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A METHOD FOR THE DEMONSTRATION OF SULPHATED MUCOPOLYSACCHARIDES IN TISSUE SECTIONS

by

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Two ion association complexes for the demonstration of acid mucopolysaccharides have been suggested by *Zugibe* (1963), and the specificity of their application was tried and compared with the occurrence of metachromasia in chemical tests in solution. However, no practical details of the method were given. The present paper deals with the theoretical and practical application of these principles to the demonstration of acid mucopolysaccharides in tissue sections.

THEORETICAL BASIS

Cetyltrimethylammonium bromide and hexadecylpyridinium chloride have been shown by *Williams & Jackson* (1956) to precipitate water and alcohol insoluble compounds with acid polysaccharides. It has also been shown by *Jones* (1953) that nucleoproteins behave similarly, and *Putnam* (1948) has shown that low concentrations of the quarternary ammonium salts do not precipitate other proteins. *Scott* (1955) showed that sulphate-containing polysaccharide complexes are insoluble above pH 0.5, and that other acid polysaccharides containing only carboxyl end groups could be excluded from precipitation by retaining the pH below 1.8. These precipitates are characterized by their great insolubility in water and alcohol.

Zugibe (1963) has shown that ferric thiocyanate will form a

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coloured ion association complex with the precipitated mucopolysaccharide/quarternary ammonium compound. He also showed that the presence of "bound" ion complex matches the occurrence of metachromasia, when tested with known acid mucopolysaccharides in solution.

In the procedure described below (Fig. 1) the quarternary ammonium/mucopolysaccharide complex is precipitated within the tissue section by treatment with hexadecylpyridinium chloride. Excess hexadecylpyridinium chloride is washed away and the ion association complex is subsequently produced by treatment with a dilute solution of ferric thiocyanate which does not contain excess thiocyanate. Since this latter ion association complex is unstable when the sections are washed in water and alcohol, and the colour is rapidly extracted by acetone, ether and other dehydrating agents, the excess $Fe(CNS)_3$ is washed away in octane-2-ol which only very slowly extracts the ion association complex. This is then stabilized by forming quantitatively the intensely red complex of a',a' dipyridil with the ferrous iron, (*Snell & Snell* 1949) which now forms part of the thiocyanatoferrate described by *Zugibe* (1963). This is stable in xylol and sections treated according to this scheme retain their colour for several days.

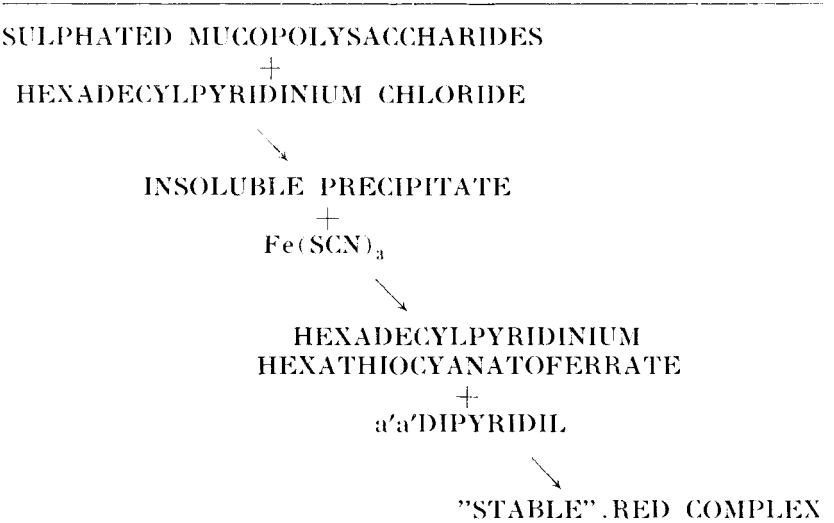

Fig. 1. Schematic representation of the reactions involved in the formation of the final coloured product.

METHOD

1. Reagents

a) 0.5 % solution of Cetavlon (ICI) or hexadecylpyridinium chloride (Fluka) in 10 % formalin with the pH adjusted with 1 N HCl to pH 1.7.

b) A solution of ferric thiocyanate made in the following manner: 30 mg NH_4CNS are dissolved in 25 ml distilled water. 30 mg $(Fe_2(SO_4)_3$ are added. After 15 minutes, the solution is adjusted to pH 2.6 with a 1 % solution of NH_4CNS. This reagent, which has a shelf life of several days, is filtered before use.

c) A 0.5 % solution of a′,a′ dipyridil in octane-2-ol.

2. Sequence

(i) Solution (a) — 15 minutes
(ii) Washed in running water — 20 minutes
(iii) Blotted dry
(iv) Solution (b) — 10 minutes
(v) Blotted dry
(vi) Washed in two changes of fresh octane-2-ol
(vii) Solution (c) — 2 minutes
(viii) Two changes of fresh octane-2-ol
(ix) Xylol and mounted in synthetic resin.

The colour produced at selected sites is orange-red.

Chemical test procedures

Chemical tests were carried out on chondroitin sulphate, heparin, starch, egg albumin, yeast ribonucleic acid (RNA), glycogen and sodium alginate.

Samples of these polysaccharides and proteins were prepared as 0.01 % solutions. Two drops of each solution were placed upon a paired series of gelatinized microscope slides and allowed to dry. One slide of each pair was immersed in the precipitating fixative, the other slide was kept unfixed as a control. After thorough washing, groups of different pairs were stained for 10 minutes each in 1 % alcian blue at pH 1.7, 0.01 % toluidine blue at pH 2.3, and the ferric thiocyanate ion association complex.

A further series of paired gelatinized test slides, one of each pair as a control, were pretreated with testicular hyaluronidase and then stained by the same procedures.

Tissue sections

Fresh, frozen 10 micron thick sections of mouse tracheal cartilage cut in a cryostat and frozen sections of the same thickness of teeth and bone cut by the method of *Nuki & Löe* (1964), were processed as two paired series, parallel to the chemical test slides.

Results

(1) *Chemical tests* (Table I): Toluidine blue metachromasia and staining by the ion complex was confined almost entirely to the sulphated mucopolysaccharides. The RNA slides though not

Table I.

Results of gelatin test slides stained with Alcian blue, toluidine blue, and ion association complex before and after treatment with testicular hyaluronidase (Intensity of staining 0–3)

Chemical test slides	Alcian Blue pH 1.9		Metachromasia		Fe. Ion Complex	
	Before	After	Before	After	Before	After
Chondroitin Sulphate	3	2	3	0	3	0
Heparin	1	1	2	0	3	0
Gelatin	0	0	0	0	0	0
Starch	0	0	0	0	0	0
Egg Albumin	0	0	1	1	0	0
RNA	0	0	0	0	3	3
Glycogen	0	0	0	0	0	0
Sodium Alginate	1	2	2	3	3	2

Fig. 2. Tracheal cartilage of mouse stained by the ion association complex.

Fig. 3. Tracheal cartilage of mouse stained in 1 % alcian blue pH 1.7.

Fig. 4. Tracheal cartilage of mouse stained in 0.01 % toluidine blue pH 2.3.

Fig. 5. Tracheal cartilage of mouse the control portion of which shows calcification. Stained by the ion association complex.

Fig. 6. Undecalcified 10 μ cut section of tooth and bone of monkey stained in 1 % Alcian blue pH 1.7.

Fig. 7. Undecalcified 10 μ cut section of tooth and bone of monkey stained in 0.01 % toluidine blue pH 2.3.

Fig. 8. Undecalcified 10 μ cut section of tooth and bone of monkey stained by ion association complex after fixation/precipitation and decalcification.

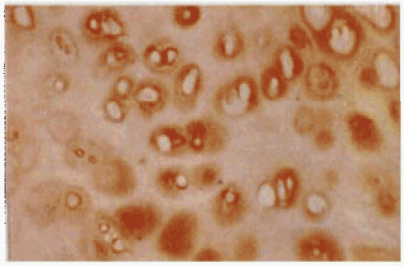

Fig. 2

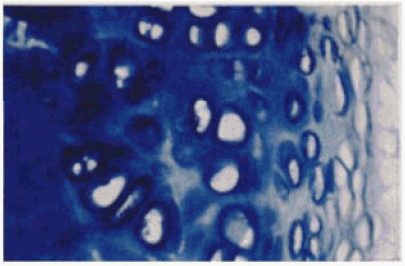

Fig. 3

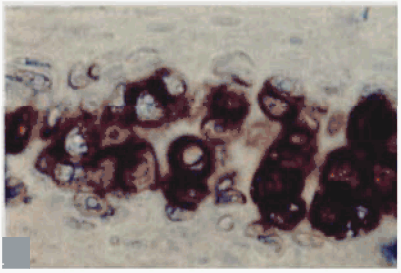

Fig. 4

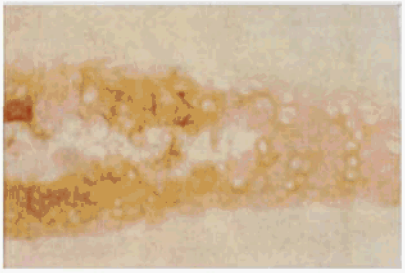

Fig. 5

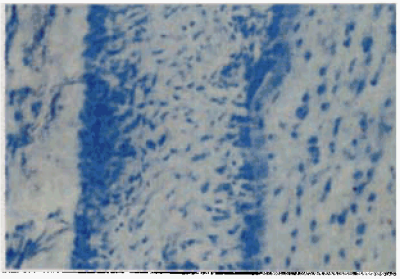

Fig. 6

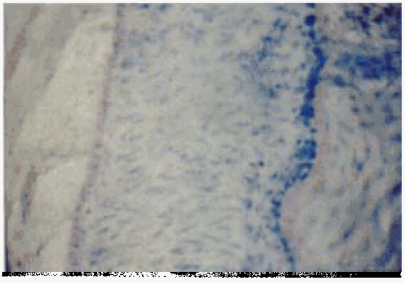

Fig. 7

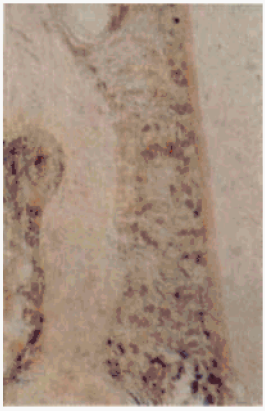

Fig. 8

exhibiting metachromasia stained intensely with the ion association complex. Both these stains were abolished by testicular hyaluronidase digestion with the exception of that produced in the sodium alginate slides. The unfixed control slides all exhibited weaker metachromasia and no deposit of the ion association complex. Alcian blue stained only the acid polysaccharides but this was not abolished by testicular hyaluronidase.

(2) *Frozen sections.* Fig. 2 shows the appearance of the tracheal cartilage of a mouse stained by the ion association complex. Fig. 3 is a similar section stained by alcian blue and Fig. 4 is such a section stained by toluidine blue. Sections incubated in testicular hyaluronidase at pH 6.8 for 48 hrs. no longer stained with the ion association complex and exhibited no metachromasia though some staining with alcian blue could still be produced.

As may be seen in Fig. 5 a partial suppression of the stain in the immediate neighbourhood of the calcified part of the tissue has occurred. However, such sections when decalcified after fixation/precipitation no longer showed this effect.

The undecalcified sections of teeth and periodontium stained weakly with alcian blue. The periodontal membrane did not stain with the exception of the cell nuclei. The calcified tissue stained evenly (Fig. 6). A slight increase was seen in the osteoid. Toluidine blue did not stain the periodontal membrane except for the metachromasia of the nuclei. Both osteoid and cementoid stained metachromatically (Fig. 7). By increasing the concentration of the dye or by prolonging the staining time an overall metachromasia of the calcified structure appeared.

Undecalcified sections of tooth and bone failed to stain with the ion association complex. However, after decalcification of the individual sections subsequent to fixation/precipitation these sections showed staining of the osteoid, the cementoid and the nuclei of the cells of the periodontal membrane (Fig. 8).

DISCUSSION

The chemical test slides as well as the tissue sections show a close parallel between metachromasia at low pH and the ion association complex. The ion association complex demonstrated in

the RNA slides and thus the nuclei of the cells shown in Figs. 6, 7, 8, is explained by the previously cited work of *Jones* (1953) of the specific precipitation of nucleoproteins by the quarternary ammonium salts.

The alcian blue stain produced and its persistence in sections treated with testicular hyaluronidase indicates its apparent non-specific nature. Several samples of alcian blue behaved more or less similarly, the intensity of stain remaining, particularly in the cartilage sections, could be dependent on the degree of impurity of the enzyme.

The explanation for the necessity to decalcify tissue sections before ion association complex staining is probably that the formation of ferric thiocyanate is suppressed by the presence of PO_4 ions as shown in solutions by *Snell & Snell* (1949).

The ion association complex is most heavily localized to the intercellular substance that lines each chondrocyte lacuna. A lesser degree of staining is found in the rest of the cartilage matrix. This localization agrees with the generally accepted distribution of sulphated acid mucopolysaccharides in cartilage.

The distribution of sulphated acid mucopolysaccharides in the periodontal tissues as demonstrated by this technique is seen to be distributed along the bone and cementum surfaces. This finding does not entirely agree with that reported by *Baratieri* (1961), in human autopsy material. This author though demonstrating alcian blue staining of the cementum, also demonstrated an equal level of stain in the periodontal fibers and throughout the alveolar bone.

The striking difference between the intensity of the stain in the intercellular substance of cartilage as compared with that of the intercellular substance of the periodontal membrane does not necessarily indicate that sulphated mucopolysaccharides are not present within the latter tissue. Since it is known that bone, dentine and periodontal membrane as well as cementum contain sulphated mucopolysaccharides it may be that the stainable groups are not readily available.

On the other hand it is possible that the decalcification of the tissue sections has unmasked some of the sulphated mucopolysaccharides of the osteoid and cementoid. This may account for

the selective staining of these tissues with the ion association complex.

In conclusion it may be stated that the ion association complex and toluidine blue metachromasia give substantially parallel results. However, whereas metachromasia is based upon ill understood and conjectured mechanisms the ion association complex is based upon a specific precipitation and a series of simple well understood chemical reactions.

SUMMARY

A step by step staining sequence is described for the demonstration of sulphated acid mucopolysaccharides in tissue sections. The test procedures undertaken indicate that the use of quarternary ammonium salts to precipitate sulphated mucopolysaccharides can be utilized to produce a true qualitative histochemical test for these substances.

The coloured complex formed localizes more specifically than alcian blue and parallels that of the occurrence of metachromasia at low pH. It is suggested that whereas the mechanism of metachromasia is ill understood the method described in the present paper is based upon well defined chemical reactions.

RÉSUMÉ

MÉTHODE POUR LA MISE EN ÉVIDENCE DES MUCOPOLYSACCHARIDES-SULFATES DANS DES COUPES DE TISSUE

Les auteurs décrivent une méthode de coloration successive pour la mise en évidence des mucopolysaccharides-sulfates dans des co .pes de tissus. Les tests entrepris indiquent que l'usage des sels quaternaires d'ammonium peut servir à élaborer un véritable test histologique qualitatif pour ces substances.

Le composé coloré formé se localise plus spécifiquement que le bleu alcian et sa localisation correspond à celle du composé produit lors de la métachromasie avec un pH bas. Il apparait que, tandis que le mécanisme de la métachromasie est mal compris, la méthode décrite dans le présent article est basée sur des réactions chimiques bien définies.

ZUSAMMENFASSUNG

EINE METHODE ZUM NACHWEIS SCHWEFELSAURER MUKOPOLYSACCHARIDEN IN GEWEBESCHNITTEN

Eine Stufenfärbung wird für den Nachweis schwefelsaurer Mukopolysacchariden in Gewebeschnitten beschrieben. Die durchgeführten Testverfahren zeigen, dass die Verwendung kvaternärer Ammoniumsalze bei der Präzipitation der schwefelsauren Mukopolysacchariden in der Erzeugung eines exakten kvalitativen histochemischen Tests für diese Substanzen zu verwerten ist.

Der gefärbte Komplex lokalisiert sich spezifischer als "alcian blue" und ist mit dem Vorkommen der Metachromasie bei niedrigem pH gleichlaufend. Es wird angenommen, dass, während der Mechanismus der Metachromasie nur schlecht verstanden ist, das oben beschriebene Verfahren sich auf wohldefinierten chemischen Reaktionen gründet.

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