Staining Properties of Hyaline Cartilage[1]

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Despite numerous attempts to develop and evalute specific methods for the histochemical demonstration of mucopolysaccharides, definitive information is not readily available. This is in spite of the notable contributions of Hale ('46); Steedman ('50); Gomori ('50); Kramer and Windrum ('54); Mowry ('56, '58); Spicer ('60); Belanger and Migicovsky ('61) and Goldstein ('62) to mention only a few.

With few exceptions (Joel et al., '56; Warren and Spicer, '61) histochemical specificity has been determined by applying the method to a variety of tissue mucopolysaccharides. While this approach is appropriate for establishing differences between tissues, it usually provides little useful information about the method itself. This is not surprising since the chemical and physical properties of both the methods and mucopolysaccharides are obscure and the material stained may vary from tissue to tissue.

An alternative approach (Davies, '52; Braden, '55; Pal and Schubert, '61; Spicer and Jarrels, '61) has been to employ "spot" or test tube methods in which purified mucopolysaccharides are utilized as the test material. The most obvious objection to this type of analysis is the assumption that the test material and the material *in situ* will exhibit similar properties. The artificiality of such an *in vitro* system renders such data open to question unless confirmed by other methods.

The more useful approach to an understanding of histochemical procedures is the direct approach, i.e., the demonstration of tissue components by the application of the method to the material *in situ*. In order to do this it is necessary that (1) the material to be examined is preserved and (2) that it is preserved in as unaltered a condition as possible. Therefore fixation should be a primary consideration when methods are to be evaluated since consistent results cannot be obtained if the tissue components are inadequately preserved.

Cetyl pyridinium chloride-formalin has been demonstrated to provide excellent preservation of acid mucopolysaccharide (Williams and Jackson, '56; Conklin, '63). Utilizing this fixative, the acid mucopolysaccharides of hyaline cartilage have been examined by histochemical methods. The excellent preservation of cartilage ground substance has in turn afforded a means of evaluating the specificity of the methods, the results of which are reported here.

MATERIALS AND METHODS

Adult monkey and adult rat tracheal cartilage served as the test material for the evaluation of the histochemical procedures. Tissue samples were fixed in 10% neutral formalin containing 8% cetyl pyridinium chloride and by freeze substitution in 10% acrolein in absolute alcohol for seven days at −70°C. Tissues fixed in the formalin derivative were washed, dehydrated in graded alcohols, cleared in cedarwood oil and paraffin embedded. Tissues fixed by freeze substitution were brought to −40°C for six hours, to 4°C for six hours, transferred to three changes of methanol for 12 hours each at 4°C, transferred to chloroform at 4°C for two hours, brought to room temperature in a second change of chloroform and paraffin embedded.

Tissue sections were stained by the following procedures:

1. Colloidal iron (Mowry's modification, '58) followed by counterstaining in 1% Bismarck brown Y, Cert. no. NN 11, in 1% acetic acid for one minute.

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2. 0.1% alcian blue 8GX (lot no. 130P, Allied Chemical Co.) in 3% acetic acid (final pH 2.0) for 30 minutes.

3. 0.01% azure A, Cert. no. NAz 17, brought to pH 2.0 and 4.0 with hydrochloric acid, for 15 minutes.

4. Periodic acid-Schiff (Mowry's modification of the Hotchkiss procedure, '58).

5. Aldehyde fuchsin (Gomori, '50) (pH 1.5) without prior oxidation.

6. 0.05% methylene blue, Cert. no. NA 23, in 0.1 M citric acid phosphate buffer, pH 5.6.

7. Two per cent light green, SF, Cert. no. NL 19, in 1% acetic acid.

Tissues stained with azure A were dehydrated in acetone. After all other methods, the tissues were dehydrated in alcohols, cleared in xylene and mounted in HSR. The several staining procedures were applied singly and in various combinations (table 1) in order to demonstrate regional differences in the cartilage matrix.

An indication of the specificity of the various methods was obtained by treating the sections with acid methylation (0.3 cm^3 concentrated HCl in 40 cm^3 methanol at 60°C for three hours), deesterification (1% KOH in 80% alcohol at 25°C for 20 minutes), acid methylation followed by deesterification, and sulfation (diethyl ether and concentrated sulfuric acid, 1:1 for five minutes) prior to staining.

For descriptive purposes the cartilage matrix was divided into five zones. These have been designated as the capsular, territorial, interterritorial (divided into amorphous and granular) and subperichondrial matrices.

RESULTS

Cetyl pyridinium chloride-formalin proved to be an excellent fixative for the preservation of cartilage mucopolysaccharides but was a poor cellular fixative at the concentration employed. Acrolein freeze-substitution gave comparable preservation of the ground substance and also resulted in excellent preservation of the various cell types (fig. 1). Since the primary objective of the study was the comparison of methods the staining characteristics to be reported are based only on cetyl pyridinium chloride-formalin fixation. The effect of acrolein fixation, with the introduction of aldehydes, on the various methods remains to be established. The only methods applied to acrolein fixed tissues were combined colloidal iron and Bismarck brown procedures. The staining which resulted was essentially the same as that observed after cetyl pyridinium chloride-formalin fixation (figs. 5 and 6).

When the several histochemical procedures were applied to hyaline cartilage, distinct zones were demonstrated within the cartilage matrix (table 1). The capsule of the lacuna exhibited certain staining characteristics distinct from the territorial matrix. In addition, the interterritorial matrix was divisible into two areas; a zone adjacent to the territorial matrix, completely lacking in structure, was designated as the amorphous matrix (fig. 6). An area containing granular material located at the junction of adjacent amorphous areas was designated as the granular matrix (figs. 2 and 5). Finally, the staining properties of the subperichondrial matrix (fig. 8) are described. The staining properties of the various zones and the effect of blockade procedures are summarized in table 1. The intensity of each reaction has been arbitrarily ranked from + to ++++.

In the untreated sections, the lacunar capsule differs from other regions by being PAS and light green negative. However, this zone becomes PAS and light green positive when methylation or demethylation sequences are employed prior to staining (fig. 7). The territorial and granular interterritorial matrices are alike in untreated sections but differ in the treated sections by the more intense light green staining in the territorial matrix after demethylation. The most marked difference between the two areas is the gamma metachromasia exhibited by the granular matrix (fig. 2). This is in contrast to the milder beta metachromasia of the territorial matrix.

The amorphous interterritorial matrix is the most distinctive zone since it is the only region which exhibits an affinity for Bismarck brown in the untreated sections (fig. 6).

The subperichondrial matrix is characterized by being the most intensely stained by the PAS reaction and exhibiting the

TABLE 1

The staining properties of hyaline cartilage

	Untreated	Methylation	Methylation deesterification	Deesterification	Sulfation
Lacunar capsule:					
Alcian blue	+++	—	—	+++	++
Colloidal iron	+++	—	—	+++	+
Aldehyde fuchsin	+++	—	—	+++	++++
Azure A (2.0)	β++	—	—	0+	0+++
Azure A (4.0)	β+	—	—	0+	0+++
Bismarck brown	—	—	—	—	++
PAS	—	+	+	+++	—
Light green	—	++	++	++	—
Territorial matrix:					
Alcian blue	+++	—	—	+++	+
Colloidal iron	+++	—	—	+	±
Aldehyde fuchsin	+++	—	—	—	++
Azure A (2.0)	β+++	—	β+	0+	0+++
Azure A (4.0)	β++	—	β+	0+	0+++
Bismarck brown	—	—	—	—	++++
PAS	++	+++	++	+	—
Light green	++	++	++	+++	—
Interterritorial matrix (amorphous):					
Alcian blue	+	—	—	++	+
Colloidal iron	—	—	—	—	±
Aldehyde fuchsin	+++	—	—	—	++
Azure A (2.0)	0+	—	—	0++	β++++
Azure A (4.0)	0++	—	—	0+	β+++
Bismarck brown	+++	—	—	—	++++
PAS	+	++	+	+	—
Light green	+	++	+	++	—
Interterritorial matrix (granular):					
Alcian blue	+++	—	—	++	+
Colloidal iron	+++	—	—	++	+
Aldehyde fuchsin	+++	—	—	++	++
Azure A (2.0)	γ++++	—	—	γ++	β++++
Azure A (4.0)	γ+++	—	—	γ+	β++++
Bismarck brown	—	—	—	—	++++
PAS	+	++	+	+	—
Light green	+	++	+	—	—
Subperichondrial matrix:					
Alcian blue	+	—	—	++	+
Colloidal iron	—	—	—	—	±
Aldehyde fuchsin	+	—	—	—	+
Azure A (2.0)	—	—	—	—	0+
Azure A (4.0)	0±	—	—	—	0+
Bismarck brown	—	—	—	—	+++
PAS	+++	++	+++	++	—
Light green	++	++	+	++	—

PAS, periodic acid Schiff; β, beta metachromasia; γ, gamma metachromasia; 0, orthochromasia; — indicates the absence of staining. 2.0, pH 2.0; 4.0, pH 4.0. The intensity of each stain has been arbitrarily ranked from + to ++++.

least affinity for azure A. Neither the subperichondrial matrix nor the amorphous interterritorial matrix are stained by colloidal iron.

Of the blockade methods, the methylation procedure consistently inhibited staining by alcian blue, colloidal iron, aldehyde fuchsin, Bismarck brown and azure A. With the exception of the staining of the territorial matrix, this inhibition was not reversed by demethylation. The sulfation procedure was consistent in inhibiting staining by PAS and light green. It reduced the intensity of alcian blue and

colloidal iron staining, intesified or introduced Bismarck brown affinity, and reduced staining by aldehyde fuchsin. The effect of sulfation on azure A staining was to reduce or inhibit metachromasia except in the amorphous matrix where metachromasia was produced.

The effect of demethylation alone is somewhat obscure since this procedure exhibited more regional variation than the other types of treatment. Demethylation intensified the PAS reaction and light green affinity of the capsule but either had no effect or reduced this staining in other areas. This treatment was without pronounced effect on the alcian blue or colloidal iron procedures although it did appear to intensify the alcian blue staining of the amorphous and subperichondrial matrices. Metachromatic staining by azure A was consistently reduced while aldehyde fuchsin staining was either inhibited (territorial and amorphous matrices) or reduced (granular matrix).

DISCUSSION

In considering the specificity of the various methods it is helpful to know the identity of the material demonstrated. The mucopolysaccharides of adult hyaline cartilage are chondroitin, chondroitin sulfates A and C, and keratosulfate (Muir, '61; Partridge et al., '61). Thus the groups which are present and may be involved in the staining reactions are carboxyl and sulfate groups and vicinal hydroxyls. Acetylated amines are also present but there is as yet no evidence to suggest their participation in the staining process.

As demonstrated previously (Conklin, '63), azure A at pH 2.0 does not stain material which is lacking in strongly acidic groups. Therefore azure A staining is indicative of the presence of either sulfate or phosphate groups. The fact that there is a regional difference (figs. 2 and 3) in the staining which occurs with azure A, i.e., orthochromasia vs. metachromasia, is probably due to a difference in either the nature or concentration of sulfate groups. It has been demonstrated that the occurrence of metachromatic staining is dependent upon the presence of a high molecular weight chromotrope (Schubert and Hamerman, '56). Methylation and a methylation-deesterification sequence consistently inhibited staining with azure A. This is in agreement with the concept that sulfate esters are methanolyzed and subsequently removed by the latter treatment (Spicer and Lillie, '59). It should be noted however that all methods except PAS and light green were also inhibited by this treatment. The inability to restore staining of certain methods after methylation occurs only in certain highly sulfated tissues such as cartilage and cornea (Spicer, '60) and in these tissues methylation-deesterification cannot be employed to distinguish between carboxyl and sulfate groups. The fact that deesterification alone reduced the degree of metachromatic staining would indicate that this treatment resulted in the partial deesterification of sulfate esters and that azure A (pH 2.0) is indeed specific for strongly acidic groups. Sulfation of cartilage prior to staining with azure A had a curious effect in that the introduction of sulfate groups produced metachromasia in areas which were previously orthochromatic and decreased metachromasia in previously metachromatic areas. This alteration of staining properties could be due to a requirement for ether optimal concentration or optimal stereochemical relationship of anionic groups in order for maximal metachromasia to occur. Since some staining with azure A (pH 2.0) occurred throughout the cartilage matrix (fig. 2) sulfate groups must also be present throughout. The greatest concentration of sulfates appears to be in the granular interterritorial matrix since this is where the most pronounced metachromasia occurs. The subperichondrial matrix seems to contain the fewest sulfate groups as indicated by the weakness of azure A staining. Azure A at pH 4.0 exhibits less specificity (fig. 3) since the amorphous interterritorial matrix is also stained by this method. However, the distribution of gamma metachromasia is the same as with azure A at pH 2.0.

It is of interest that the subperichondrial matrix exhibits the most prominent PAS staining. This finding is in agreement with the concept that adjacent acidic groups may prevent the demonstration of aldehydes produced by the periodate oxidation

of vicinal hydroxyls (Spicer, '61). The fact that sulfates may interfere with the PAS reaction is further supported by the observation that sulfation completely inhibited the PAS reaction in all areas of cartilage and that methylation-deesterification induced PAS staining in the lacunar capsule. The distribution of light green staining was quite similar to the location of the PAS positive material. Since both methods will stain collagenous fibers, it is possible that they are demonstrating the same material.

The concept that aldehyde fuchsin, when employed without oxidation, is specific for sulfate groups was suggested by Scott and Clayton ('53) and supported by Sulkin ('60) although Spicer ('60) has observed that sialic acid-containing and nonsulphated mucins exhibit a weak affinity for aldehyde fuchsin. In the present study, rather equivocal results were obtained with aldehyde fuchsin. The fact that all areas of the cartilage except the subperichondrial matrix (fig. 8) exhibited a strong affinity for the stain would indicate that sulfate groups are involved in the staining reaction. This is further supported by the fact that deesterification reduced aldehyde fuchsin staining with the exception of that in the lacunar capsule. However, sulfation reduced aldehyde fuchsin staining in all areas with the exception of the lacunar capsule where staining was enhanced. While this leaves the nature of aldehyde fuchsin staining unsettled, it does point up the fact that the lacunar capsule is distinctive in its response to this pretreatment.

The amorphous interterritorial matrix also displays a distinctive staining characteristic in that it is the only area which is Bismarck brown positive in untreated sections (fig. 6). Staining is inhibited by deesterification and occurs in previously negative areas after sulfation. This would indicate that the stain is demonstrating sulfate groups. However, these groups must differ from those in other areas since, except for the subperichondrial matrix, this area has the least affinity for azure A. It is of interest that developing hyaline cartilage does not stain with Bismarck brown but there is an increasing affinity for the stain with increasing age of the cartilage (unpublished observation). It should also be noted that Bismarck brown staining is pronounced only after fixation with either formalin-sodium acetate, formalin-cetyl pyridinium chloride or acrolein.

While it has been suggested that the alcian blue and colloidal iron procedures stain the same substances (Pearse, '60), their behavior in the staining of cartilage is not the same. Both methods will stain mucopolysaccharide which contains only carboxyl groups (Conklin, '63). Spicer ('60) has suggested that alcian blue may also stain certain sulfate groups while Belanger and Migicovsky ('61) maintain that colloidal iron will demonstrate unbound sulfate groups. The observation that neither method is positive after methylation-deesterification does not argue for the role of sulfates in these reactions since as previously noted this method is not discriminatory in sulfate-rich areas. Deesterification was either without effect on alcian blue staining (lacunar capsule and territorial matrix), enhanced the staining (amorphous and subperichondrial matrices) or reduced the staining (granular matrix). Colloidal iron staining was either unaffected by this treatment (lacunar capsule) or reduced (territorial and granular matrices). In untreated sections some alcian blue staining occurred in the amorphous and subperichondrial matrices while the colloidal iron reaction was negative in these areas. All of the above observations would indicate that both methods will demonstrate sulfate groups, in addition to carboxyl groups. The fact that deesterification reduced the colloidal iron staining in certain areas (fig. 10) would support Belanger and Migicovsky's concept that the method may be demonstrating unbound sulfate esters. The occurrence of variable staining with both methods after sulfation would suggest that some degree of stereospecificity of sulfate groups may be involved. Goldstein ('62), on the other hand, has suggested that the specificity of these methods may be a function of the size of the dye particle and the density of the cartilage matrix. The distribution of staining in the present study would not support this concept.

While some of the regional differences in staining such as the variation in metachromasia are undoubtedly the result of a variation in the concentration of certain groups, it is also possible that there are differences in the distribution of the several mucopolysaccharides. This is particularly suggested by the distribution of Bismarck brown and the acquisition of affinity for this stain with increasing age. In a study of cartilage from individuals of various ages, Kasavina and Zenkevich ('61) have reported a gradual increase in keratosulfate. It therefore seems possible that Bismarck brown is selectively demonstrating the distribution of keratosulfate. This concept is also supported by the fact that this area is weakly metachromatic; a finding which correlates with the apparent frequency of keratosulfate in the isolated mucopolysaccharide-protein complex of cartilage (Partridge et al., '61). The distribution of other sulfate groups would suggest that chondroitin sulfate is present throughout the cartilage matrix although present in the least quantity in the subperichondrial matrix. This is indicated by the weak staining in this area with all methods except PAS and light green. If the colloidal iron method is indeed indicative of unbound sulfate groups, this would suggest that in the territorial matrix and lacunar capsule most of the chondroitin sulfate is not complexed with protein but exists in a free state. This may not be too surprising since this is the area where cellular production of the mucopolysaccharide is occurring. This would also suggest that complexing between the acid mucopolysaccharide and protein is a secondary occurrence and not a product of cellular secretion.

SUMMARY

The mucopolysaccharides of hyaline cartilage have been demonstrated by histochemical methods following fixation of the tissues in cetyl pyridinium chloride-formalin. With the methods employed it was possible to demonstrate five zones within the cartilage matrix which exhibited distinctive staining characteristics. These have been designated as the lacunar capsule, territorial, granular and amorphous interterritorial, and subperichondrial matrices. The distribution of the various stains within the matrix suggests that chondroitin sulfate is present throughout the cartilage matrix while keratosulfate may be limited to the amorphous interterritorial matrix.

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LITERATURE CITED

Belanger, L. F., and B. B. Migicovsky 1961 A comparison between different mucopolysaccharide stains as applied to chick epiphyseal cartilage. J. Histochem., *9:* 73–78.

Braden, A. W. H. 1955 The reactions of isolated mucopolysaccharides to several histochemical tests. Stain Techn., *30:* 19–26.

Conklin, J. L. 1963 Staining reactions of mucopolysaccharides after formalin-containing fixatives. Ibid. (In press.)

Davies, D. V. 1952 Specificity of staining methods for mucopolysaccharides of the hyaluronic acid type. Ibid., *27:* 65–70.

Goldstein, D. J. 1962 Correlation of size of dye particle and density of substrate, with special reference to mucin staining. Ibid., *37:* 79–94.

Gomori, G. 1950 Aldehyde fuchsin: a new stain for elastic tissue. Amer. J. Clin. Path., *20:* 665–666.

Hale, C. W. 1946 Histochemical demonstration of acid polysaccharides in animal tissues. Nature, *157:* 802.

Joel, W., Y. F. Masters and M. R. Shetlar 1956 Comparison of histochemical and biochemical methods for the polysaccharides of cartilage. J. Histochem., *4:* 476–478.

Kasavina, B. S., and G. D. Zenkevich 1961 Mucopolysaccharides of the bone tissue under normal and pathological conditions. Clin. Chim. Acta, *6:* 874–882.

Kramer, H., and G. M. Windrum 1954 Sulfation techniques in histochemistry with special reference to metachromasia. J. Histochem., *2:* 196–208.

Mowry, R. W. 1956 Alcian blue technics for the histochemical study of acidic carbohydrates. Ibid., *4:* 407.

——— 1958 Improved procedure for the staining of acidic polysaccharides by Müller's colloidal (hydrous) ferric oxide and its combination with the Fuelgen and the periodic acid-Schiff reactions. Lab. Invest., *7:* 566–576.

Muir, H. 1961 Chondroitin sulphates and sulphated polysaccharides of connective tissue. Biochem. Soc. Symp. No. 20, University Press, Cambridge.

Pal, M. K., and M. Schubert 1961 Specific adsorption of metachromatic compounds of chondroitin sulfate by insoluble calcium salts. J. Histochem., *9:* 673–680.

Partridge, S. M., H. F. Davis and G. S. Adair 1961 The constitution of the chondroitin sulphate-protein complex in cartilage. Biochem. J., *79:* 15–26.

Pearse, A. G. E. 1960 Histochemistry, theoretical and applied. Little, Brown and Co., Boston.

Schubert, M., and D. Hamerman 1956 Metachromasia; chemical theory and histochemical use. J. Histochem., *4:* 159–189.

Scott, H. R., and B. P. Clayton 1953 A comparison of the staining affinities of aldehyde-fuchsin and the Schiff reagent. Ibid., *1:* 336–352.

Spicer, S. S. 1960 A correlative study of the histochemical properties of rodent acid mucopolysaccharides. Ibid., *8:* 18–34.

——— 1961 The use of various cationic reagents in histochemical differentiation of mucopolysaccharides. Amer. J. Clin. Path., *36:* 393–407.

Spicer, S. S., and R. D. Lillie 1959 Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J. Histochem., *7:* 123–125.

Spicer, S. S. and M. H. Jarrels 1961 Histochemical reaction of an aromatic diamine with acid groups and periodate engendered aldehydes in mucopolysaccharides. Ibid., *9:* 368–379.

Steedman, H. F. 1950 Alcian blue 8GS: A new stain for mucin. Quart. J. Micr. Sci., *91:* 477–479.

Sulkin, N. M. 1960 The distribution of mucopolysaccharides in the cytoplasm of vertebrate nerve cells. J. Neurochem., *5:* 231–235.

Warren, L., and S. S. Spicer 1961 Biochemical and histochemical identification of sialic acid containing mucins of rodent vagina and salivary glands. J. Histochem., *9:* 400–408.

Williams, G., and D. S. Jackson 1956 Two organic fixatives for acid mucopolysaccharides. Stain Techn., *31:* 189–191.

PLATE 1

EXPLANATION OF FIGURES

All figures are of hyaline cartilage. × 125.

1 Hyaline cartilage fixed by acrolein freeze-substitution and stained with colloidal iron and Bismarck brown. Contrast with figure 6 which is cetyl pyridinium chloride fixed and stained with the same methods.

2 Azure A, pH 2.0. Note the staining of the lacunar capsule, territorial matrix, and granular interterritorial matrix (arrow). Only the latter zone exhibits gamma metachromasia. Photographed through a Zeiss interference green filter.

3 Azure A, pH 4.0. The staining is more diffuse at this pH and also occurs in the amorphous interterritorial matrix (upper half of figure). Photographed as figure 2.

4 Alcian blue. The lacunar capsule, territorial matrix, and granular interterritorial matrix are stained. Photographed through a Wratten no. 22 filter.

5 Colloidal iron and Bismarck brown. The distribution of colloidal iron is the same as alcian blue (fig. 4). Photographed as figure 4.

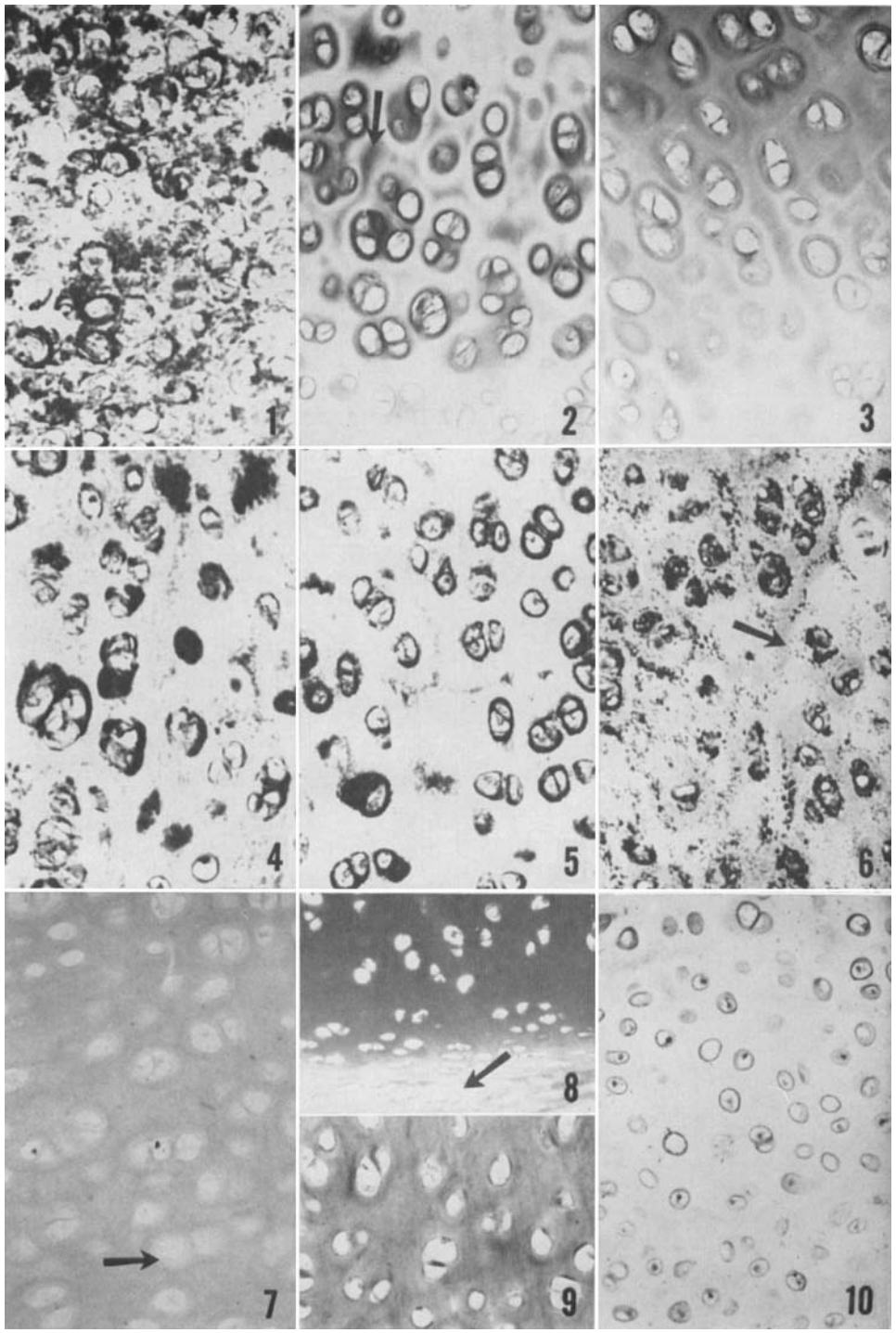
6 Colloidal iron and Bismarck brown. A portion of the Bismarck brown stained amorphous interterritorial matrix is visible (arrow). Photographed through a no. 25 Wratten filter.

7 Hyaline cartilage treated by methylation and stained by the PAS reaction. The lightly stained lacunar capsule (arrow) may be distinguished from the territorial matrix. Interference green filter.

8 Aldehyde fuchsin. Note the intense staining of all areas except the subperichondrial matrix (arrow).

9 Hyaline cartilage treated by deesterification and stained with Alcian blue. Note the general staining throughout the matrix. No. 22 Wratten filter.

10 Hyaline cartilage treated by deesterification and stained with colloidal iron. Staining is most prominent in the lacunar capsule. No. 22 Wratten filter.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