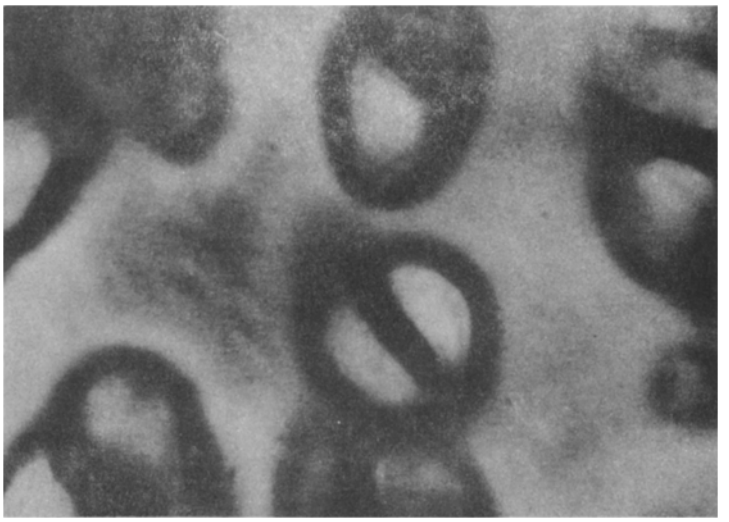

Fig. 1. Hyaline cartilage of the rabbit's trachea. Structures of the ground substance are revealed but the cartilage cell are not stained. Basic brown without counterstaining. Photomicrograph. Magnification: objective 100 x, ocular K-12.

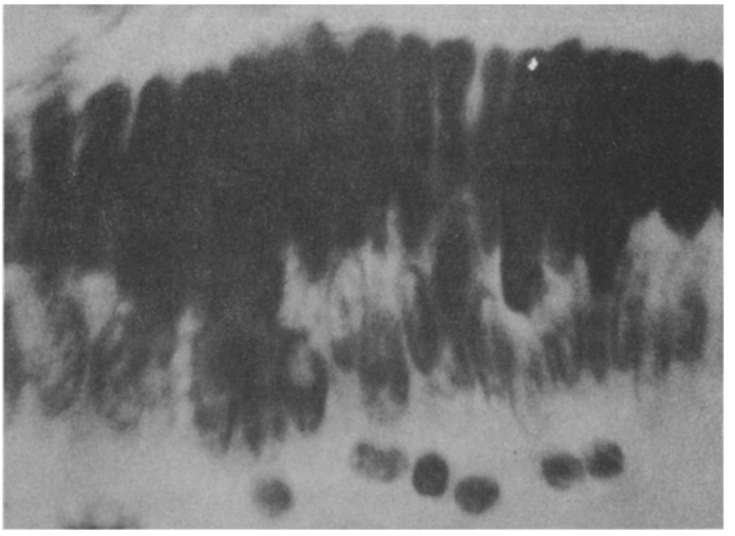

Fig. 2. Mucous membrane of a rabbit's stomach. Mucoid in the apical part of the epithelial cells covering the mucosa is stained with basic brown and the nuclei are counterstained with gallocyanin. Photomicrograph. Magnification: objective 100 x, ocular K-12.

Deparaffinized sections and fixed films are taken through to 70° alcohol and placed in a solution of the dye for 1-2 hours (in most cases 1 hour is sufficient). The dye solution is prepared by the following formula: basic brown 0.5 g, ethyl alcohol 80 ml, 1 N hydrochloric acid 20 ml.* Before it is used for the first time the solution must be filtered. After removal of the preparation from the dye it is taken through three changes of 70° alcohol, for one or two minutes in each, after which it is dehydrated, cleared and mounted in balsam.

* The introduction of a certain amount of hydrochloric acid is important in principle for it ensures the elective staining of the acid mucopolysaccharides by removing the stain from other basophilic substrates.

When this staining method is used an intensive brown coloration is shown by the ground substance of cartilage, the mucoid material secreted by the surface epithelium of the stomach, the mucin in the submandibular and sublingual salivary glands, the mucin in the goblet cells and mucous glands of the trachea, the mucin in the goblet cells of the small and large intestine and the efferent ducts of the pancreas, the cytoplasmic granules of the mast cells and the chromotropic substance of the blood vessels (Figs. 1, 2 and 3). All the structures enumerated above are known to contain different acid mucopolysaccharides containing a sulfate group [1, 4, 3, 15].

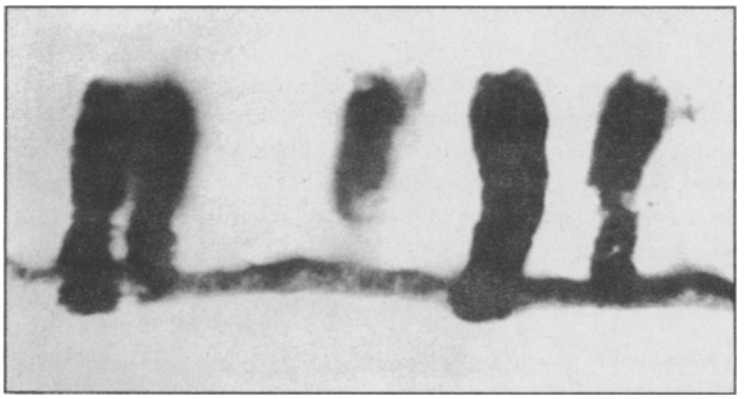

Fig. 3. Epithelium of a rabbit's trachea. Mucin in the goblet cells and on the surface of the mucous membrane is stained with basic brown, without counterstaining. Photomicrograph. Magnification: objective 100 x, ocular K-12.

Other basophilic substances, including nucleic acids, are not stained by this technique. For instance, the nuclei of cells containing deoxyribonucleic acid remain colorless. The tigroid of the neurons and the basal part of the cytoplasm of the secretory cells of the pancreas, which contain ribonucleic acid, also are unstained. The volutin of bacteria (we investigated the diphtheria bacillus and the bacterium of lactic acid fermentation), which gives a marked metachromasia when stained with thiazine dyes and consists, as shown recently [2], of high-polymer metaphosphate, is not stained by our method. No staining is shown by neutral polysaccharides, for example liver and muscle glycogen, or by the mucopolysaccharide secreted by the cells of the neck of the fundal glands of the stomach, which according to data in the literature [15] is not acid in character.

At present it is difficult to give full details of the chemical basis of the staining of acid mucopolysaccharides with basic brown. There is no doubt that a fundamental feature is the combination of the dye with acid groups of the acid mucopolysaccharides. The absence of staining of other basophilic substrates shows that the acid groups of these substrates (the phosphate groups of nucleic acids, the carboxyl groups of proteins) do not combine with basic brown. Comparison of the results of staining with data relating to the chemical composition of the stained substances suggests that the acid group which combines with basic brown is the sulfate group esterifying the mucopolysaccharide. This group is in fact present in all the substrates taking up the stain. Acid mucopolysaccharides not containing a sulfate group are not stained by this method. An example of this is hyaluronic acid, present in the ground substance of connective tissue, which does not stain with basic brown. Control experiments in which sections were treated with hyaluronidase (the preparation "lidase" in a concentration of 1 mg/ml, 3 hours at 37°) showed that hyaluronic acid does not participate in staining with basic brown.

In order to verify our hypothesis that staining is related to the presence of a sulfate group in the mucopolysaccharide molecule in an ester bond, experiments were carried out involving methylation and subsequent saponification of the sections. The method of treatment was as follows: 1) Methylation (deparaffinized sections were kept for 2 hours at 60° in methanol containing 0.1 g. eq. hydrochloric acid); 2) Saponification (after methylation, the sections were kept for 20 minutes at room temperature in a 1% solution of caustic potash in 80° ethanol).

During treatment of the sections with methanol containing 0.1 g. eq. hydrochloric acid, a methanolytic "desulfatation" of the acid mucopolysaccharides takes place, i.e., the irreversible splitting of the sulfate group from the mucopolysaccharide to form a polysaccharide free from sulfate. If the above-mentioned experimental

conditions are observed, nucleic acids are not affected by methylation and their staining properties are unchanged. During methylation the carboxyl groups of proteins are esterified, which leads to the disappearance of their inherent basophilia. Saponification causes decomposition of the esters and the reappearance of basophilia, due to the carboxyl groups of the proteins [11, 12, 16, 17].

The experiments show that treatment of sections with methanol containing 0,1 g. eq. hydrochloric acid prevents the staining of all structures staining with basic brown. Subsequent saponification does not lead to the reappearance of staining. These facts give grounds for the assertion that the staining of acid mucopolysaccharides with basic brown is due to the presence of an ester-bound sulfate group in the composition of the mucopolysaccharide.

Our suggested method of staining of acid mucopolysaccharides is characterized by its essential specificity and it gives standard, reproducible results. The method is simple in use and does not require reagents in short supply. By combining staining with basic brown with treatment by other histochemical methods (the Feulgen reaction, Shabadash's method, staining with chrome alum-gallocyanin, etc.) it is possible to obtain multicolored preparations in which neutral polysaccharides, nucleic acids and other substances are revealed in addition to acid mucopolysaccharides.

SUMMARY

The author suggests a method of selective staining of acid (sulphated) mucopolysaccharides in histological preparations. Sections or film preparations are passed through alcohols, gradually increasing in grade, finally reaching the 70° grade. Then they are put into a solution of basic brown and left there for 1-3 hours (stain – 0.5 gm, alcohol – 80 ml, 1 N hydrochloric acid solution – 80 ml). This procedure is followed by passing the section through 3 containers with 70° alcohol (1-2 minutes in each), dehydration, clearing and embedding in balsam. Experiments with methylation and subsequent saponification of the sections demonstrate the dependence of the staining upon the presence of mucopolysaccharides of the ether-bound sulphate group in the molecule.

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* Original Russian pagination. See C. B. translation.