APPLICATION OF PERIODATE OXIDATION TO THE HISTOCHEMICAL IDENTIFICATION OF ACIDIC POLYSACCHARIDES

a thesis submitted by

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ABSTRACT

This thesis is concerned with the application of periodate oxidation to the histochemical identification of the acidic polysaccharides (glycosaminoglycans) of connective tissues, especially cartilage.

When fresh frozen sections of bovine nasal septal cartilage are given prolonged exposure to sodium periodate solution a great deal of basophilic sulphated material is lost. This was characterised by staining with Alcian blue in 0.40M $MgCl_2$ to be mainly chondroitin sulphates.

The loss was shown to be due specifically to the periodate ion and most occurs during the first hour of exposure of sections.

Two main sites of action of periodate in cartilage were considered. These are the network of collagen fibrils and the interfibrillar proteoglycans. Periodate was shown to cause great decreases in viscosity for proteoglycan and glycosaminoglycuronan solutions.

Propan-1-ol, a presumed free-radical scavenger, was found to diminish loss of stainable material from cartilage sections and the rate of decrease of viscosity of glycosaminoglycuronans in periodate solution. It is proposed that free-radicals are present in periodate solution and that they cause depolymerization of glycosaminoglycuronans, thus facilitating their loss by diffusion. In addition, periodate causes a general loosening-up of the matrix of collagen fibres.

Methods of preventing the loss from cartilage sections were investigated. Formaldehyde fixation had a little effect. Cetylpyridinium, a precipitant for acidic polymers such as chondroitin sulphates, was much more effective. Treating sections with solutions of low pH produced a fixation which could not be reversed. The use of free-radical scavengers was considered.

It was not possible to determine the effect of cetylpyridinium complex formation upon the rate of periodate oxidation of glycosaminoglycuronans by performance of model experiments, with these substances spotted on strips of 'paper'. Ordinary cellulose paper was periodate reactive, cellulose acetate paper gave anomalous results and polysaccharide would not remain on glass-fibre paper when exposed to periodate solution.

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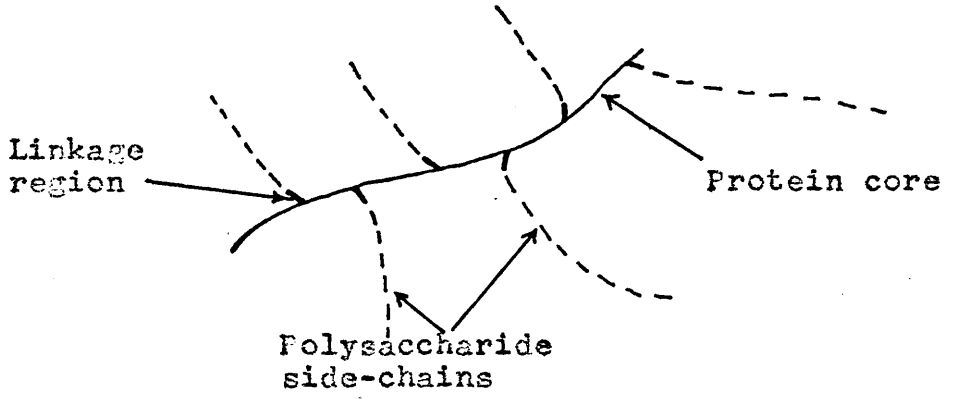
INTRODUCTION

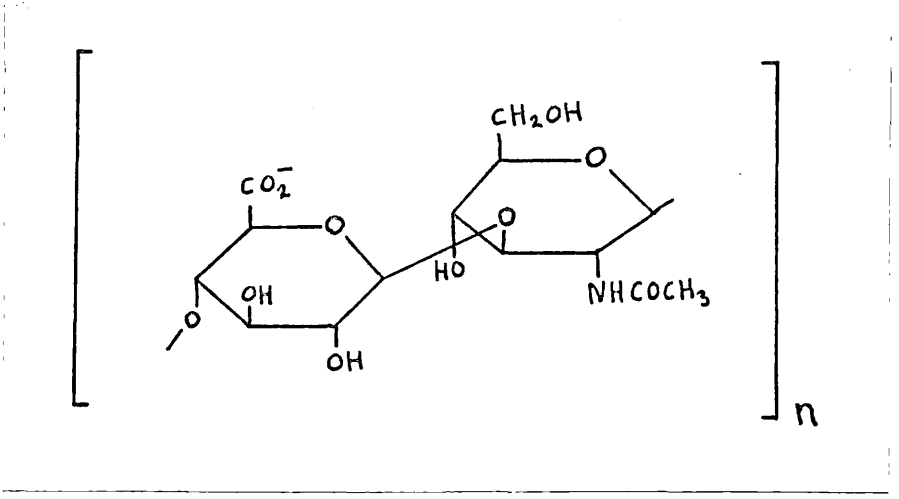
This thesis is concerned with the application of periodate oxidation to the histochemical identification of the acidic polysaccharides (glycosaminoglycans) of connective tissues, especially cartilage.

There are seven major vertebrate glycosaminoglycans. Some but not all occur in the form of proteoglycans. A proteoglycan has a protein core with polysaccharide side-chains covalently linked to it.



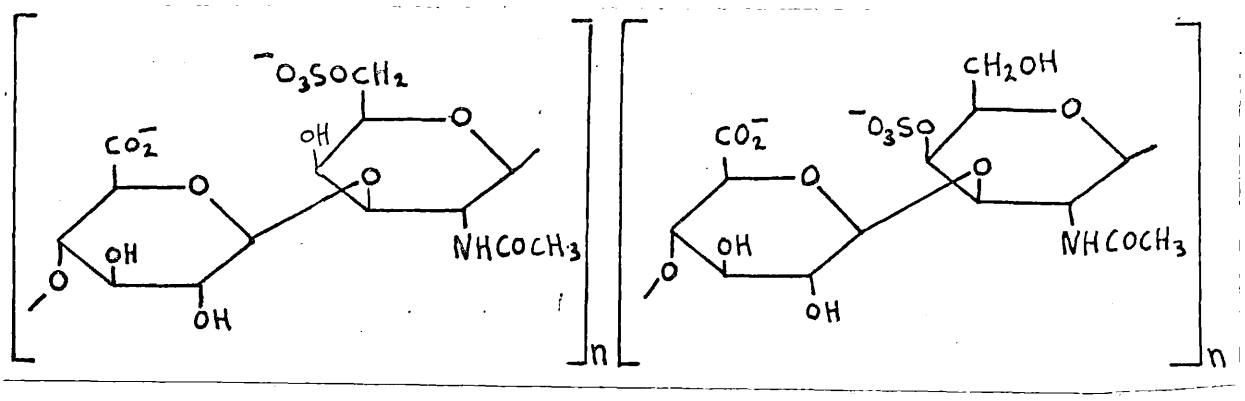
The structures of the repeating disaccharide units for individual glycosaminoglycans and of proteoglycans are described below. These formulae are reproduced from Balazs (1970).

Hyaluronic acid



Chondroitin 4- and 6- sulphate

There is variation in the degree of sulphation and in the possible occurrence of chondroitin-4-sulphate and chondroitin-6-sulphate hybrids.

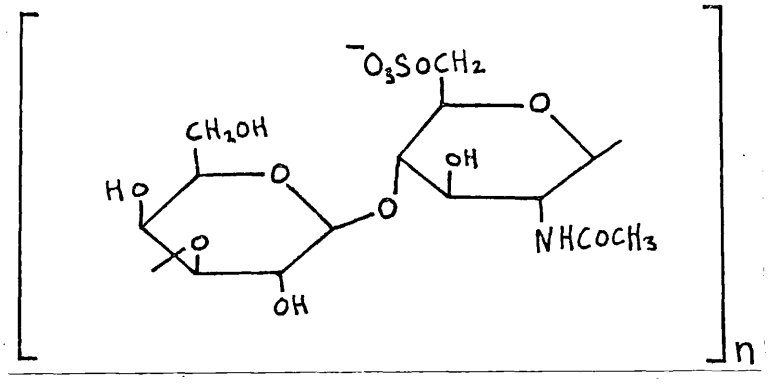


Chondroitin-6-sulphate
(Chondroitin sulphate C)

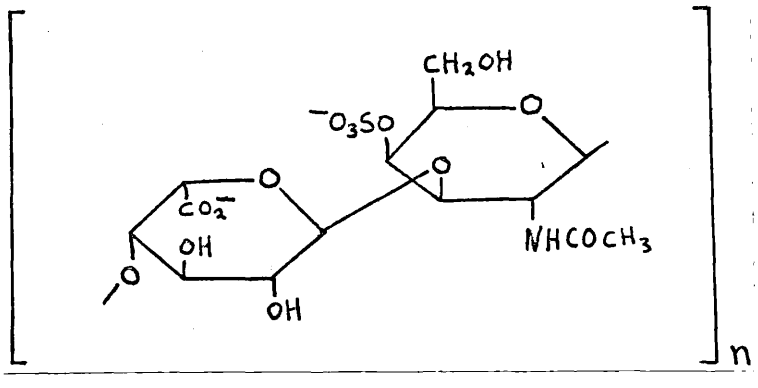
Chondroitin-4-sulphate
(Chondroitin sulphate A)

Keratan sulphate

Keratan sulphate differs from the other glycosaminoglycans in the absence of a uronic acid component.

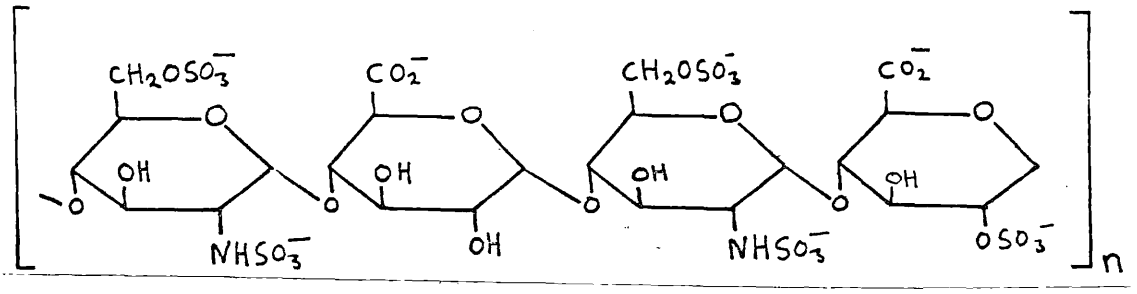


Dermatan sulphate (Chondroitin sulphate B)



Heparin and Heparan sulphate

The generally accepted structure of heparin is illustrated by the tetrasaccharide below.



"This tetrasaccharide is not meant to be strictly conceived as a repeating unit, but rather as a fragment expressing the characteristic structural features of the molecule". (Lindahl, 1970).

Heparin and heparan sulphate differ with regard to sulphate and acetyl content, heparan sulphate having more N-acetyl and fewer N- and O- sulphate groups than heparin.

Proteoglycans

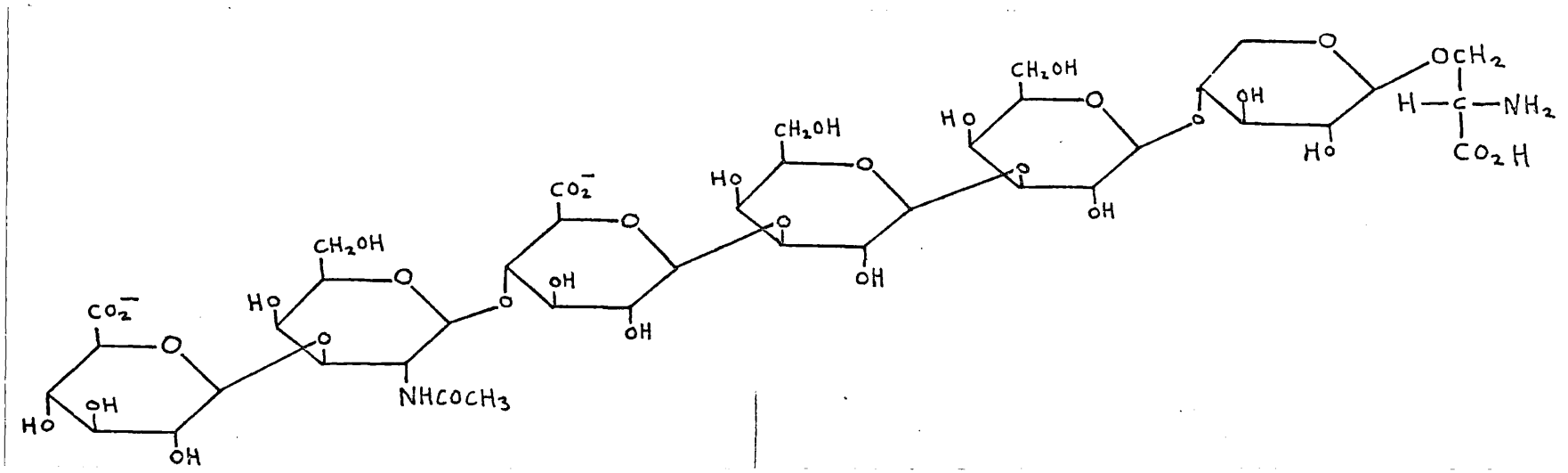
Chondroitin -4 and -6 sulphates exist in the form of proteoglycans. The linkage from the protein to the polysaccharide side-chains occurs through serine residues in the protein core. The oxygen atom of the hydroxyl group in serine is linked to the potentially reducing end of either chondroitin sulphate by way of a specific oligosaccharide which differs in composition from the characteristic repeating unit of the chondroitin sulphate chain. This oligosaccharide is 3-O-B-D-galactosyl-4-O-B-D-galactosyl-D-xylose (Roden & Smith, 1966). See the diagram overleaf.

In cartilage, chondroitin sulphate proteoglycans exist which contain no keratan sulphate but keratan sulphate always occurs together with chondroitin sulphate (Pedrini, 1969).

There appears to be a close similarity between the carbohydrate-protein linkages of dermatan sulphate and chondroitin sulphate (Stern, 1968).

"Both heparin and heparan sulphate, when isolated after proteolysis, contain the specific polysaccharide-protein linkage region and a residual peptide, and therefore it is reasonable to assume that they both occur in the native state as multichain proteoglycans". (Lindahl, 1970).

No linkage region has been isolated and most protein can be removed from hyaluronate by mild physical methods which suggests that it is not covalently linked to form proteoglycans. (Laurent, 1970).



Structure of the Carbohydrate-Protein Linkage Region of Chondroitin Sulphate

Periodic-Acid Schiff reaction

In order to understand the role which a group of substances play in the functioning of a tissue it is not enough to know the gross composition of that tissue. The distribution of these substances must be known. This knowledge can be gained by microanalysis and/or the application of a histochemical method so that the substances become coloured or marked in some way and their distribution in tissue sections can be observed under the microscope.

A commonly used reaction for the histochemical demonstration of carbohydrate containing materials is the Periodic Acid Schiff (PAS) reaction. Substances containing a glycol group can be oxidised by periodate to give a dialdehyde structure which can subsequently be reacted with Schiff reagent to give a red-coloured product. The usual PAS procedure involves exposure of tissue sections to 10g/l periodic acid for 10 minutes before staining with Schiff reagent (Pearse, 1968).

The glycosaminoglycans, excluding keratan sulphate and fully sulphated heparin, contain a glycol group in their uronic acid moieties and might be expected to be demonstrable by the PAS reaction. Until recently there was much confusion as to whether the glycosaminoglycans were oxidisable by periodate and therefore demonstrable by the PAS reaction.

This situation was clarified when Scott showed that they were slowly but specifically oxidised in their uronic acid moities. Scott (Scott & Harbinson, 1968) demonstrated that the intense negative electrostatic field of acidic polysaccharides excludes negatively charged periodate ions from the domain of the polysaccharide, thus reducing the rate of oxidation. It was found that polysaccharides containing 1:4-linked D-glucuronic acid (hyaluronate, chondroitin sulphates A and C) were less easily oxidised than those containing 1:4-linked L-iduronic acid (chondroitin sulphate B). Different conformations for the D- and L-uronide were proposed to account for the different rates of oxidation (Scott & Harbinson, 1969).

Scott and Dorling (1969) developed a PAS technique for chondroitin sulphates and other glycosaminoglycuronans employing sodium periodate as oxidant. Rapidly oxidisable substrates were oxidised during a 1 hour primary oxidation. They were then reduced to non-Schiff reactive primary alcohols before exposing the section overnight to oxidise the glycosaminoglycuronans (Fig.1.).

During that investigation it was noted that fresh frozen sections of bovine nasal septal cartilage lost much basophilic material in sodium periodate solution. This was characterised, using Alcian blue in the critical electrolyte concentration technique, as mainly chondroitin sulphates.

It was proposed to investigate the reasons for the loss and to develop methods for preventing it.

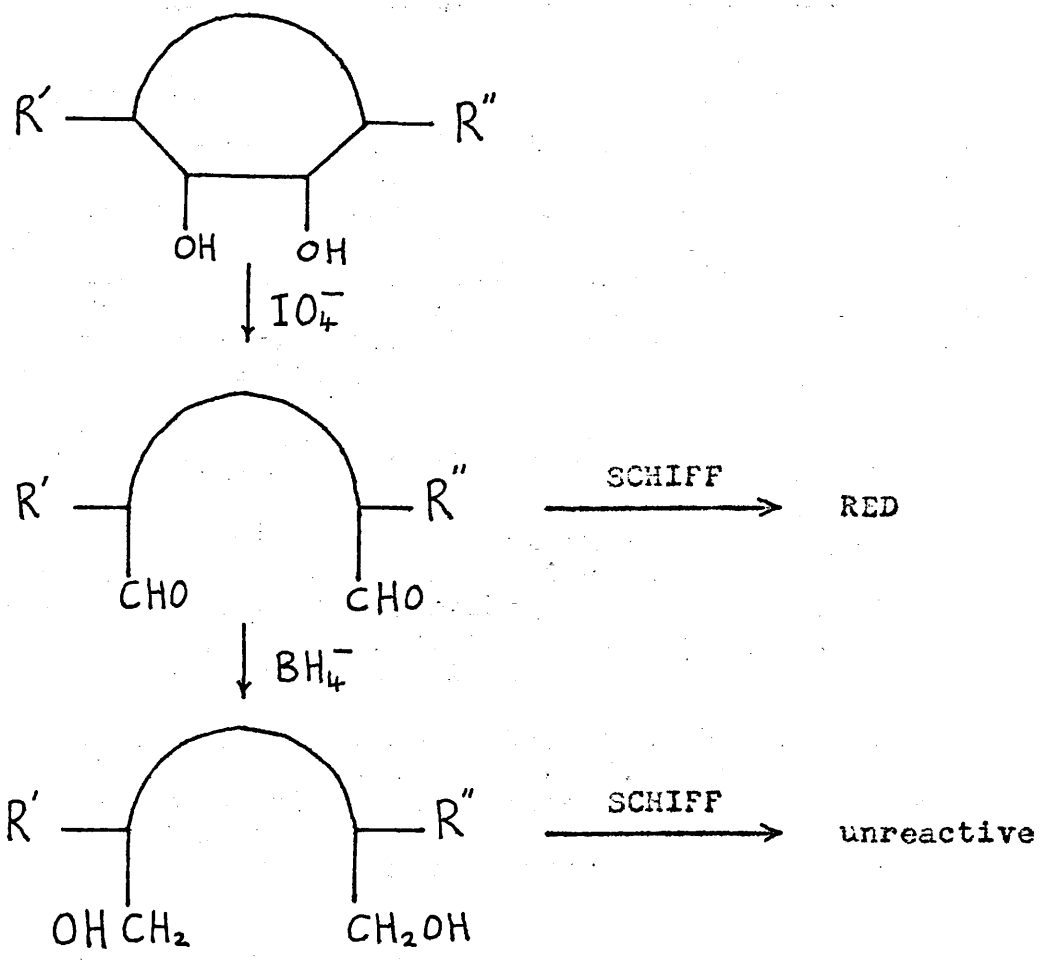


FIG.1.

The borohydride (BH_4^-) blockade of periodate oxidised glycans. R' and R" are non-oxidisable groups or substituents (glycosidic bonds, in the present context). Taken from Scott & Dorling (1969).

DISCUSSION

LOSS OF CHONDROITIN SULPHATES FROM CARTILAGE SECTIONS

Time course of loss

All investigations were performed with fresh frozen sections of bovine nasal septal cartilage. Sections left for one or twenty-four hours in 20g/l sodium periodate at 30°C showed that loss of basophilia, as demonstrated by Alcian blue in 0.4CM $MgCl_2$ which stains mainly chondroitin sulphates, decreased only slightly from one hour onwards. This indicated that most loss from sections occurs during the first one hour of exposure to sodium periodate solution.

Demonstration that loss is due to the periodate ion

(See experimental details on p.38)

There are a number of alternative ways in which an aqueous sodium periodate solution could be active in causing loss of chondroitin sulphates from cartilage sections. In the first instance it might simply be due to leaching out of these substances in aqueous solution.

The situation could be more complicated than this and loss be due to the pH of the solution (around 5.8 for aqueous sodium periodate), the salt concentration or an effect due specifically to sodium or periodate ions. Another alternative is that iodate ions produced in reduction of periodate by glycols

$$IO_4^- + \begin{matrix} -\dot{C}\text{-OH} \\ -\dot{C}\text{-OH} \end{matrix} \rightarrow IO_3^- + \begin{matrix} >C=O \\ >C=O \end{matrix} + H_2O$$

are active.

Basophilia in sections was greatly diminished on exposure to 20g/l(0.094M) aqueous sodium periodate at 30°C for 1 hour (Fig.3), compared with controls that were either (a) not treated at all (Fig.2), (b) exposed to acetate buffer, pH 5.7 (0.025M), (c) exposed to sodium perchlorate solution (0.094) (d) exposed to sodium potassium iodate solution (0.10M).

This showed that it was the periodate ion which was responsible for the loss.

Extraction of cartilage sections with periodate solution and demonstration of uronic acid in that extract

(See experimental details on pp.41-43)

Loss in basophilia (Alcian blue in 0.4CM $MgCl_2$) of cartilage sections strongly suggests, but does not prove, that loss of chondroitin sulphates has occurred. It was proposed to obtain direct evidence for this by measuring the uronic acid content of a periodate-solution extract from cartilage sections.

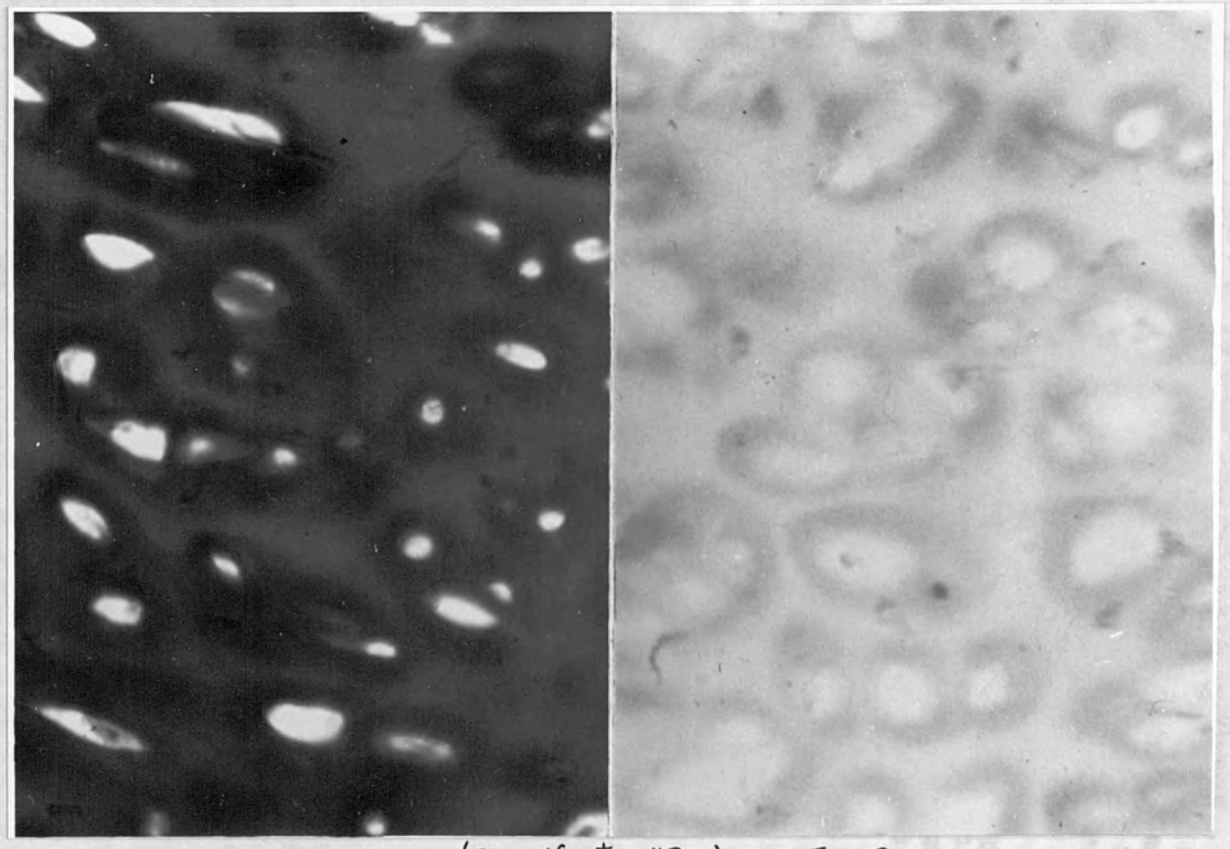


Fig. 2 (magnification X300) Fig. 3

Figure 2. Control section of cartilage stained, without pretreatment, in 0.50g/l Alcian blue in 0.025M acetate buffer, pH 5.7, containing 0.40M $MgCl_2$.
Figure 3. Section stained as in Fig.2 but pretreated with aqueous 0.094M sodium periodate, at 30°C, for 1 hour.

Taken from Scott, Tigwell & Sajdera (1972)

Cartilage sections were incubated with a 20g/l sodium periodate solution for 1 hour and a uronic acid determination, by the Dische carbazole method, was performed on the extracting solution. Control sections were incubated with sodium perchlorate solution.

Both periodate and perchlorate extracts gave 'normal' uronic acid spectra. Twice as much material was present in the periodate extract as in the perchlorate extract. This must be an underestimate of the amount of polyuronide extracted in periodate solution, although not an underestimate for perchlorate extraction, because some uronic acid residues would be oxidised and destroyed during the 1 hour exposure of sections to periodate solution.

The uronic acid determination confirmed in a direct manner that periodate caused loss of uronic acid containing material from cartilage sections. Alcian blue staining showed that it was sulphated material i.e. chondroitin sulphates, that was lost.

MECHANISM OF LOSS

Sites of action of periodate

Chondroitin sulphates occur in cartilage in the form of proteoglycans which lie in the spaces between the three-dimensional network of collagen fibres. Most can be extracted by mechanical or physico-chemical methods which may be assumed to indicate that covalent links are not the main reason for them staying in position. Aggregation of proteoglycans occurs in the presence of a glycoprotein (Hascall & Sajdera, 1969). The structure of proteoglycans, especially with regard to the linkage region between polysaccharide and protein, has already been considered (pp.10-11).

Hardingham & Muir (1972) observed that hyaluronic acid from umbilical cord increased the apparent size, as measured by gel chromatography, of proteoglycans from pig laryngeal cartilage. They suggested that each hyaluronic acid molecule interacted with a large number of proteoglycan molecules. Following this observation, Hascall (1973) proposed that hyaluronic acid plays an important role in causing aggregation of proteoglycans in bovine nasal septal cartilage.

The site of attack of periodate upon cartilage could be the collagen fibres or the interfibrillar proteoglycans. Since the staining properties of the material extracted from cartilage, in periodate solution, are the staining properties of proteoglycans it was decided to look at the effect of periodate upon these substances.

Action of periodate on proteoglycans

(See experimental details on pp.44-55)

It was found that solutions of proteoglycan decreased in viscosity when exposed to sodium periodate solution, in the presence of sodium perchlorate as supporting electrolyte (Figs. 4 & 9). The apparent dependence on concentration of the reduced viscosity of proteoglycan sub-unit and proteoglycan complex (p.18) is probably due to the inconstant shear rate (diminishing with increasing concentration), according to the run-out time. The viscosity of proteoglycan complex is dependent on shear rate (Sajdera & Hascall, 1969).

It was apparent from the results above that periodate was acting on the proteoglycans. Periodate could act upon the protein cores of proteoglycans, the polysaccharide side-chains or the linkage regions between polysaccharide and protein.

The effect of periodate upon the viscosity of several glycosaminoglycans was examined. Solutions of hyaluronate, chondroitin-4-sulphate and shark cartilage chondroitin sulphate (Figs. 5, 6 & 7) showed marked decreases in viscosity in the presence of sodium periodate. Periodic acid and sodium periodate were about equally effective in reducing the viscosity of hyaluronate.

Specific viscosities for solutions of glycosaminoglycans and proteoglycans and changes therein brought about by periodate

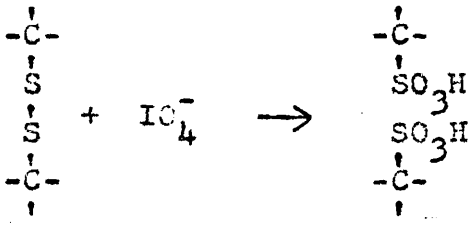
Substrate polyanion	Polyanion concentration (g/l)	η_{sp}	η_{sp}/c (ml/g.)	% Decrease in η_{sp} in 1 hour in presence of $NaIO_4$	M $NaIO_4$
Chondroitin-4-sulphate (from bovine nasal septal cartilage)	3.0	0.069	23	23	0.1
Chondroitin-6-sulphate (from shark cartilage)	3.0	0.171	57	15	0.01
Hyaluronate	3.0	3.85	1,283	47	0.01
Proteoglycan subunit	5.7* 2.3* 0.9*	2.3 0.585 0.2	398 253 217	28	0.1
Proteoglycan complex	2.3* 1.55* 1.0*	0.865 0.40 0.27	370 258 264	52 20 23	0.1 0.003 0.01

*Determined from uronic acid content, using factors of 30% and 29.5% for proteoglycan and cartilage extract respectively (Sajdera & Hascall, 1969)

Data (Table 2) taken from Scott, Tigwell & Sajdera (1972)

These viscosity decreases showed that periodate was acting to degrade the polysaccharides in some way or to produce a change in molecular shape. Either activity could produce loss from cartilage sections by diffusion.

Disulphide bonds in the protein cores of proteoglycans are known to be necessary to maintain their shape so that aggregation between proteoglycans can occur (Hascall & Sajdera, 1969). Periodate would be expected to oxidise and cleave these bonds with the formation of sulphonic acids.



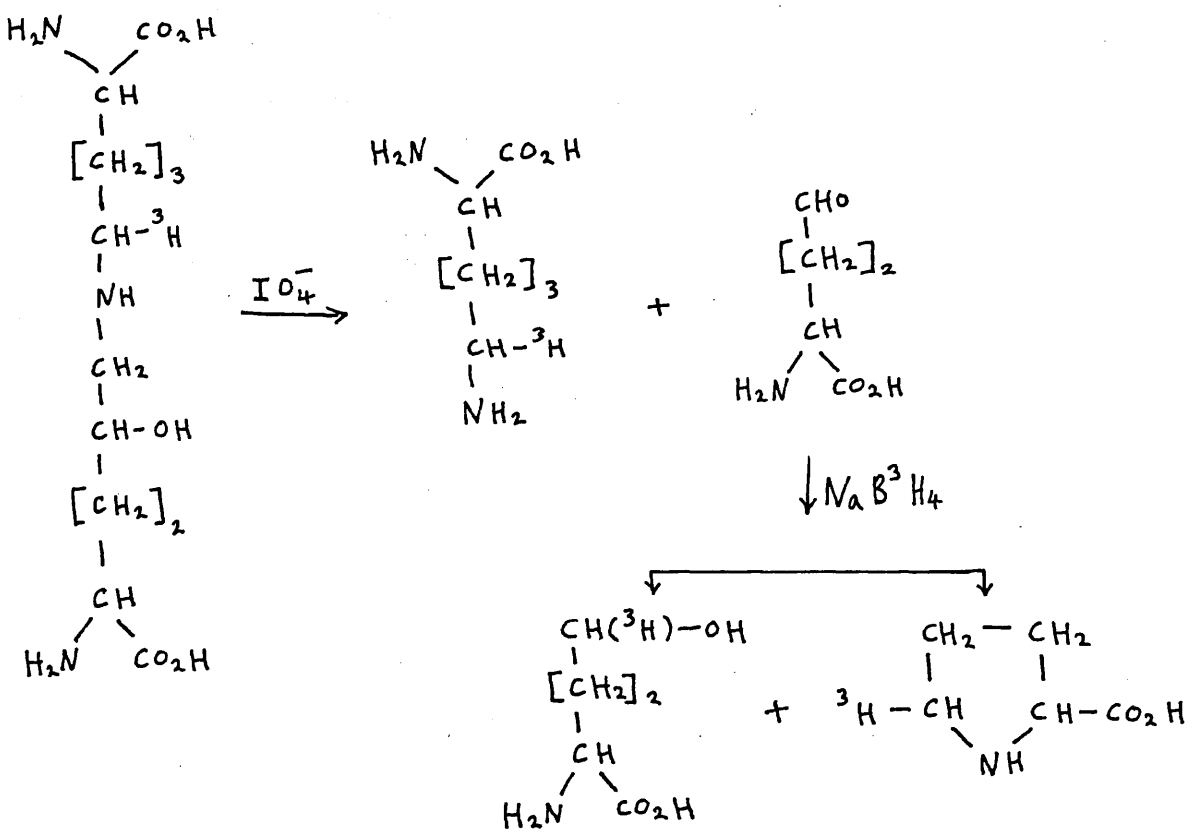
If this is important in causing loss of proteoglycans from cartilage sections then thiol agents, which could also break the disulphide bonds, should also reduce basophilia. The thiol agents in the table on p. 59 were not effective.

These observations, plus the biochemical results of Sajdera (1969) who found that treatment of cartilage with dithiothreitol did not extract proteoglycan complex, suggest that splitting of disulphide bonds by periodate is not important in causing loss from tissue sections. There are two further reasons for thinking that periodate has little effect on protein cores by any mechanism. Proteoglycans showed a generally rather slower rate of viscosity decrease compared with glycosaminoglycuronans. The viscosity of a polypeptide, gelatine, was not affected in sodium periodate solution (Fig.8).

Two glycol-containing structures are found in the linkage region which do not occur in the side-chain (see pp. 10-11). These are glucuronic acid linked to galactose and xylose linked to serine. Rates of glycol cleavage and stabilities of the cleaved products are not known.

Action of periodate on collagen

Bailey (1970) isolated from reduced collagen two new amino acids believed to be involved, in their non-reduced form, as inter-molecular cross-links stabilizing the collagen fibre. One of the cross-links was shown to be cleaved by periodate.



Periodate may be expected to cause the network of collagen fibres in cartilage to become more 'open'. This in turn would facilitate the loss of the product from the action of periodate on proteoglycan complex.

Mechanism of action of periodate on glycosaminoglycuronans

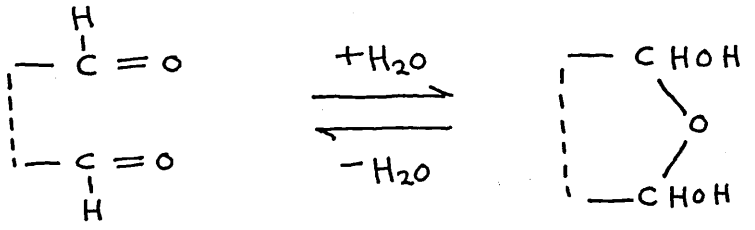
Glycol cleavage

In 0.001M sodium periodate, the specific viscosity of hyaluronate solution dropped by 50% in 100 minutes although consumption of periodate was at most 3% of the theoretical (Fig.7). There was only enough periodate to oxidise 14% of the glycols present if all was consumed. (See calculation on p.56.)

Breaking of a hyaluronate chain would not occur from glycol cleavage alone. It follows that cleavage of a very few glycols produces a large change in shape and hydrodynamic interaction with the solvent (Scott, Tigwell & Sajdera, 1972). It is possible that a monomer, in the polysaccharide chain, containing a cleaved glycol is more flexible than before that cleavage took place.

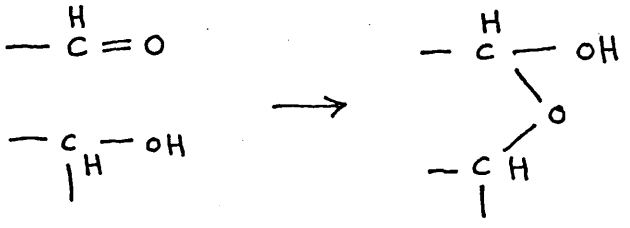
The precipitous decrease in molecular stiffness for alginate molecules, following introduction of only 1 or 2% periodate-oxidised units, was accounted for by the creation of centres of high local flexibility. This flexibility was associated with rotation about the three bonds adjoining C-4, C-5, the original ring-oxygen atom, and C-1 of the oxidised hexuronic-acid residues (Smidsrod & Painter, 1973).

However, there are further processes that could take place after cleavage which would limit flexibility at the oxidised monomer rather than increase it. In the first case the newly formed aldehydes could react with each other (Guthrie, 1961) to form hemialdal groups. This group is formed by the addition of the elements of a molecule of water across two aldehyde groups.



Hemialdal

A second alternative is that put forward particularly for alginate (Painter & Larsen, 1971). Aldehyde groups could react with hydroxyl groups on neighbouring sugar residues to give hemiacetals.



Hemiacetal

Labile bonds

Another activity of periodate solutions, besides oxidation of glycol groups, which splits the polymer chains needs to be considered. This splitting of the chain could occur by attack upon specific links labile to periodate and which would only need to occur infrequently throughout the molecule.

The presence of special labile bonds in hyaluronate has been proposed (Swann, 1969) to account for the observation that ascorbic acid degraded hyaluronate into sub-units

whose size could not be reduced by further treatment with ascorbic acid. However, a number of proposals have been made that depolymerisation of hyaluronate and other polymers by ascorbic acid and a range of reducing agents (the oxidative-reductive depolymerisation reaction) is mediated through free radicals. These act by causing random oxidative cleavage of glycosidic bonds (Pigman, Rizvi & Holley, 1961).

Free radicals

Free radical degradation was postulated to be responsible for the observed viscosity reducing power of sodium periodate upon alginate solutions (Painter & Larsen, 1971). Alginate oxidised to its limit in the presence of propan-1-ol, an assumed free-radical scavenger, was found to have an intrinsic viscosity of 4.0dl/g compared with 0.17dl/g in the absence of propan-1-ol. Measurements were made in 0.01M NaCl on the product isolated after oxidation.

The effect of propan-1-ol on cartilage sections was investigated (p.60). Sodium periodate treatment of sections in the presence of propan-1-ol for 4.5 hours at 30°C caused decreasing losses of basophilia as the concentration of propan-1-ol rose from 50ml/l to 150ml/l, at which concentration staining was practically as intense as in the control (exposed to buffer only). On overnight exposure of sections to periodate propan-1-ol had relatively less effect in preserving basophilia. This effect is not likely to be due to the influence of propan-1-ol on rate of uronic acid oxidation since, in a separate experiment (Fig.10), the rate of glycol oxidation (pinacol) was found to be unaffected in the presence of 150ml/l propan-1-ol. Pinacol was chosen as a simple glycol whose oxidation rate is not too fast to be easily followed. In addition, propan-1-ol was found to slow the rate of decrease of viscosity of proteoglycan and hyaluronate solutions exposed to periodate (Figs. 5 & 9).

It seems reasonable to suppose that the action of propan-1-ol on cartilage, cartilage proteoglycan and hyaluronate is to scavenge free radicals produced in periodate solutions.

Hydroxyl free radicals from periodate solutions

Hydrogen peroxide was found to be as active as sodium periodate in decreasing basophilia of cartilage sections (p.59). This reagent is well known to produce free radicals e.g. $^{\cdot}OH$, particularly in the presence of trace amounts of heavy metals (Fenton's reagent) such as would be present in tissues, and these have been shown to depolymerise polysaccharides and other polymers. Hydrogen peroxide is not generally active in oxidising glycols. Periodate and hydrogen peroxide solutions may act in a similar way, through the production of free radicals which then act on tissue polymers.

Symons (1955) performed various tests, thought to be diagnostic of the presence of active free radicals, upon potassium periodate solutions. Periodate solutions containing respectively acrylonitrile and methyl methacrylate were irradiated by means of a mercury arc from which light of wavelength shorter than 365nm was filtered. Polyacrylate and polymethacrylate were isolated from these solutions after a time. Irradiated periodate solutions were found to hydroxylate benzoic acid. Similar products were formed by using non-irradiated periodate solution containing ferrous ions.

Symons drew a parallel between the behaviour of periodate in these ractions and that of hydrogen peroxide in similar reactions. He proposed that hydroxyl radicals are formed both in the reaction of periodate with ferrous ion and in the photochemical decomposition:

$$H_4IO_6^- + h\nu \rightleftharpoons H_3IO_5^{\bullet -} + {}^{\bullet}OH \qquad (1)$$

$$H_3IO_5^{\bullet -} \rightarrow IO_3^- + H_2O + {}^{\bullet}OH \qquad (2)$$

$$H_4IO_6^- + {}^{\bullet}OH \rightarrow H_2O_2 + H_3IO_5^{\bullet -} \qquad (3)$$

$$H_4IO_6^- + H_2O_2 \rightarrow IO_3^- + 3H_2O + O_2 \qquad (4)$$

and for reaction with ferrous ions

$$Fe^{2+} + H_4IO_6^- \rightleftharpoons FeOH^{2+} + H_3IO_5^{\bullet -}$$

$H_3IO_5^{\bullet -}$ may then break down as in (2) above.

PREVENTION OF LOSS OF CHONDROITIN SULPHATES FROM CARTILAGE SECTIONS

Formaldehyde fixation

"The aim of fixation is the preservation of cells and tissue constituents in a condition identical to that existing during life and to do this in a way that will allow the preparation of thin, stained sections." (Drury & Wallington, 1967).

Formaldehyde is a widely used fixative for the preservation of proteins. It functions by forming bridging links between tissue proteins. The most frequently encountered reaction of formaldehyde (Pearse, 1968, Chapter 5) is its addition to a compound containing a reactive hydrogen atom with the formation of a hydroxy methyl compound

$$RH + CH_2O \rightleftharpoons R.CH_2(OH)$$

This compound may condense with a further H atom to form a methylene bridge ($-CH_2-$).

$$R.CH_2(OH) + HR^1 \rightleftharpoons R - CH_2 - R^1 + H_2O$$

Fixation of proteins brings about the mechanical trapping of other tissue components.

The effect of formaldehyde fixation in preventing loss of chondroitin sulphates, from cartilage sections, was investigated by Scott and Dorling (personal communication). They found that it had a small effect in diminishing loss from cartilage sections exposed to periodate solution.

Cetylpyridinium precipitation

(See experimental details on p.63)

Cetylpyridinium chloride ($C_{21}H_{38}NCl.H_2O$) is a precipitant for acidic polymers (Scott, 1960). It was anticipated that treatment of cartilage sections with this reagent would prevent, or diminish, loss of acidic polysaccharides from them upon subsequent exposure to periodate solution.

The use of cetylpyridinium chloride (CPC) to fix acidic polysaccharides in tissues is not new. It was first employed in aqueous formaldehyde solutions (Williams & Jackson, 1956) though not as a pretreatment for periodate oxidation.

Zugibe (1970) performed prolonged periodic acid oxidation of CPC-acidic polysaccharide smears on glass slides noting that 16-24 hours oxidation were required before they could be demonstrated with Schiff reagent. Sections of cock's comb and human umbilical cord, fixed with cetylpyridinium in formalin, required similar oxidations before their acidic polysaccharides could be demonstrated.

CPC treatment prevented loss of large amounts of Schiff stainable material from cartilage sections (Fig.12). Localisation of chondroitin sulphates in the territorial regions of chondrocytes was found to be particularly sharp following the two-step PAS procedure. A reason for this is that neutral PAS-positive substances may diffuse out from tissue sections during the long exposure to aqueous solution because only the polyanionic chondroitin sulphates are fixed.

Pretreatment of cartilage sections with cetylpyridinium chloride greatly diminished loss upon subsequently being put through the 2-step PAS procedure. However, cetylpyridinium may be of less use when applied to other tissues, as model experiments suggest. When acidic polysaccharides were spotted on glass-fibre paper, CPC treatment did not prevent total loss of polysaccharides from the paper upon subsequent exposure to sodium periodate solution (p.78).

Acid fixation

When periodic acid was substituted for sodium periodate in the 2-step PAS procedure very good Schiff uptake, by fresh frozen sections of bovine nasal septal cartilage, was observed (p.36). This suggested that less loss of chondroitin sulphates occurred in periodic acid compared with sodium periodate solution. Treating sections respectively for 1 hour with 20g/l sodium periodate or 0.1M periodic acid solution, before staining with Alcian blue in 0.40M $MgCl_2$, showed that a greater loss of chondroitin sulphates occurred from those sections exposed to sodium periodate solution (p.65). An investigation was carried out to find whether this was an effect of low pH and/or due to periodic acid being an oxidising agent. Experiment showed (pp.66-68) that treatment of cartilage sections for 1 hour with 0.1M hydrochloric, acetic or orthophosphoric acid, as well as 0.1M periodic acid, would prevent subsequent loss of chondroitin sulphates upon prolonged exposure to sodium periodate solution. It appeared that the observation made initially with periodic acid was a pH effect.

This effect might be due to electrostatic interaction between the polyanionic chondroitin sulphates and the tissue proteins which would be polycations at low pH. Exposure of acid-treated sections to neutral buffer or to high concentration salt solution (1.0M $NaClO_4$) should reverse this effect (Scott, Dorling & Stockwell, 1968). The acid effect could not be reversed under the experimental conditions tried.

Sajdera (1969) found that pretreatment of cartilage with 0.1M hydrochloric acid prevented subsequent extraction of proteoglycan in concentrated salt solution. A similar effect was observed by Obrink (1971) for cross-linked collagen gels which were permeated with chondroitin sulphate in solution. At lower pH levels quite a lot of chondroitin sulphate was found which was difficult to elute by raising the pH or increasing the eluting salt concentration to values where an electrostatic complex could not be formed.

One of the main features of the separation of cartilage extracts by anion-exchange chromatography (How, 1969) is that, after application of polyanions to the matrix, upon addition of salt the matrix shrinks and traps absorbed polyanions.

By analogy, an explanation for the irreversibility of the pH effect is that at low pH levels the collagen network in cartilage sections is 'opened-up' and strong electrostatic interaction occurs between the proteoglycans and the tissue protein - particularly collagen. When the pH or salt concentration are raised then shrinkage of the collagen fibrils produces mechanical entrapment of the proteoglycan which then cannot be extracted.

Free-radical scavengers

(See experimental details on p.60 and Figs. 5 & 9)

Propan-1-ol, an assumed free-radical scavenger, when present in 20g/l sodium periodate solution at a concentration of 150ml/l was very effective in diminishing loss from sections exposed for 4½ hours but not so effective for more prolonged exposure. Propan-1-ol also slowed the rate of decrease of viscosity for proteoglycan and hyaluronate solutions exposed to periodate.

Other, more effective, free-radical scavengers might be found to be incorporated in sodium periodate solution. It would be important that any agent used in this way did not itself consume periodate.

MODEL EXPERIMENTS. PERIODATE OXIDATION OF POLYSACCHARIDES ON PAPER

Cetylpyridinium chloride (CPC) treatment of fresh frozen bovine nasal septal cartilage sections increased Schiff reactivity following performance of the 2-step PAS reaction. This would appear to be because CPC causes retention of glycosaminoglycuronans in the sections.

It was proposed to determine the effect of CPC complex formation upon rates of periodate oxidation of glycosaminoglycuronans by performance of model experiments, with these substances spotted on strips of paper. Polysaccharide spots on paper represent a less complex situation than that existing in tissue sections.

Ordinary cellulose paper

(See experimental details on pp.69-71)

An attempt was made to investigate the effect of CPC upon the rate of periodate-oxidation of glycosaminoglycuronans spotted on filter paper. Results could not be interpreted because the paper itself was deeply coloured by Schiff reagent following exposure to periodate solution. This was presumably due to oxidation of cellulose. As a way round this problem an attempt was made to obtain completely periodate-oxidised paper which, after reduction with sodium borohydride solution, would give a non-Schiff reactive support. Four hours exposure to periodate solution was not long enough to complete the oxidation of the paper since after reduction and subsequent exposure to sodium periodate solution, the paper was still Schiff reactive.

The use of longer exposures to periodate solution could have been investigated but it was decided to use cellulose acetate paper as an alternative to ordinary cellulose paper.

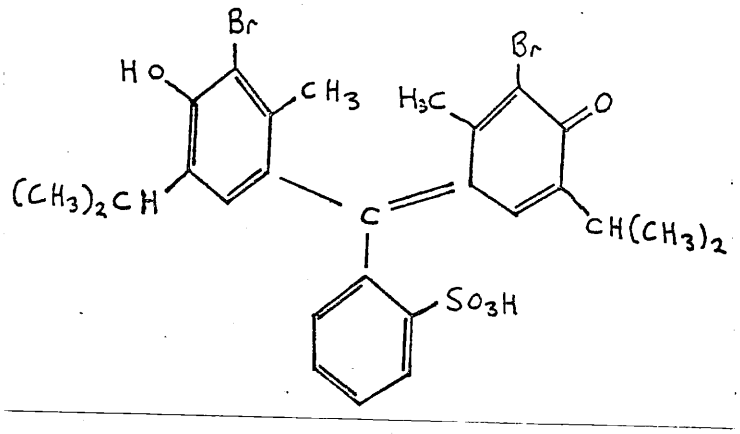
Cellulose acetate paper

(See experimental details on pp.72-73)

Acetylation of hydroxyl groups of cellulose in cellulose acetate paper should prevent them from being attacked by periodate and consequently this paper should not be as Schiff reactive as filter paper, following exposure to periodate. While using this paper it became necessary to be able to stain CPC-polysaccharide complexes to ascertain whether they stayed on cellulose acetate paper exposed to sodium periodate solution.

A method of staining CPC-polysaccharide complexes was developed using the indicator/dye Bromo-thymol blue. Bromcresol green has been used for this purpose (Kelly, Bloom & Scott, 1963).

Bromo-thymol blue has the formula:



Under conditions of high pH the sulphonic acid group in the dye competes with the glycosaminoglycan molecules for their complexed cetylpyridinium. First experiments were concerned with the choice of base in the staining solution. Comparison was made of (a) Tris buffer (b) sodium carbonate and (c) ammonia in the Bromo-thymol blue staining solution. Similar results were obtained with each of the three buffers consequently any of them could be used in the staining solution. However, the pH of the Tris buffer was a little low, as evidenced by the green staining of sections upon being removed from the staining solution. Ammonia has the disadvantage that the gas is lost from solution and therefore the concentration of ammonia in solution is not accurately known. This may affect the reproducibility of the technique. Sodium carbonate would be the best buffer because its concentration in solution can be controlled and its solutions have a high pH. Consequently, sodium carbonate was used in the subsequent preparation of Bromo-thymol blue staining solutions.

Lack of staining for Heparin and CSA spots on cellulose acetate paper when exposed to Bromo-thymol blue in 0.01M sodium carbonate solution was the reason for trying higher base concentrations. A possible explanation for this lack of staining was considered to be due to the fact that Bromo-thymol blue is a sulphonic acid compound. Consequently cetylpyridinium has little to gain by dissociating from sulphate ester groups, on the glycosaminoglycans, and combining with the dye. Increasing the salt content of Bromo-thymol blue solution should aid dissociations and therefore improve CSA and Heparin staining on paper.

It was found that, for spots on cellulose acetate paper, raising the base concentration in the Bromo-thymol blue solution did <u>not</u> increase staining for CSA and Heparin. However, they did stain well on Whatman 54 paper. This suggested that poor staining on cellulose acetate paper was something to do with the nature of the paper or the application of polysaccharide spots to the paper.

It became apparent that the lack of Bromo-thymol blue staining for sulphated polysaccharides, described above, was due to poor spotting technique. Those polysaccharides less viscous than hyaluronate or alginate were diffusing through the cellulose acetate sheet to the support (beaker or paper tissue) beneath the sheet.

Although Bromo-thymol blue staining showed that CPC-glycosaminoglycuronan complexes stayed on cellulose acetate paper exposed to periodate solution they did not stain with Schiff reagent after periodate treatment. This anomaly was investigated.

Pre-oxidised polygalacturonate was found to be Schiff reactive in solution and when spotted on Sartorius cellulose acetate paper. CPC treatment increased the clarity of staining for pre-oxidised polygalacturonate and alginate spots on this paper.

The anomaly, that CPC treated pre-oxidised polysaccharide spots on Sartorius cellulose acetate paper were Schiff reactive but that CPC treated polysaccharide spots were not Schiff reactive after being exposed to periodate solution, could not be explained at this time. Possibly the cellulose acetate was interacting with the aldehyde groups, on the polysaccharide, as they were being formed in periodate solution (p.21). The use of this paper was abandoned and further investigations performed with glass-fibre paper which may be expected to be chemically inert under PAS conditions.

Glass-fibre paper

(See experimental details on pp.78-80)

Periodate caused complete loss of glycosaminoglycuronans from glass-fibre paper even after CPC treatment. Consequently, the use of model experiments was not investigated further.

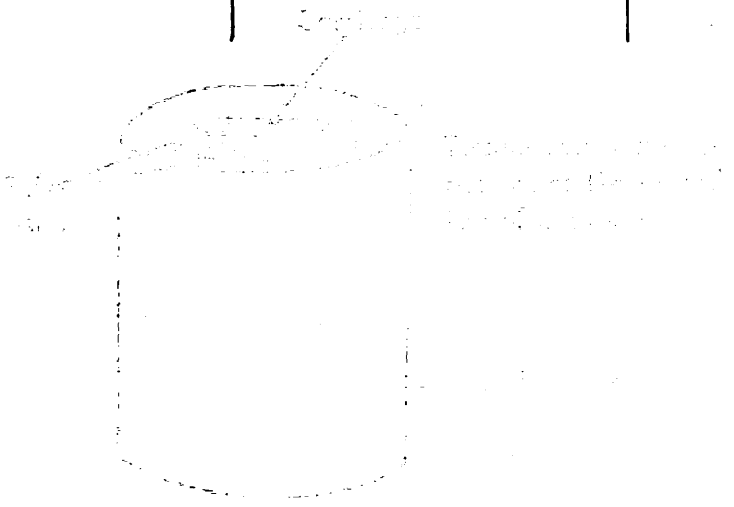
This loss from glass-fibre paper is further evidence for the depolymerising action of periodate solutions (see pp.20-23) In addition, it suggests that CPC may be of less use when applied to other more 'open' tissues compared with cartilage e.g. liver or brain. These tissues, and glass-fibre paper, do not have a tight fibrous network to trap CPC-precipitated polysaccharides.

Flow diagram of results for model experiments

Cellulose	Cellulose acetate	Glass-fibre
Attempted investigation of CPC treatment on rate of IO_4^- oxidation of polysaccharide spots on circles of Whatman no.1 paper. Red background, due to oxidation of filter paper, prevented interpretation of results		
↓		
Attempted to obtain a fully IO_4^- oxidised filter paper which after reduction with $NaBH_4$ would give a non-Schiff reactive support. Unsuccessful - even 4 hr exposure to IO_4^- solution was not long enough to fully oxidise the paper		
↘		
Evolved technique of staining CPC-polysaccharide complexes on cellulose acetate and Whatman 54 paper using Bromo-thymol blue in 0.5M Na_2CO_3 solution. Found that hyaluronate, alginate, CSA and heparin could be stained on Whatman 54 paper but only hyaluronate and alginate on cellulose acetate paper		
	↓	

Cellulose	Cellulose acetate	Glass-fibre
	Employed an improved technique for application of polysaccharide spots to Sartorius cellulose acetate paper. Hyaluronate, alginate, CSA and Heparin all stained with Bromo-thymol blue. Staining with this dye showed that all CPC treated polysaccharides stayed on the paper when exposed to periodate solution but none of them were Schiff reactive after periodate treatment	
	↓	
	Pre-oxidised polygalacturonate was shown to react with Schiff reagent in solution and when spotted on Sartorius cellulose acetate paper	
	↓	
	Pre-oxidised polygalacturonate and alginate spots on Sartorius paper. CPC and non-CPC treated. Both spots stained with Schiff on CPC treated paper. On non-CPC treated papers only polygalacturonate stained and staining was more diffuse than for the CPC papers. This showed that CPC treatment does not interfere with Schiff reactivity	
	↘	

Cellulose	Cellulose acetate	Glass-fibre
		Performance of PAS reaction for polysaccharide spots on glass-fibre paper - with and without CPC treatment. No spots were Schiff reactive. Bromo-thymol blue staining showed that CPC treated spots did not stay on paper exposed to periodate solution. This loss was caused by the IO_4^- ion because IO_3^- solution did not cause loss of CPC treated spots



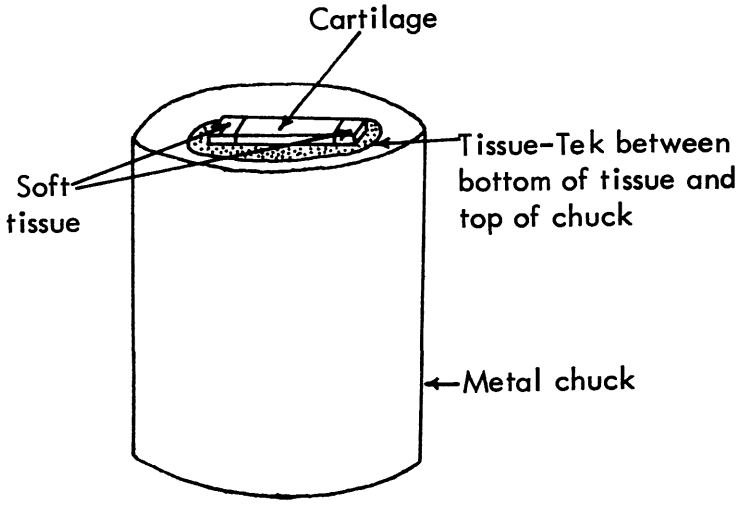
EXPERIMENTAL

Cutting fresh frozen sections of bovine nasal septal cartilage

Pieces of bovine nasal septum were quenched, after slaughter, in iso-pentane cooled with liquid nitrogen. They were stored at -70°C.

Sections were cut from these pieces of tissue using a Slee 'Pearse' cold microtome (cryostat) type 'H' fitted with a Cambridge rocker microtome. The knives for use in this instrument were sharpened by A. Young and Sons Ltd. of Edinburgh.

Before cutting sections from a piece of nasal septum it was trimmed with a scalpel. It was then supported on the detachable chuck, from the cryostat, using Tissue Tek (OCT, Ames Ltd.) between the tissue and chuck. Carbon dioxide gas was used to freeze the piece of tissue and solidify the Tissue Tek. The chuck was then inserted in the cryostat.



The cryostat was set to cut 6μ thick sections and a check was made that the intended thickness was being cut by listening to the number of clicks made per stroke of the cryostat handle. Two clicks were produced for each 1μ thickness of section consequently a 6μ section gave twelve clicks per stroke.

Sections were cut with the temperature of the cryostat cabinet at -20°C. The first few sections obtained from the piece of tissue were discarded. Then sections were picked up from the cryostat knife-blade on acid-alcohol washed glass slides. The dry slides were held by a rubber sucker on the end of a long glass rod. The sections were dried in a stream of air then stored at -10°C, if not used on the day that they were cut.

Cartilage was found to be a difficult tissue from which to cut good sections i.e. sections of uniform thickness. A sharp knife was found to be essential otherwise alternate thick and thin sections were obtained.

Soft tissue was left attached to either side of the cartilage for two reasons (i) soft tissue anchors the section to the glass slide because it adheres well to the glass, in contrast to the cartilage which adheres poorly; (ii) soft tissue contains substances demonstrable by the 'normal' PAS method (10 minute periodate oxidation) which contrast in behaviour with the slowly oxidisable chondroitin sulphates in cartilage.

2-step Periodic Acid Schiff (P.A.S) reaction

The theoretical background to this method is given on pp. 12-13 and in Scott and Dorling (1969).

Materials

Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Sodium borohydride $NaBH_4$ (British Drug Houses)
Lillie's 'Cold Schiff' (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses)
A stock solution was stored at 4°C
Bisulphite rinses were according to Lillie (1965)
D.P.X. (British Drug Houses)
Xylol

Procedure

The sections on slides were immersed in 50ml of 20g/l sodium periodate for 1 hour for performance of the primary oxidation. This solution was contained in a glass Coplin jar standing in a water bath at a temperature of 30°C.

At the end of 1 hour the sections were removed from the periodate solution and washed with three changes of distilled water. For these washes the slides were supported in metal racks and immersed in water contained in glass dishes.

50ml of 10g/l sodium borohydride solution were contained in a Coplin jar on the bench, at room temperature. This solution was always made up not longer than ½ hour before it was used. The sections were exposed to the borohydride solution for 3 minutes then given 3 changes of distilled water wash as before.

Sections were returned to the 20g/l sodium periodate solution, in the 30°C bath, for a secondary oxidation of 24 hours duration. Upon completion of this oxidation they were again washed with distilled water.

50ml of Lillie Cold Schiff were added to a Coplin jar on the bench at room temperature. Sections were immersed in this reagent for ½ hour before being put through 3 washes with bisulphite rinse for respectively 1, 2 and 2 minutes. The 3 x 50ml bisulphite rinses were contained in Coplin jars on the bench at room temperature. The lids of the Coplin jars containing Schiff reagent and bisulphite rinses were smeared at their edges with silicone grease (supplied by Edwards High Vacuum Ltd.) to prevent escape of sulphur dioxide dissolved in these solutions.

Finally, sections were washed for 10 minutes in running tap water before being dehydrated and mounted.

Dehydrating and mounting

Sections were washed in ethanol/water mixtures, ethanol and xylol contained in glass dishes. The order of the washes was 700 ml/l ethanol, 900 ml/l ethanol, 1st ethanol, 2nd ethanol, 1st xylol, 2nd xylol.

Upon being removed from the last xylol wash a glass cover-slip, with a drop of DPX mountant on it, was placed over the sections and a gentle pressure applied.

When the DPX was dry the excess mountant, which had exuded from under the cover-slip and subsequently solidified, was trimmed away with a scalpel.

Substitution of periodic acid for sodium periodate in the 2-step PAS procedure

Materials

Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Sodium borohydride $NaBH_4$ (British Drug Houses)
Lillie's 'Cold Schiff' (Lillie, 1965 was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses)
A stock solution was stored at 4°C
Bisulphite rinses were according to Lillie (1965)
D.P.X. (British Drug Houses)
Xylol
Periodic acid $HIO_4.2H_2O$ 50%w/w (British Drug Houses)
Fresh frozen sections of bovine nasal septal cartilage, 6μ thick, mounted on glass slides

Procedure

Some cartilage sections were put through the normal 2-step PAS procedure. At the same time, other sections were put through this procedure with the substitution of 0.10M periodic acid for 20g/l (0.094M) sodium periodate solution.

Sections were stained together in Schiff reagent and their appearance compared after mounting.

Result

Those sections for which periodic acid was used as the oxidant stained much more intensely with Schiff reagent compared to those which had the normal 2-step PAS procedure, with sodium periodate as oxidant.

The periodic acid oxidised sections exhibited a more uniform distribution of stained material, without clear localisation, compared to sodium periodate oxidised sections. In the 2-step PAS procedure employing sodium periodate, and also with Alcian blue in 0.40M $MgCl_2$, the stained material (chondroitin sulphates) is characteristically localised in the vicinity of the chondrocytes. (See Fig.2 on p.15.)

The use of Alcian blue in 0.4OM $MgCl_2$ for staining chondroitin sulphates

Alcian blue is a cationic copper-containing phthalocyanine dye. Its use in the staining of glycosaminoglycans is based upon its differential affinity for these substances in the presence of different electrolyte concentrations. Scott & Dorling (1965).

The dye was used in the presence of 0.4OM $MgCl_2$. In this concentration of $MgCl_2$ mainly chondroitin sulphates, plus a small amount of keratan sulphate, are stained in cartilage sections.

Materials

Alcian blue 8GX was a gift from I.C.I. Ltd.
Magnesium chloride $MgCl_2$ (Analar, British Drug Houses)
Sodium acetate $CH_3.COONa$ (British Drug Houses)
1.OM HCl solution made by dilution of British Drug Houses ampoule CVS containing 36.46% hydrochloric acid

The composition for 50ml of Alcian blue in 0.4OM $MgCl_2$ was:

5.0ml 4.0M $MgCl_2$ solution
30.0ml H_2O
12.5ml 0.1OM Na acetate/HCl buffer pH5.7
2.5ml 10g/l Alcian blue 8GX

mixed thoroughly.

Procedure

Sections were exposed to the staining solution in a Coplin jar, at room temperature, overnight. They were then removed and washed with 3 changes of distilled water before being dehydrated with ethanol, cleared with xylol and mounted in DPX in the manner described on pp.35-36.

Determining specificity of sodium periodate in respect of causing loss of basophilic sulphated material from cartilage sections

Materials

0.10M $K.NaIO_3$ prepared by dissolving KIO_3HIO_3 in water, adjusting to pH 5.4 with 1.0M NaOH solution using an electrode and making up to a known total volume of solution with water
0.025M Acetate buffer pH 5.7 - of composition used for Alcian blue staining solutions
Alcian blue in 0.4M $MgCl_2$
Sodium metaperiodate $NaIO_4$ and sodium perchlorate $NaClO_4$ (Analar, British Drug Houses)
Fresh frozen sections of bovine nasal septal cartilage, 6μ thick, mounted on glass slides

Procedure

Coplin jars were filled respectively with solutions of (i) 20g/l $NaIO_4$ (ii) 0.025M acetate buffer pH 5.7 (iii) 0.10M $NaClO_4$ (iv) 0.10M $K.NaIO_3$. They were stood in a water bath at 30°C.

Cartilage sections were immersed in these solutions for 1 hour durations before being washed with 3 changes of distilled water then stained with Alcian blue in 0.40M $MgCl_2$. Control sections, not exposed to aqueous solution, were also stained.

Results

Test or control solution	Control (not exposed to any solution)	20g/l $NaIO_4$	0.1M $NaClO_4$	0.025M acetate buffer pH 5.7	0.1M $K.NaIO_3$
Intensity of staining (Alcian blue 0.40M $MgCl_2$) for sections	4+	1+	3+	3+	3+

Comment

Loss of basophilic sulphated material was shown to be caused specifically by the periodate ion.

Uronic acid determination

Uronic acid was determined by the carbazole reaction of Dische in the borate modification of Gregory according to Bitter and Muir (1962). Glucurone was used as standard.

Materials

D-Glucuronolactone (D-Glucurone) $CO.(CHOH)_2.CH(O).CHCH.CHO$
(British Drug Houses)
Sulphuric acid. Microanalytical reagent grade (British Drug Houses)
Carbazole (L.Light & Co.)
1.0 Molar Potassium Tetraborate, prepared by dissolving boric acid (Analar, British Drug Houses) and potassium hydroxide (Analar, British Drug Houses) in water, mixing and making up to an appropriate volume. This solution was filtered through Whatman 54 paper.

Procedure

The reaction was performed in glass-stoppered test-tubes of capacity 15ml. Each test-tube contained a glass bead for mixing. To each test-tube was added 0.45ml test solution, glucurone standard solution or water as a blank plus 0.08ml 1.0 Molar potassium tetraborate solution. Glucurone standard solutions of concentrations 0.025 and 0.050g/l were used. The test solutions were diluted to concentrations to fall within the limits of the standard concentrations.

The sulphuric acid was contained in a dispenser (from Exelo) calibrated to deliver 3.0ml.* This quantity of sulphuric acid was added in turn to each test-tube. After shaking vigorously, using the glass beads to mix the viscous sulphuric acid solutions, each test-tube was heated in a boiling water bath for 20 minutes. Addition of sulphuric acid and commencement of heating was done at 30 second intervals between each test-tube. Stoppers were placed loosely in place at first then pressed home tight 30 seconds after commencement of heating. This was to allow for expansion of air in the test-tube which otherwise expelled the stopper with explosive force.

The test-tubes were removed at 30 second intervals into cold running tap water, to cool them thoroughly.

0.10ml 1.0g/l carbazole in absolute ethanol was added to each of the test-tubes when they were cool. They were heated in the boiling water bath for 10 minutes. Upon being removed from the boiling water, test-tubes were again cooled in running water.

The extinctions of the solutions were read directly, without further dilution, against water in 1cm silica cells at 530nm. Extinction measurements were made with a Perkin-Elmer-Hitachi UV-Visible recording spectrophotometer Model 124.

*It delivered 3.08ml and for this reason 0.08 instead of 0.075ml borate solution were taken.

The extinction of the blank solution was subtracted from the extinctions of the test and standard solutions. Corrected standard solution extinctions were plotted against glucurone concentrations to give a straight line graph. From this graph the concentration of uronic acid in the test solutions could be obtained, using their corrected extinctions.

Additional note

The glass-stoppered test-tubes and beads were washed, before use, successively with chromic acid, hot tap water and distilled water before drying in an oven at a temperature of 120°C.

Extraction of cartilage sections with periodate solution and subsequent uronic acid determination

Materials

Sodium metaperiodate $NaIO_4$ and sodium perchlorate (Analar, British Drug Houses)
Absolute ethanol
Alcian blue in 0.40M $MgCl_2$
Glucuronolactone, microanalytical sulphuric acid and carbazole as described on p.39.

Cartilage sections

Fresh frozen sections of bovine nasal septal cartilage were cut and mounted on glass slides in the manner described previously (p.33) but with certain modifications.

The soft tissue on each side of a piece of cow nasal septal cartilage was dissected off with a scalpel. Any material extracted from this soft tissue in periodate solution would complicate the measurement of material extracted from the cartilage. Tissue-Tek was applied to cover the bottom of the cartilage so that it was not cut with the sections.

Sections were cut with the cryostat set to produce a section thickness of 20µ. It was found impossible to prevent the cryostat cutting alternate thick and thin sections. This was presumably because of the magnitude of the attempted thickness of section. The thin sections were discarded and the thick sections taken, three to a slide. Each section was 1cm square.

Procedure

After the sections were dry, liquid paraffin wax from a heater-dispenser was applied to the slides in such a manner as to create a 'well' around each section. Two slides were taken and supported on a wet sponge in a glass dish with a lid. The glass dish was stood in a water bath at a temperature of 30°C; this temperature being the one at which oxidation is performed under the conditions of the 2-step PAS procedure.

Onto each of the sections on one slide was pipetted 0.5ml of 20g/l sodium periodate solution while 0.5ml of 0.10 Molar sodium perchlorate solution were measured on to each of the three sections on the other slide. The wet sponge kept the atmosphere in the dish humid thereby diminishing evaporation of water from solutions covering sections.

The slides were left for one hour. At the end of this period the periodate and perchlorate solutions were taken off, with Pasteur pipettes, and added to respective 50ml centrifuge tubes containing 15ml each of absolute ethanol and mixed. This was to dissolve the sodium periodate, or perchlorate, in ethanol while at the same time precipitating the uronic acid containing materials extracted from cartilage.

The cartilage sections were washed in three changes of distilled water. Periodate and perchlorate treated sections were washed in separate histological glass dishes. Sections floated off their slides during the water washes because they had no soft tissue to anchor them. Consequently, after their final wash, the sections had to be placed back on their respective slides with the aid of a thin glass rod.

The slides were then put in a Coplin jar containing Alcian blue in 0.40M $MgCl_2$. In this jar, the slides with sections on them were separated by additional glass slides so that sections would not get mixed if they came off their slides. Sections were stained overnight and washed, dehydrated and mounted next day in the usual manner (p.37). For this procedure the periodate and perchlorate incubated sections were treated separately so as not to get them mixed.

The ethanol suspensions were centrifuged for 15 minutes. The supernatants were tipped off before washing the deposits with a further 15ml of absolute ethanol each. After centrifugation for 15 minutes, the supernatants were tipped off and the centrifuge tubes inverted on paper tissues to allow ethanol to drain from the deposits. There was more deposit in the centrifuge tube containing the periodate extract than in that containing the perchlorate extract.

Each deposit was dissolved in 2ml of distilled water, stirring with a glass rod. These solutions were centrifuged for 15 minutes and the supernatants tipped off. The uronic acid content of the supernatants was measured directly, without diluting them further. The procedure for these determinations was as described on pp.39-40.

Results

Solution	Extinction recorded at 530nm
Blank (1) Blank (2)	0.025 0.02
Standard (1) 0.025g/l Standard (2) 0.025g/l	0.275 0.275
Standard (1) 0.050g/l Standard (2) 0.050g/l	0.525 0.54
$NaClO_4$ extract (1) $NaClO_4$ extract (2)	0.145 0.145
$NaIO_4$ extract (1) $NaIO_4$ extract (2)	0.30 0.30

Alcian blue staining showed the presence of much less basophilic sulphated material in the sections incubated with sodium periodate compared to those incubated with sodium perchlorate solution. This is in accord with experience described earlier (p.38).

The calculated uronic acid contents of the extracts were:

2.0ml $NaIO_4$ extract found to contain 0.0275g/l uronic acid

2.0ml $NaClO_4$ extract found to contain 0.01225g/l uronic acid

Comment

More material was extracted in $NaIO_4$ solution than in $NaClO_4$ solution.

The application of viscometry to measurement of change in molecular weight of macromolecules

The viscosity of a dilute solution of a polymer in a suitable solvent varies in a regular manner with the molecular weight of the polymer. The higher the molecular weight, in a homologous series of linear polymers, the greater the viscosity for a given weight concentration of polymer. The relationship between viscosity and molecular weight was originally derived empirically (H. Staudinger 1928-1933) but has been shown to have a theoretical basis.

The presence of large molecules in a liquid medium introduces inhomogeneities which increase the resistance to flow of the molecules of liquid and this increases the viscosity. If η_o and η are the viscosities of the solvent and solution, respectively, $\frac{\eta}{\eta_o}$ is the relative viscosity, η_r of the solution, the specific viscosity, η_{sp}, is equal to $\eta_r - 1$,

$$\frac{\eta - \eta_o}{\eta_o} = \frac{\eta}{\eta_o} - 1 = \eta_r - 1 = \eta_{sp}$$

The ratio η_{sp}/c, where c is the concentration of polymer is a function of the molecular weight. Since this ratio varies with the concentration, it is necessary to extrapolate a plot of η_{sp}/c against c to infinite dilution. The value then obtained is the intrinsic viscosity, $[\eta]$, so that

$$[\eta] = \lim_{c \to o} \left(\frac{\eta_{sp}}{c}\right)$$

Since η_{sp} is dimensionless, $[\eta]$ has the units of a reciprocal concentration and since c is usually expressed in g per ml of solution, $[\eta]$ is given in ml per g. The relationship between the intrinsic viscosity and the molecular weight of the dispersed polymer is

$$[\eta] = KM^a$$

where K is a constant for a given polymer solvent system, M is the molecular weight of the polymer and a depends on the shape of the molecules.

It can be seen from the relationship above that measurement of viscosity is a sensitive indicator of change of molecular weight for macromolecules.

The viscosity of a liquid decreases markedly if the temperature is raised. The variation of the viscosity of a liquid with temperature may be expressed by the exponential equation

$$\eta = Ae^{E/RT}$$

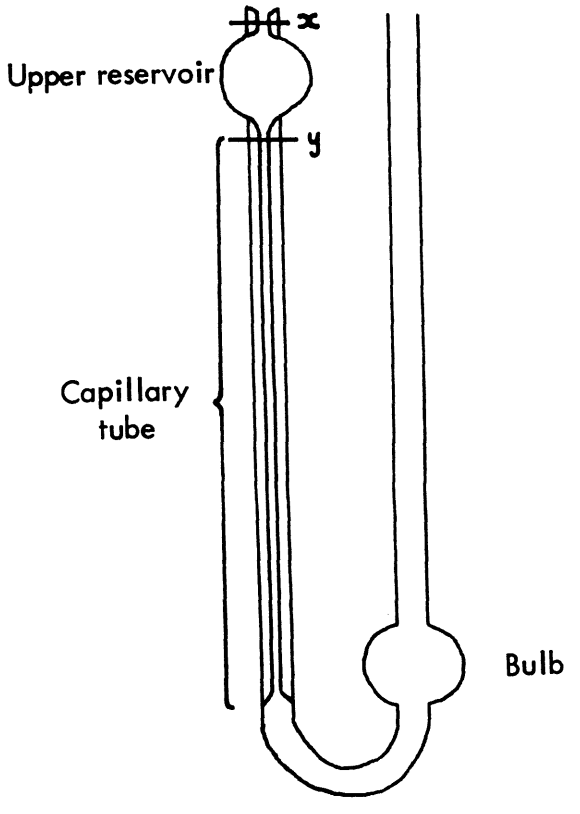
where A and E are constants for the given liquid. Consequently in the measurement of viscosity it is necessary to maintain the temperature of the solution under test constant.

At a constant temperature, the relationship between the viscosity and the rate of flow of the liquid through a tube is given by the Poiseuille equation for streamline (non-turbulent) flow

$$\eta = \frac{\pi r^4 t p}{8VL}$$

where V ml is the volume of liquid flowing in t sec through a narrow tube of radius r cm and length L cm, under a driving pressure of p dynes per sq cm.

A simple form of viscometer is named after W. Ostwald and its use is based on the Poiseuille equation.



The solution under test is introduced into the bulb and then sucked into the upper reservoir. The time t is observed for the solution to flow through the capillary tube between the marks x and y. Referring to the Poiseuille equation, it can be seen that for the Ostwald viscometer the radius r and length L of the capillary tube, and the volume V of the bulb are constant. The pressure p depends on the head of solution and its density; the former is constant but the latter will be dependent on the composition of the solution under test. For a solution of constant composition, density is constant and the measured run-out time t is directly proportional to the viscosity of that solution since from the Poiseuille equation

$$\frac{\pi r^4 p}{8VL} = \text{a constant, K.}$$

so,

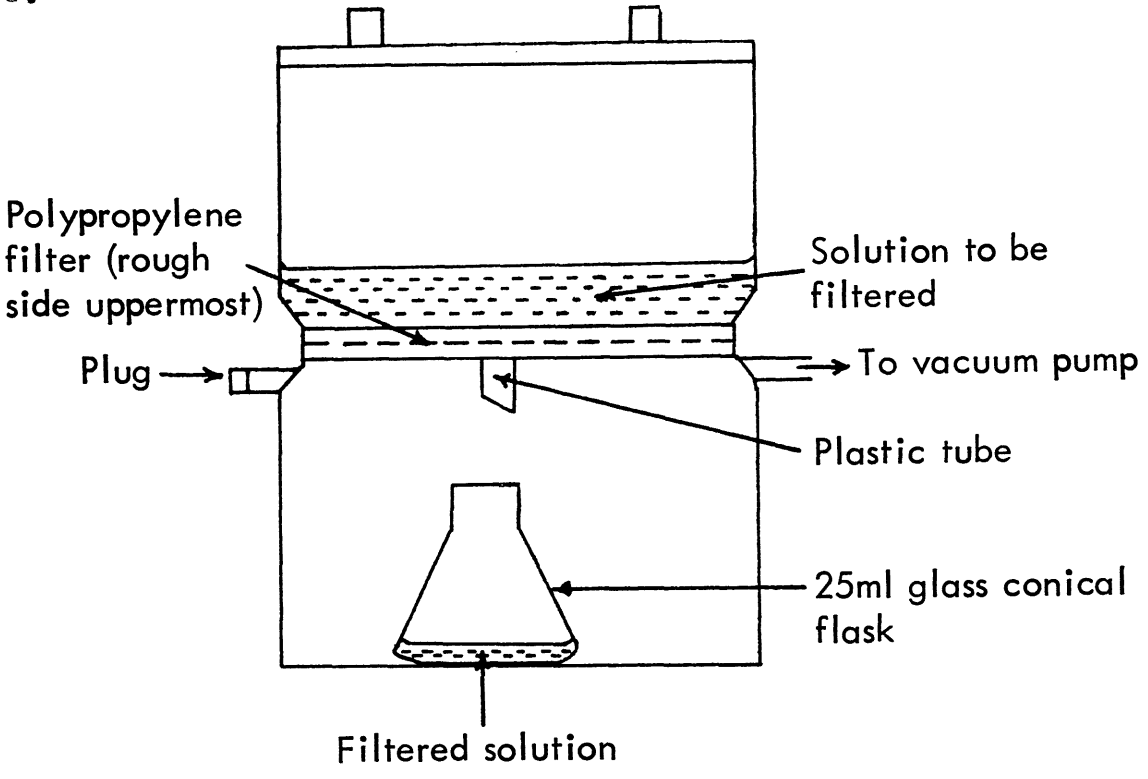
$$\eta = K.t$$

In the measurement of the viscosity of polyelectrolyte solutions an additional factor has to be considered. This is the ambient electrolyte concentration. In the absence of electrolyte, polyanions or polycations are maximally extended as a result of the mutual repulsion of their bound charges, consequently interacting 'frictionally' most strongly with the solvent. Interpretation of results is made simpler by dissolving the polyelectrolyte in a high concentration of supporting electrolyte. The counter ions then screen the mutual repulsion between the bound charges of the polyelectrolyte. Another reason for working at a high electrolyte concentration is to avoid the Donnan effect. The intense negative electrostatic field of the unscreened polyanion charges repels mobile anions such as periodate (Scott & Harbinson, 1968).

Viscometric determinations of proteoglycans, polysaccharides and gelatine

General viscometric procedure

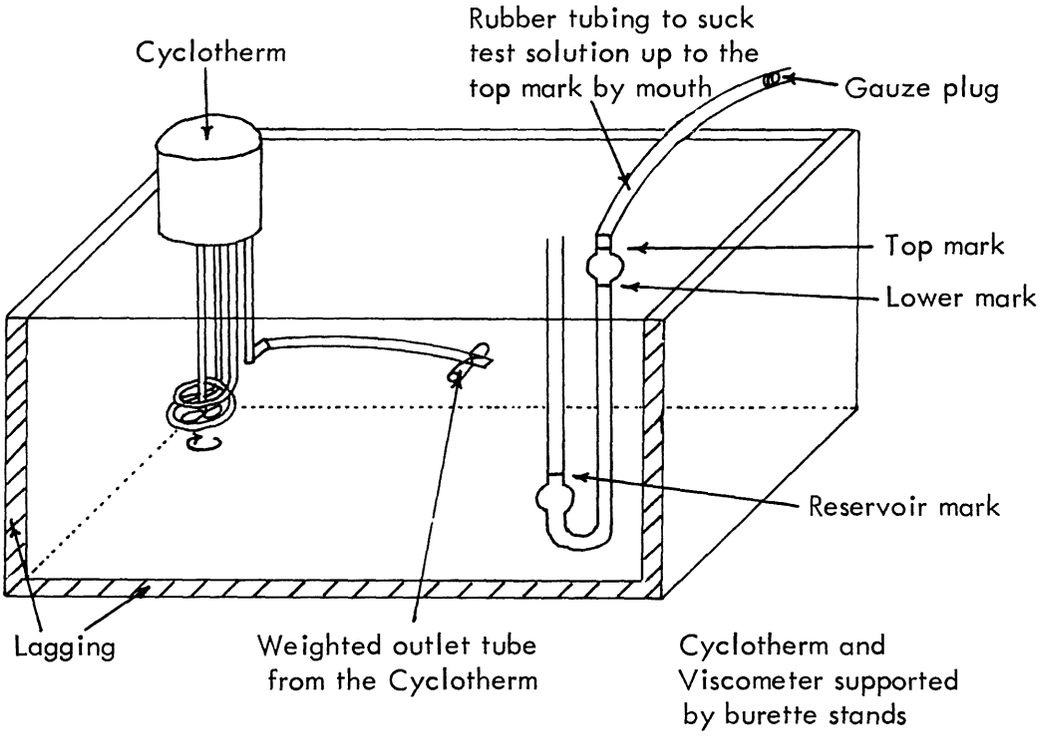
Polymer, sodium periodate and sodium perchlorate solutions were filtered before use in a plastic Millipore filter (type XX1104706). This was fitted with a 47mm diameter polypropylene filter (part no.61757) from Gelman Instrument Co. Ann Arbor, Michigan, U.S.A. These filters had 10 micron diameter pore size.



The solution to be filtered was poured into the upper part of the Millipore filtration device. A conical flask collected the filtered solution.

Viscometric measurements were made in Ostwald U-tube viscometers (type BS/U/M, M_2 and M_3, Baird & Tatlock Ltd. London). Before recording the run-out time for each solution, the viscometer was washed with distilled water, filtered methanol and filtered ether (filtration was performed through sintered glass). Air was sucked through the viscometer to dry it. This was done by attaching one limb of the viscometer to a water pump while plugging the other limb with gauze, to filter the air.

Measurements were made at a constant temperature of 25°C. This was achieved by supporting the viscometer in a water bath (fish-tank) containing water continuously warmed and circulated by means of a Cyclotherm (from Shandon). The bath was lagged with polystyrene sheets and the temperature of the water was checked periodically. In order to prevent the growth of moulds, which would diminish visibility, the water in the bath contained a little cupric sulphate (tip of medium sized spatula).



The run-out time was the time taken for the meniscus of the solution to fall from the Top to the Lower mark. This time was measured with a stop-watch.

Sodium perchlorate was the supporting electrolyte and was used at 0.50 Molar for control solutions or mixed with sodium periodate to give a final sodium concentration of 0.50 Molar for test solutions. Solutions were brought up to 25°C, before mixing, by supporting them in vessels in the water bath. Aliquots of reagents were measured and solutions were delivered to the viscometer with pipettes that had been washed with filtered methanol and ether to remove dust.

For the lowest-viscosity polymer solutions the viscometer with the slowest solvent run-out time (M2) was used so that the contribution from the polymer to the viscosity of the solution could be most accurately measured.

Solutions were mixed before being delivered to the reservoir limb of the viscometer, by means of a Pasteur pipette. Excess solution was withdrawn from the viscometer, using an extra long Pasteur pipette, to bring the meniscus to the reservoir mark.

The solvent run-out time for the viscometer was frequently measured so as to check the efficiency of the filtration procedure and also that the viscometer capillary was not becoming occluded. It was found that the presence of very little dust in solutions gave erratic run-out times.

Periodate-containing solutions in the viscometer were screened from light between measurements of run-out times.

In the plotting of viscosity/time curves, 'time after start' was taken as the time when the meniscus of the solution under test passed an imaginary line midway between the Top and Lower marks of the viscometer. This was done because it was not possible to measure the viscosity at a definite moment in time since each measurement took a few minutes to perform i.e. for the meniscus to fall between the Top and Lower marks.

Materials used in viscometry

Sodium metaperiodate $NaIO_4$ and sodium perchlorate $NaClO_4$ (Analar, British Drug Houses)
Periodic acid $HIO_4.2H_2O$ 50% w/w (British Drug Houses)

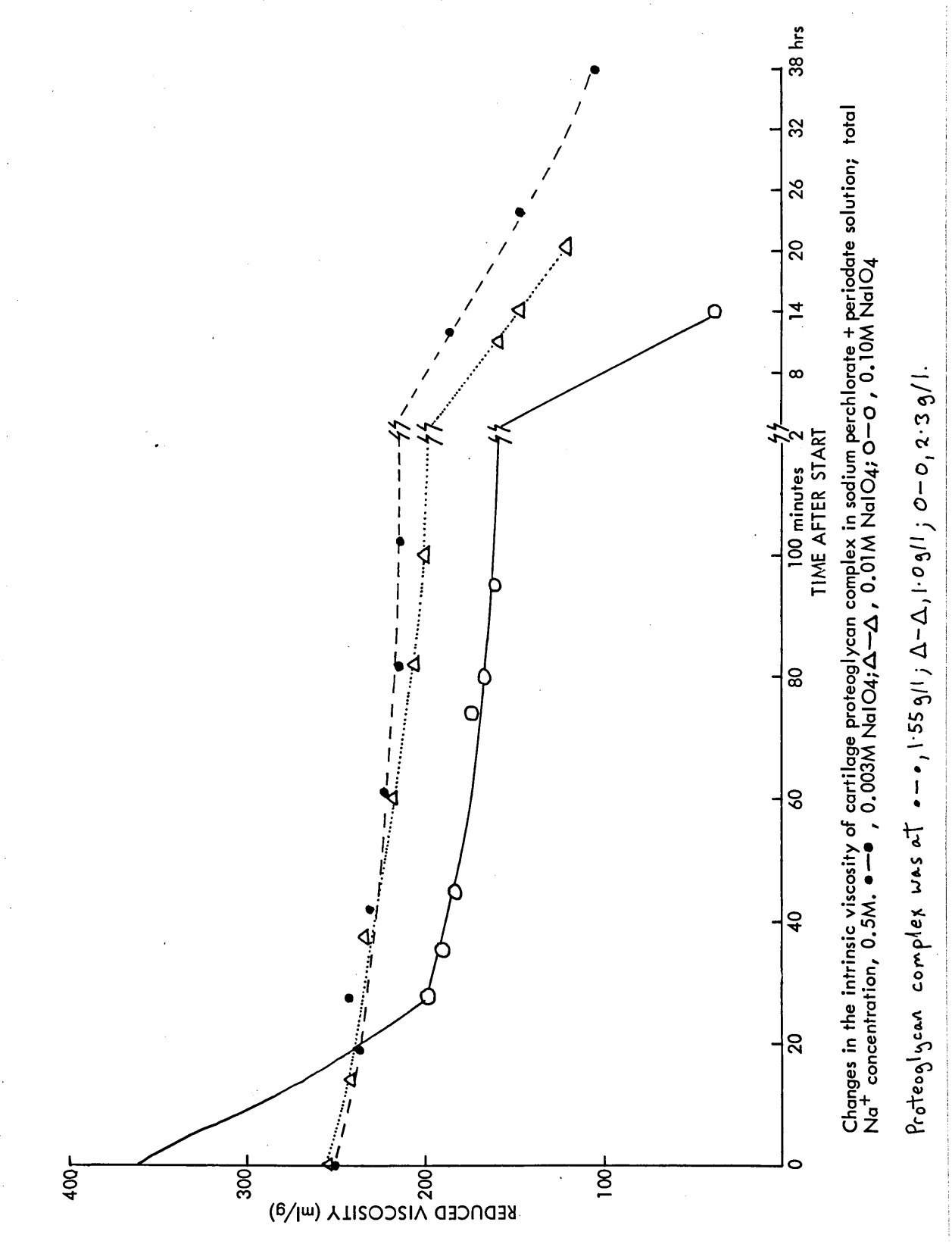
Sodium hyaluronate and sodium chondroitin-4-sulphate were prepared from human mesothelioma fluid and bovine nasal septum respectively, by the method of Scott (1960). Shark cartilage chondroitin sulphate was from Koch-Light.

Proteoglycan and proteoglycan complex were prepared, as described (Sajdera & Hascall, 1969), by S.W. Sajdera from bovine nasal septal cartilage and were a gift. The concentrations of these materials were determined from their uronic acid contents which were measured by the Dische-Carbazole method (pp. 39-40).

Gelatine 246 (from acid processed pig-skin, a gift from the British Leather Research Association).

Measurement of periodate consumption by hyaluronate undergoing viscometry

Aliquots of the test solution, in a flask supported in the water bath, were taken periodically and diluted with water. The periodate content, from which consumption was calculated, was measured by the spectrophotometric absorbance at 240nm. Correction for iodate absorption and for absorption of hyaluronate in sodium perchlorate solution was made. General spectrophotometric procedure was as described on p. 61. See also Scott and Harbinson (1968).



Changes in the intrinsic viscosity of cartilage proteoglycan complex in sodium perchlorate + periodate solution; total Na^+ concentration, 0.5M. •—• , 0.003M $NaIO_4$; Δ—Δ , 0.01M $NaIO_4$; o—o , 0.10M $NaIO_4$

Proteoglycan complex was at • - - •, 1·55 g/l; Δ-Δ, 1·0 g/l; o-o, 2·3 g/l.

Figure 4.

Taken from Scott, Tigwell & Sajdera (1972)

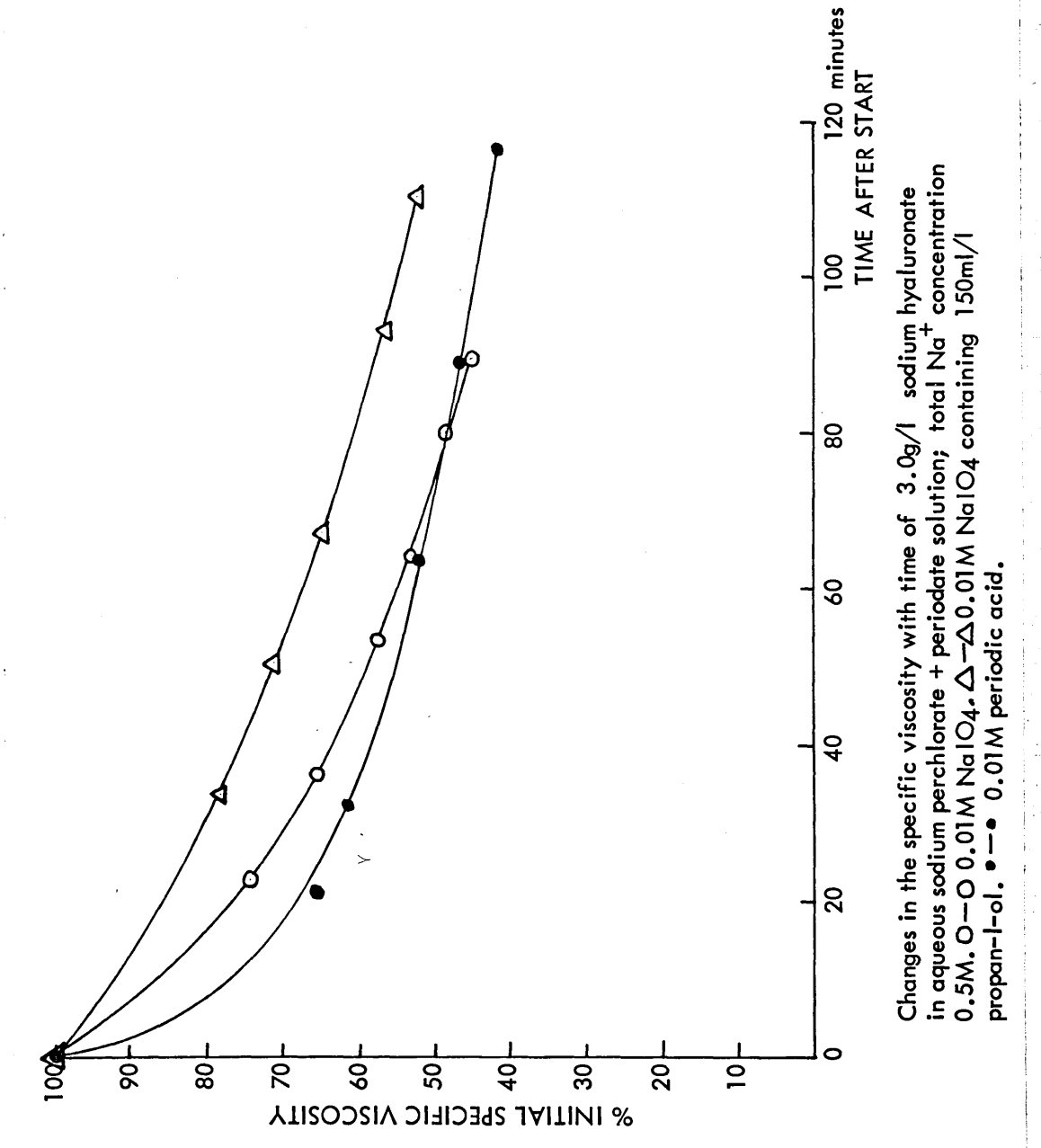
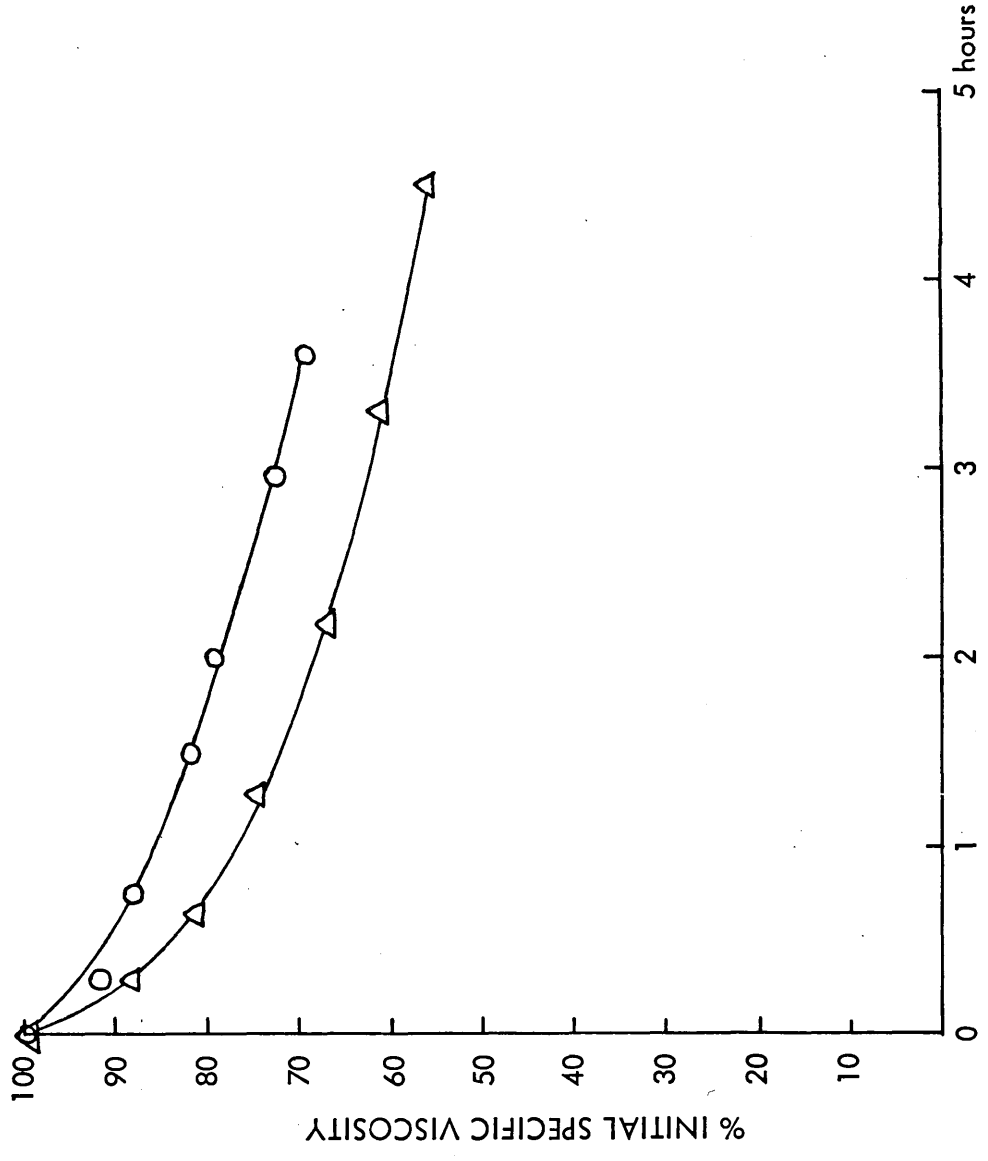


Figure 5.

Taken, in part, from Scott, Tigwell & Sajdera (1972)



Changes in the specific viscosity with time of 3.0g/l chondroitin sulphates in aqueous sodium perchlorate + periodate solution; total Na^+ concentration 0.5M. O—O Shark cartilage chondroitin sulphate in 0.01M $NaIO_4$. Δ—Δ Bovine nasal septal CSA in 0·1M $NaIO_4$.

Figure 6.

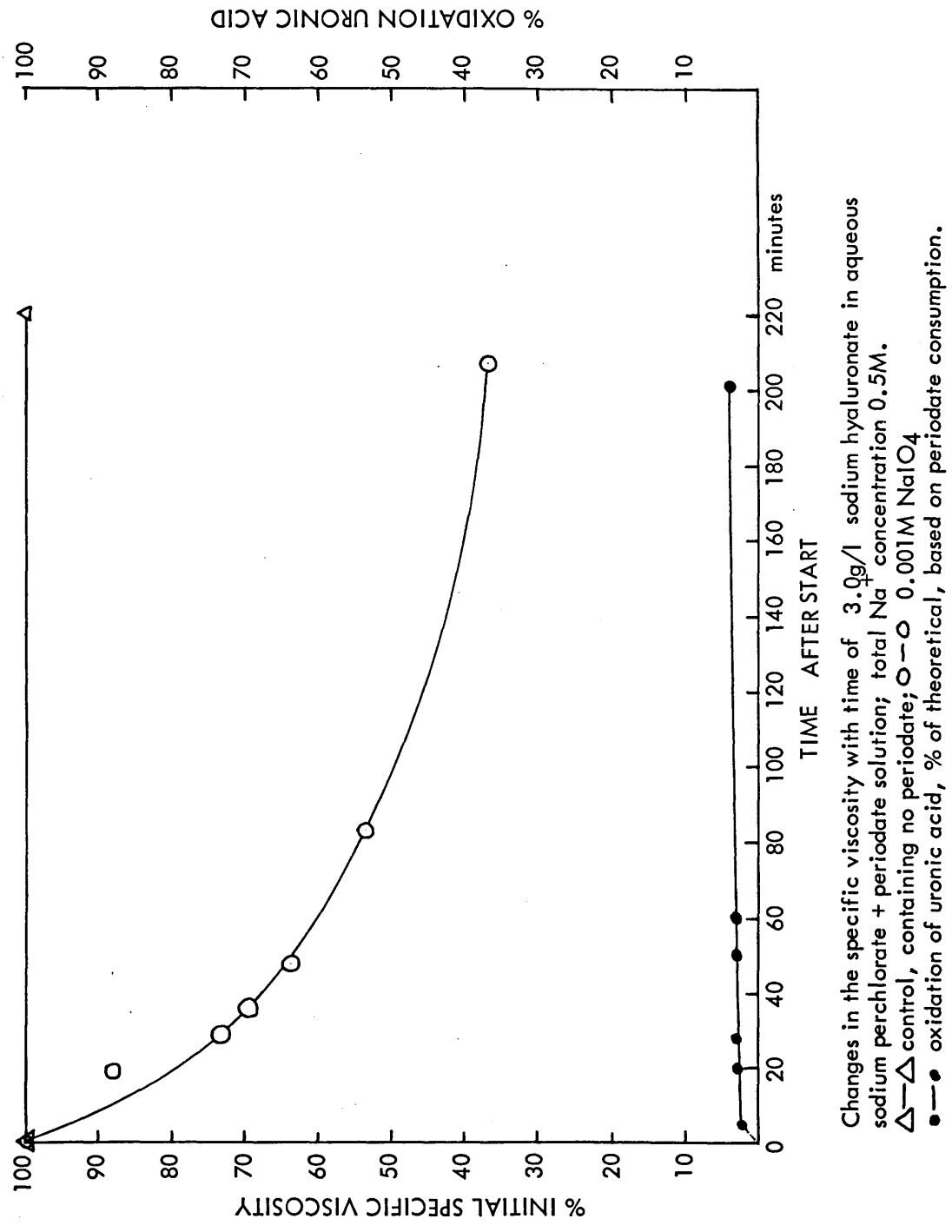
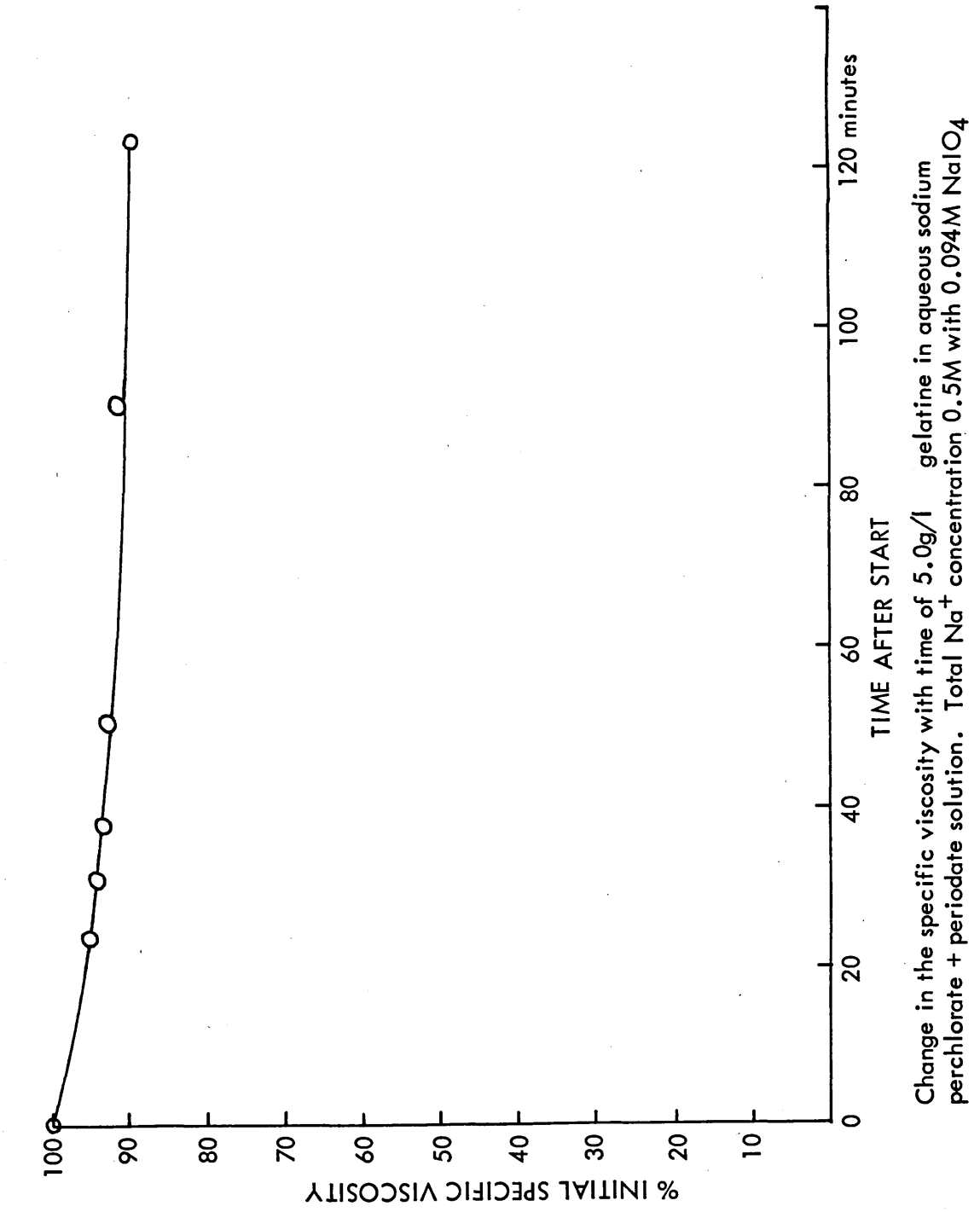


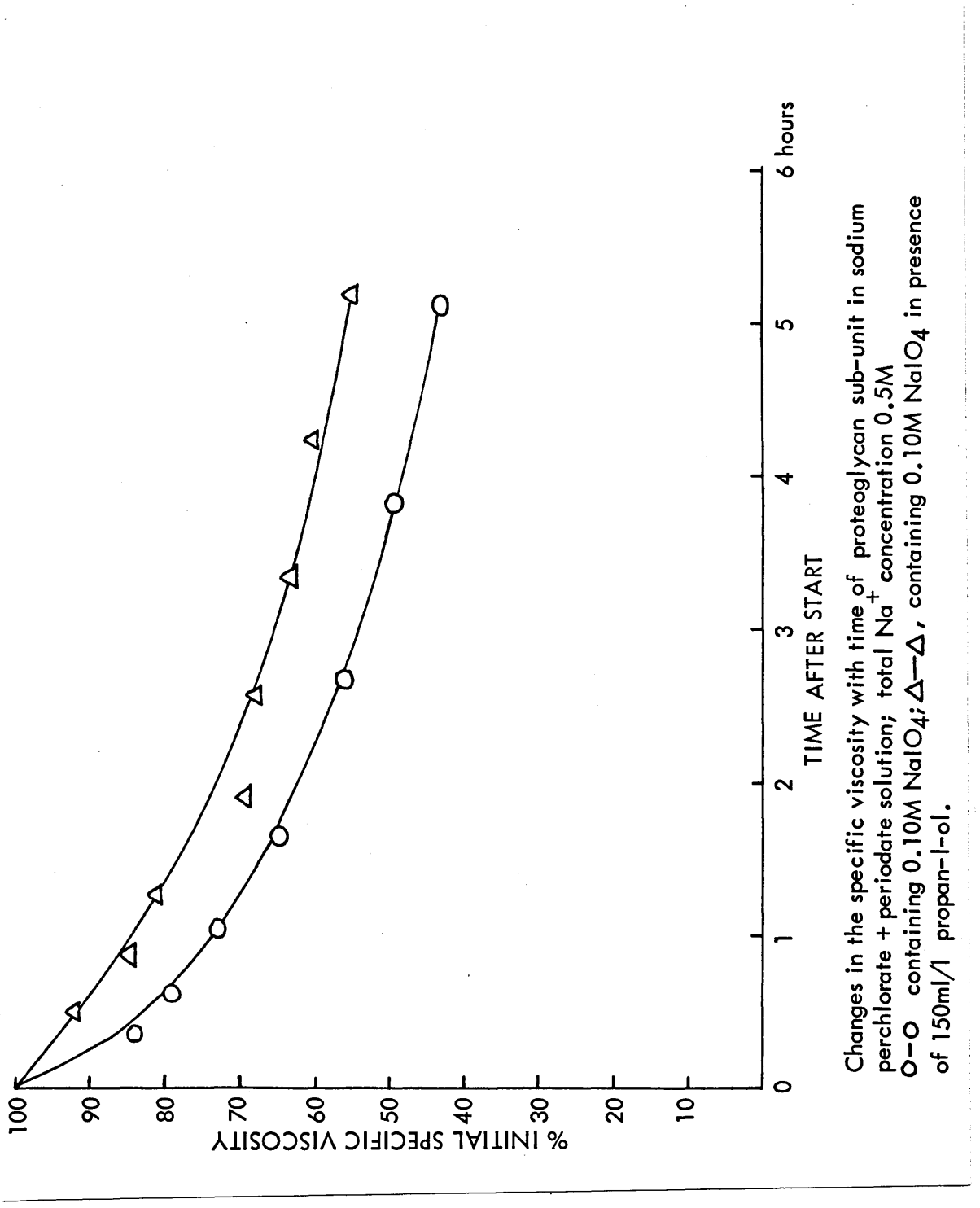
Figure 7.

Taken from Scott, Tigwell & Sajdera (1972)



Change in the specific viscosity with time of 5.0g/l gelatine in aqueous sodium perchlorate + periodate solution. Total Na^+ concentration 0.5M with 0.094M $NaIO_4$

Figure 8.

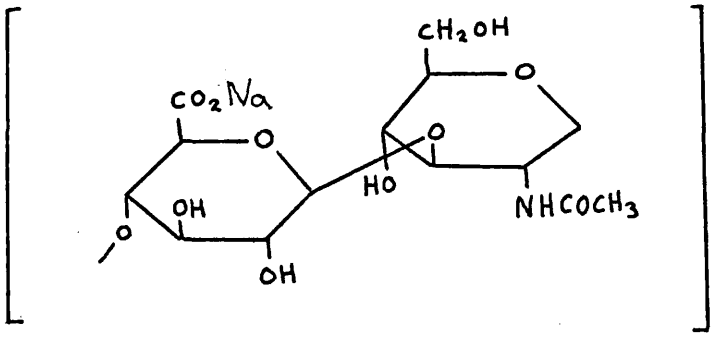


Changes in the specific viscosity with time of proteoglycan sub-unit in sodium perchlorate + periodate solution; total Na^+ concentration 0.5M
O–O containing 0.10M $NaIO_4$; Δ–Δ, containing 0.10M $NaIO_4$ in presence of 150ml/l propan-1-ol.

Figure 9.

Calculating the ratio $\frac{\text{moles glycol}}{\text{moles periodate}}$ in a solution of 3.0g/l Na hyaluronate in the presence of 0.001M $NaIO_4$

The molecular formula for the repeating unit in hyaluronate is:



Molecular weight of repeating unit ($C_{14}H_{20}O_{12}N_1Na_1$) = 417

There is one glycol group per repeating unit.

1 μ mole repeating unit ≡ 417×10^{-6} gm sodium hyaluronate

∴ concentration of repeating unit in 3.0g/l solution = $\frac{3}{417}$ Molar

i.e. $\frac{3}{417 \times 1000}$ moles per ml

or $\frac{3000}{417}$ μ moles per ml = 7.2 μ moles per ml

0.001M $NaIO_4$ solution contains 1 μ mole per ml

$\frac{\text{moles of glycol}}{\text{moles of periodate}}$ in a solution of 3.0g/l Na hyaluronate in presence of 0.001M $NaIO_4$ = $\frac{7.2}{1}$

i.e. there is enough periodate present to oxidise only 13.9% of the glycol groups.

Extraction of cartilage sections with solutions of oxidising and reducing agents

Materials

Fresh frozen sections of bovine nasal septal cartilage, 6u thick, mounted on glass slides
Alcian blue in 0.4M $MgCl_2$
Acetate buffer pH 5.7 of same composition as used in Alcian blue solution.

2-Mercaptoethanol ($HS.CH_2.CH_2OH$) (L. Light & Co.)
Molecular weight = 78.13. Weight per ml = 1.12gm
A stock 1.0M solution was prepared by dissolving 2-mercaptoethanol in water. An aliquot of this solution was further diluted, with water and 0.1M acetate buffer solution, to produce a 0.2M 2-mercaptoethanol solution in 0.025M acetate buffer. The pH of this solution, measured with an electrode, was 5.7.

5952h Dithiothreitol $(CHOH.CH_2SH)_2$ (Koch-Light)
Prepared 0.02M solution in water then used this stock solution to prepare 0.01M dithiothreitol in 0.025M acetate buffer.

L-Cysteine Hydrochloride $CH_2(SH).CH(COOH).NH_2HCl$ (British Drug Houses)
Prepared 0.25M solution in water then 'neutralised' some of this solution with 1.0M NaOH to give a 0.20M cysteine solution of pH 5.85 (measured with electrode)
Used the 'neutral' 0.20M cysteine solution to prepare a solution of 0.10M in 0.025M acetate buffer.

20 volume Hydrogen Peroxide solution was diluted to give 2 volume hydrogen peroxide (0.10M) in 0.025M acetate buffer.

Procedure

Cartilage sections were incubated with, respectively:

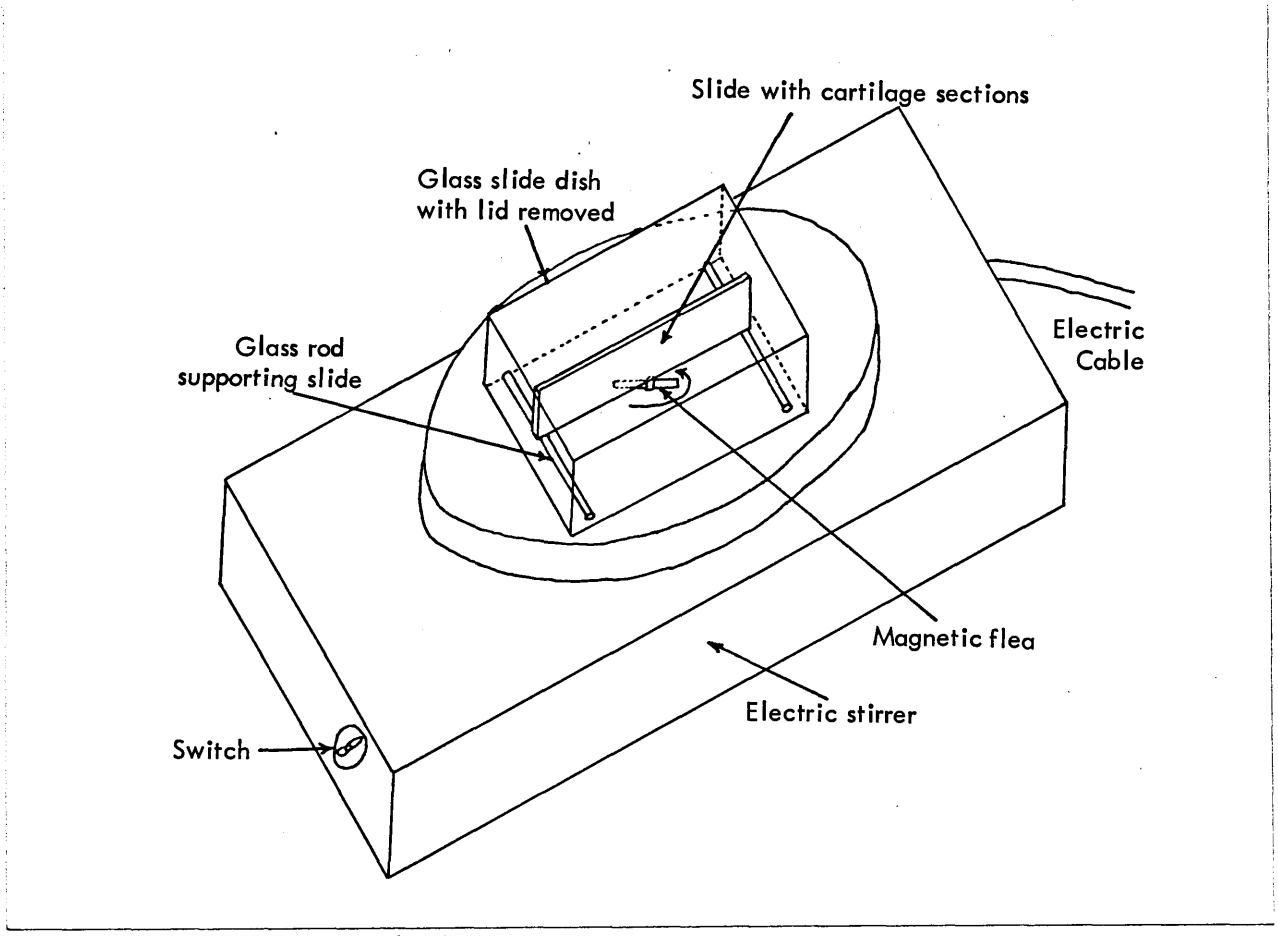
(i) 0.025M Acetate buffer pH 5.7
(ii) 0.20M Mercaptoethanol in 0.025M acetate buffer pH 5.7
(iii) 0.10M Cysteine in 0.025M acetate buffer pH 5.7
(iv) 0.01M Dithiothreitol in 0.025M acetate buffer pH 5.7
(v) 0.10M Hydrogen peroxide in 0.025M acetate buffer pH 5.7

At the end of incubations the sections were washed with 3 changes of distilled water before being stained with Alcian blue in 0.4M $MgCl_2$. Control sections, not exposed to any solution, were also stained.

The solutions of buffer, buffered cysteine, buffered dithiothreitol, and buffered mercaptoethanol were contained in Coplin jars on the bench, at room temperature. The mercaptoethanol solution was stirred by a magnetic flea. Slides in the Coplin jar were supported free of the flea in the bottom of the jar by means of short lengths of glass rod.

The hydrogen peroxide solution was contained in a 100ml capacity glass slide dish. Slides were supported on lengths of glass rod (see diagram below). The solution was stirred by a magnetic flea. It was particularly important to thoroughly stir this solution to prevent the formation of bubbles of oxygen on the surface of the sections. These would prevent the solution from coming into direct contact with the cartilage sections.

The duration of exposure of sections to respective solutions was overnight i.e. approx 16 hours.



Results

Composition	Intensity of staining (Alcian blue 0.4M $MgCl_2$) for sections
None	4+
Sodium acetate/HCl buffer pH 5.7, 0.025M	3+
Mercaptoethanol (0.2M) in 0.025M acetate buffer pH 5.7	2-3+
Dithiothreitol (0.01M) in 0.025M acetate buffer, pH 5.7	2-3+
L-cysteine HCl in 0.025M acetate buffer, pH 5.7	2-3+
Hydrogen peroxide (2 vols) in 0.025M acetate buffer pH 5.7	1+

Comment

Hydrogen peroxide caused a great loss from sections but thiol agents were relatively ineffective.

Incubation of cartilage sections with sodium periodate solutions containing propan-1-ol

Materials

Fresh frozen sections of bovine nasal septal cartilage, 6μ thick, mounted on glass slides
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Propan-1-ol (British Drug Houses)
Alcian blue in 0.40M $MgCl_2$.

Procedure

50ml aliquots of 20g/l sodium periodate solution containing respectively 0, 10, 50, 150 and 250ml/l propan-1-ol were freshly prepared. These solutions were contained in Coplin jars standing in a 30°C water bath.

Sections were immersed in the solutions for 4½ hours, and washed with 3 changes of distilled water before being stained with Alcian blue in 0.40M $MgCl_2$. Control sections, not exposed to any solution, were also stained.

In addition, some sections were exposed to 150ml/l propan-1-ol in 20g/l $NaIO_4$ overnight before being stained with Alcian blue.

Results

Test solution	Duration (hours) of exposure to test solution	Intensity of staining (Alcian blue 0.40M $MgCl_2$) for sections
20g/l $NaIO_4$ alone	4½	1+
20g/l $NaIO_4$ in 10ml/l propan-1-ol	4½	2+
20g/l $NaIO_4$ in 50ml/l propan-1-ol	4½	3+
20g/l $NaIO_4$ in 150ml/l propan-1-ol	4½	4+
20g/l $NaIO_4$ in 250ml/l propan-1-ol	4½	4+
20g/l $NaIO_4$ in ~~250~~ 150ml/l propan-1-ol	16	2+
None		4+

Comment

Propan-1-ol was effective in decreasing loss from sections over 4½ hours but less effective over 16 hours.

Comparison of rate of oxidation of glycol (pinacol) by periodic acid in water with rate in the presence of 150ml/l propan-1-ol

Introduction

The periodate ion absorbs at 222.5nm. Consequently, the rate of oxidation of a substrate can be followed by monitoring the decrease at this wavelength; provided the oxidation products do not interfere.

Aspinall and Ferrier (1957) introduced the use of a correction for the absorption due to the iodate ion produced during the oxidation of glycols.

In general, particularly for oxidation of carbohydrates, high concentrations or large excesses of periodate are to be avoided since such conditions favour over-oxidation and other non-selective oxidations.

Periodate oxidations are normally carried out in the dark since light promotes the non-selective oxidation of certain compounds.

Non-oxidisable lids or stoppers are used for periodate containing solutions i.e. not cork or rubber.

Materials

Pinacol. Anhydrous pure (4730h Koch-Light Ltd.)
Propan-1-ol (British Drug Houses)
Periodic acid. $HIO_4.2H_2O$ 50% w/w (British Drug Houses)
Iodic acid. HIO_3. (Analar, British Drug Houses)

Procedure

Extinction measurements were made with a Perkin-Elmer-Hitachi UV-Visible recording spectrophotometer, Model 124.

Control and test solutions were prepared in test-tubes covered with small glass beakers and kept in a dark cupboard, at room temperature, between readings.

Pinacol was at a concentration of 0.002 Molar and periodic acid at 0.001 Molar; in the presence and absence of 150ml/l propan-1-ol.

Aliquots were taken and diluted 1 in 10 with water. Spectra were read, immediately after dilution, against water in 1 cm silica cells.

Spectra were also recorded of propan-1-ol and of iodic acid solutions. Calculations on percentage of periodate consumed were performed on the basis of the extinction at 222.5nm. At this wavelength, iodate was found to have an extinction equivalent to 13% of the extinction of an equimolar periodate solution. This factor was used in the calculation of the results. The shape of the test spectra did not depart from the 'normal' during the course of the oxidation.

The results are expressed graphically overleaf.

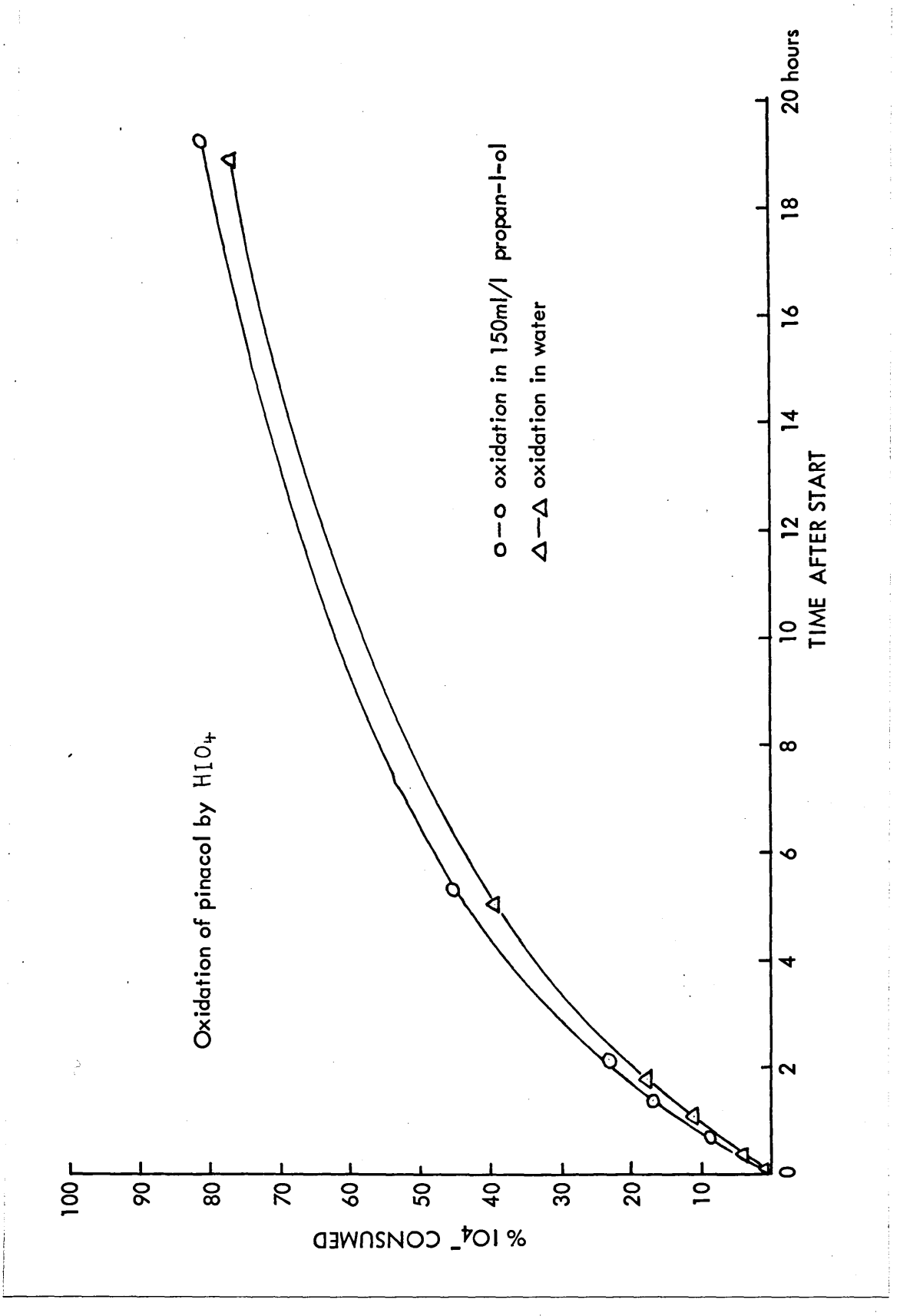

Oxidation of pinacol by HIO_4
o—o oxidation in 150ml/l propan-l-ol
Δ—Δ oxidation in water
% IO_4^- CONSUMED
TIME AFTER START
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20 hours

Figure 10.

Cetylpyridinium chloride treatment of sections

Materials

Fresh frozen sections of bovine nasal septal cartilage, 6u thick, mounted on glass slides
Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Sodium borohydride $NaBH_4$ (British Drug Houses)
Lillie's 'Cold Schiff' [4] (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses). A stock solution was stored at 4°C.
Bisulphite rinses were according to Lillie (1965)

Procedure

The procedure for the performance of the 2-step Periodic Acid Schiff (P.A.S.) reaction has already been described (p.35).

A Coplin jar was filled with 25g/l CPC solution then stood in a 30°C water bath. (CPC comes out of solution at room temperature on cold days.) CPC treatment of sections involved immersing them in this solution for 1 hour. Then sections were washed in 3 changes of distilled water.

Some cartilage sections were treated with CPC for 1 hour before being put through the normal 2-step PAS procedure. Other sections were given a primary oxidation then treated with CPC for 1 hour before continuing with the 2-step PAS procedure. At the same time control sections were given the normal 2-step PAS procedure without CPC treatment at all. This enabled the effectiveness of CPC treatment, before and after the primary oxidation, to be assessed.

It was also proposed to investigate the effect of CPC upon the staining of rapidly oxidisable substrates. For this purpose, some sections were treated with CPC for 1 hour then given a primary oxidation only before Schiff staining. Controls did not have CPC treatment before the oxidation and Schiff staining.

Results

2-step PAS procedure

CPC treatment before the primary oxidation caused a marked increase in intensity of Schiff staining for cartilage sections compared with controls that had no CPC treatment. This increase occurred particularly in the territorial regions around the chondrocytes.

Schiff staining was not much more intense than for control sections if CPC treatment was performed after the primary oxidation.

Primary oxidation only

Schiff staining after the primary oxidation was found to be diminished in intensity if CPC treatment had preceded the oxidation.

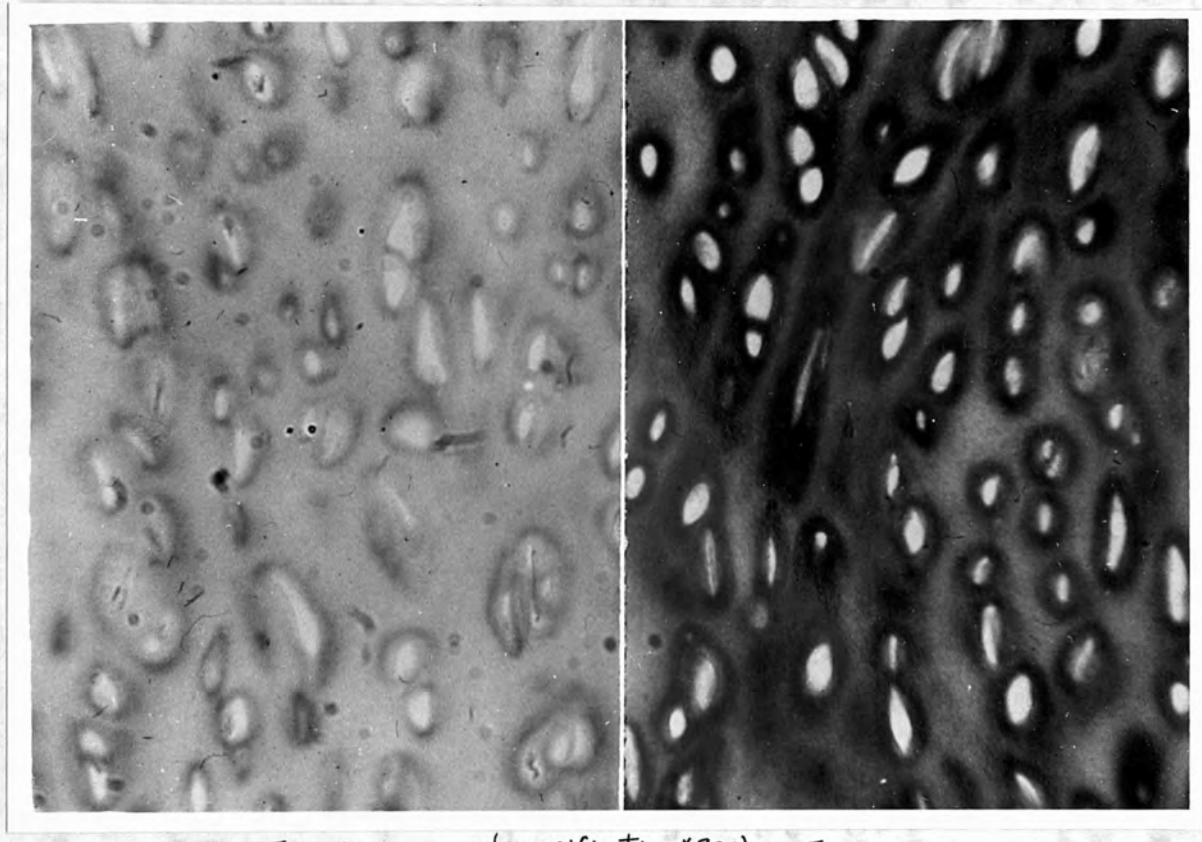


Fig.11 (magnification X300) Fig.12

Figure 11. Control section of cartilage stained, without pretreatment, by the two-stage periodate-Schiff procedure.
Figure 12. Cartilage stained as in Fig.11 but after pretreatment in 25.0g/l aqueous cetylpyridinium chloride solution

Taken from Scott, Tigwell & Sajdera (1972)

Comparison of sodium periodate and periodic acid solutions in respect of causing loss of chondroitin sulphates from cartilage sections

Materials

Fresh frozen sections of bovine nasal septal cartilage, 6μ thick, mounted on glass slides
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Periodic acid $HIO_4.2H_2O$ 50% w/w (British Drug Houses)
Alcian blue in 0.4OM $MgCl_2$

Procedure

Three Coplin jars were filled with (i) 20 g/l sodium periodate solution (ii) 0.10M periodic acid solution (iii) distilled water. They were supported in a water bath at 30°C.

Cartilage sections were immersed in these solutions for 1 hour before being washed with 3 changes of distilled water then stained with Alcian blue in 0.4OM $MgCl_2$.

Control sections, not exposed to water or aqueous solution, were also stained.

Results

Test or control solution	Control (untreated)	20g/l $NaIO_4$	0.1M HIO_4	H_2O
Intensity of staining (Alcian blue in 0.4M $MgCl_2$) for sections	4+	1+	4+	3+

Comment

No loss occurred from sections in periodic acid solution.

Treatment of cartilage sections with acid solution before exposing them to sodium periodate solution

Materials

Glacial acetic acid (Analar, British Drug Houses)
Periodic acid $HIO_4.2H_2O$ (British Drug Houses)
Orthophosphoric acid H_3PO_4 (Analar, British Drug Houses)
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
1.0M Hydrochloric acid prepared by dilution of British Drug Houses ampoule CVS containing 36.46% hydrochloric acid
Tris neutral buffer prepared using:

1.0M NaOH

Tri (Hydroxymethyl) methylamine (Puriss, Koch-Light)
Maleic acid (British Drug Houses)

Alcian blue in 0.4OM $MgCl_2$
Acetate buffer pH 5.7 of composition used in Alcian blue staining solutions
Sodium perchlorate $NaClO_4$ (Analar, British Drug Houses)
Fresh frozen sections of bovine nasal septal cartilage, 6μ thick, mounted on glass slides

Procedure

The following solutions were prepared, transferred to Coplin jars and kept at 30°C in a water bath:

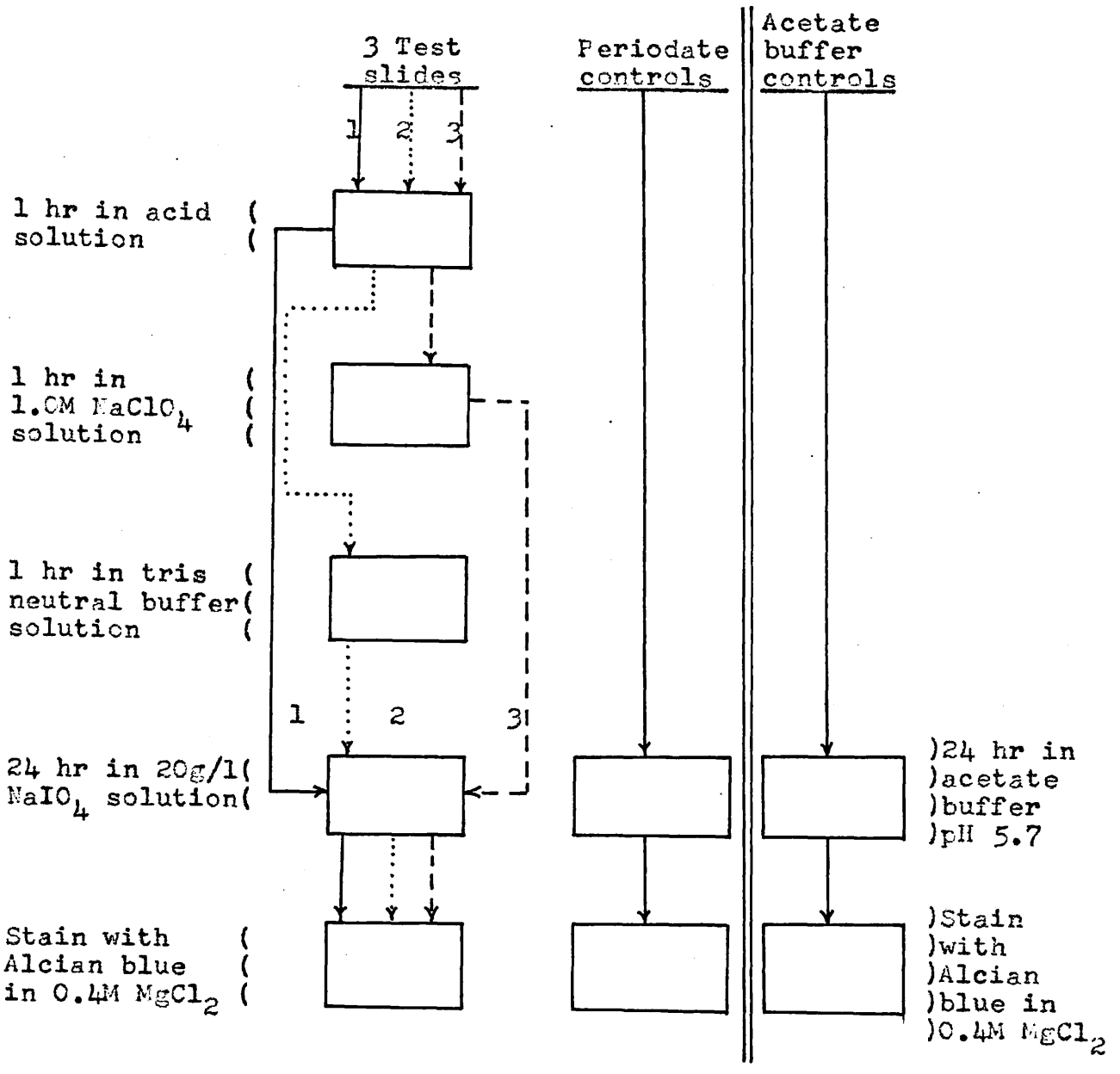
0.1M HIO_4
0.1M HCl
0.1M H_3PO_4
0.1M CH_3COOH

0.1M Tris neutral buffer (Measured pH with electrode = 6.9)
1.0M $NaClO_4$

20g/l $NaIO_4$

0.025M acetate buffer pH 5.7

The procedure adopted is illustrated in the flow diagram on the next page.



Into each 0.1M acid solution was placed 3 slides. They were left for 1 hour before being removed and washed with 3 changes of distilled water.

The slides were then put through the remainder of the scheme above. After exposure to each solution they were washed with 3 changes of distilled water.

Absolute control sections, not exposed to any solution, were stained with Alcian blue as were periodate and acetate control slides depicted in the scheme above.

Results

CONTROL SOLUTIONS

Treatment before staining	Intensity of staining (Alcian blue 0.4M $MgCl_2$) for sections
NONE	4+
24 hr in acetate buffer pH 5.7	2+
24 hr in 20g/l sodium periodate solution	½+

TEST SOLUTIONS

Acid (1 hr exposure)	Treatment before 24 hr in 20g/l sodium periodate solution	Intensity of staining (Alcian blue 0.4M $MgCl_2$) for sections
HCl	NONE	3+
	1 hr in Tris buffer(0.05M) pH 7.0	3+
	1 hr in 1.0M sodium perchlorate	3+
CH_3COOH	NONE	3+
	1 hr in Tris buffer(0.05M) pH 7.0	3+
	1 hr in 1.0M sodium perchlorate	3+
H_3PO_4	NONE	3+
	1 hr in Tris buffer(0.05M) pH 7.0	3+
	1 hr in 1.0M sodium perchlorate	3+
HIO_4	NONE	3+
	1 hr in Tris buffer(0.05M) pH 7.0	3+
	1 hr in 1.0M sodium perchlorate	3+

Comment

Treatment of sections with any of the four acid solutions produced a fixation which could not be reversed by subsequent exposure to neutral buffer solution or solution of high salt concentration.

Effect of cetylpyridinium upon rate of periodate oxidation of glycosaminoglycuronans spotted on filter paper

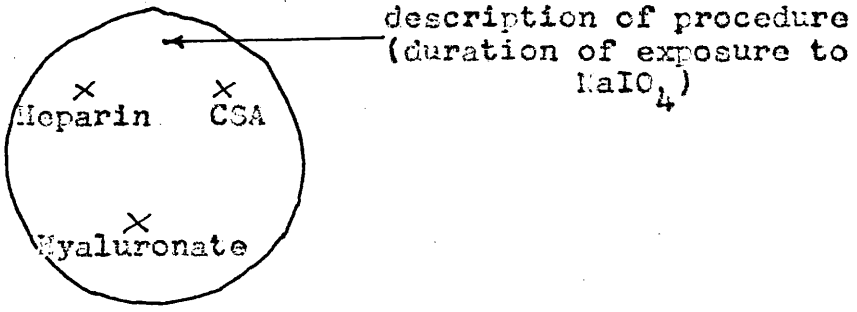
Materials

Whatman no.1 filter paper circles, 4.25 cm diameter
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Lillie's 'Cold Schiff' (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses). A stock solution was stored at 4°C.
Bisulphite rinses were according to Lillie (1965)
Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Sodium hyaluronate and sodium chondroitin-4-sulphate were prepared from human mesothelioma fluid and bovine nasal septum respectively, by the method of Scott (1960)
Mucous heparin, 5,000 I.U. in 5ml. Without preservative (Evans Medical Ltd.)

Procedure

Spots of 10g/l polysaccharide solutions were applied, from melting-point tubes, to circles of filter paper. Crosses and labels were marked on the paper with a pencil.

Sodium periodate and CPC solutions were at concentrations of 20g/l and 25g/l respectively. 50ml aliquots of reagents were contained in Coplin jars.



Five circles were prepared, to be oxidised in sodium periodate solution, at room temperature, for respectively ½, 2, 4, 8 hours and overnight. In addition, a circle was prepared as a control for Schiff staining.

All circles were soaked in CPC solution, at room temperature, for ½ hour then washed with 3 changes of distilled water before blotting between paper tissues. Circles were placed in sodium periodate solution at staggered times and removed together. They were washed with 3 changes of distilled water and blotted between paper tissues.

Finally the paper circles were stained with Schiff reagent, put through bisulphite rinses and washed in running tap water in the manner adopted in the 2-step PAS procedure for tissue sections (p.35).

Results

The control circle, stained with Schiff but not exposed to periodate, was white while the rest of the circles were red. The longer the exposure to periodate solution the more intense the staining of the paper circles as a whole. The overnight and 8 hour oxidised papers were black.

Comment

The background staining of the filter paper made it impossible to compare intensities of staining of polysaccharide spots.

Attempted preparation of fully periodate-oxidised Whatman no.1 filter paper

Materials

Whatman no.1 filter paper circles 4.25 cm diameter
Lillie's 'Cold Schiff' (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses). A stock solution was stored at 4°C.
Disulphite rinses were according to Lillie (1965)
Sodium metaperiodate $NaIO_4$. Analytical reagent grade (British Drug Houses)
Sodium borohydride $NaBH_4$ (British Drug Houses)

Procedure

20g/l sodium periodate solution was used at 30°C and 10g/l sodium borohydride solution used at room temperature.
50ml aliquots of reagents were contained in Coplin jars.
Circles of Whatman no.1 filter paper were cut into halves to provide duplicates.

It was proposed to have 3 series of duplicates.

(1) Papers oxidised for respectively 1, 2, 3, 4 hours then stained with Schiff.

(2) Papers oxidised for respectively 1, 2, 3, 4 hours reduced with sodium borohydride then stained with Schiff.

(3) Papers oxidised for respectively 1, 2, 3, 4 hours reduced with sodium borohydride, oxidised for a further 1 hour then stained with Schiff.

Duplicates were placed in sodium periodate solution at staggered times so that they were all removed together and washed with 3 changes of distilled water. They were then blotted between paper tissues.

Series (1) papers were then stained with Schiff reagent.

Series (2) and (3) papers were immersed in sodium borohydride solution for 3 minutes. Then washed with 3 changes distilled water before blotting between paper tissues.

Series (2) papers were then stained with Schiff. At the same time control duplicates, not exposed to periodate, were also stained.

Series (3) papers were given a further 1 hour in sodium periodate solution, washed and blotted before being stained with Schiff reagent.

Results

All papers, except controls, were stained red. It was apparent that even 4 hours exposure to sodium periodate solution was not long enough to completely oxidise the papers. Therefore a fully oxidised Whatman no.1 paper had not been obtained.

Development of Bromo-thymol blue staining technique for demonstration of CPC-glycosaminoglycan complexes

Choice of base

Proposed to compare use of a) Tris buffer b) sodium carbonate Na_2CO_3 and c) ammonia in the Bromo-thymol blue staining solution.

Materials

Bromo-thymol blue pH 6.0—7.6 (British Drug Houses)
100ml of 0.5g/l solutions of this dye were prepared in the presence of the three bases

Bromo-thymol blue in 0.01M Tris

0.2M Tris buffer prepared using:

Tri (Hydroxymethyl) methylamine (Puriss, Koch-Light)

0.05gm of the solid Bromo-thymol blue were weighed in a small beaker. Most of the solid was transferred to a 100ml volumetric flask. 5ml of 0.2M Tris buffer were added to the solid remaining in the beaker. The contents of the beaker were transferred to the flask, washing with distilled water from a wash-bottle. Finally the solution in the 100ml flask was made up to the mark with distilled water.

Bromo-thymol blue in 0.01M sodium carbonate

Anhydrous sodium carbonate Na_2CO_3 (Merck)

0.05gm of Bromo-thymol blue were weighed in a smallbeaker and a 0.5g/l solution prepared in a similar manner as for the Tris solution, using 5ml of 0.2M sodium carbonate solution.

Bromo-thymol blue in 0.01M ammonia

Ammonia solution sp.gr.0.88. (Analar, British Drug Houses)
Prepared a 0.2M solution by diluting 3.35ml Analar ammonia in 250ml distilled water.

0.05gm of Bromo-thymol blue were again weighed in a small beaker and a 0.5g/l solution prepared in a similar manner as with the Tris solution only this time using 5ml of 0.2M ammonia solution.

A little Bromo-thymol blue remained undissolved in each of the above solutions.

The pH of an aliquot of each of the three solutions was measured with an electrode:

Tris buffered Bromo-thymol blue	pH = 8.70
Sodium carbonate buffered Bromo-thymol blue	pH = 10.25
Ammoniacal Bromo-thymol blue	pH = 9.9

Used unfixed sections of fresh frozen bovine nasal septal cartilage 6μ thick, mounted on glass slides.

Mountant was a modified Apathy medium; composition of this is:

Gum arabic	50gm
Cane sugar	50gm
Distilled water	50ml
Thymol	0.05gm

The ingredients were dissolved with the aid of a gentle heat
The mountant sets by evaporation
5ml of this mountant were mixed with 0.20ml Analar ammonia sp.gr.0.88

50ml 25g/l CPC solution were used at 30°C in a Coplin jar.

Procedure

Sections were immersed in 25g/l CPC at 30°C for 1 hour. They were then washed with 3 changes of distilled water.

50ml aliquots of each of the three Bromo-thymol blue staining solutions were transferred to separate Coplin jars. Pairs of slides were placed in each of the Bromo-thymol blue staining solutions for 5 minutes at room temperature. Upon being removed from the staining solutions they were washed in dilute ammonia solution (few drops of Analar 88 ammonia in 500ml distilled water) before being mounted in ammoniacal apathy medium. They were then examined.

This procedure was repeated next day using the Bromo-thymol blue staining solutions which had been kept on the bench overnight.

Result

When the sections stained with Tris buffered Bromo-thymol blue were removed from their staining solution they were coloured green but changed to blue on being put in dilute ammonia wash. The sections stained in the other two Bromo-thymol blue solutions were blue on being removed from their staining solutions.

There was very little difference in appearance of sections stained with Bromo-thymol blue in the presence of the three buffers. Leaving staining solutions on the bench overnight made no difference to staining of sections. For all sections the cartilage was stained moderately intensely.

Concentration of base

Materials

Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Bromo-thymol blue pH 6.0 – 7.6 (British Drug Houses)
Anhydrous sodium carbonate Na_2CO_3 (Merck)
Sodium hyaluronate and sodium chondroitin-4-sulphate were prepared from human mesothelioma fluid and bovine nasal septum respectively, by the method of Scott (1960)
Mucous heparin, 5,000 I.U. in 5ml. Without preservative (Evans Medical Ltd.)
Sodium alginate (British Drug Houses)
Whatman 54 and Oxoid cellulose acetate papers

Procedure

Applying polysaccharide spots to paper:

Sheets of cellulose acetate and Whatman 54 paper were soaked in distilled water for 5-15 minutes, then laid on moist paper tissues. Spots of 10g/l polysaccharide solutions were applied to these sheets from melting-point tubes. (Allowed solution from 1 mm length of the melting-point tube to flow by capillarity into the paper.) Sheets were then cut into strips.

Staining polysaccharide spots on paper:

Strips were immersed in CPC solution for ½ hour at 30°C. They were separated in the Coplin jar of CPC solution by means of glass slides. Then immersed pairs of cellulose acetate and Whatman 54 paper strips in Bromo-thymol blue solutions respectively containing 0.01, 0.10 and 0.50M sodium carbonate solution. After 5 minutes exposure, washed them in dilute ammonia solution and examined. Control strips, with no polysaccharide spots, were also stained.

Results

0.01M Na_2CO_3

Cellulose acetate:

Control - Apparent staining of spots in positions in which they had not been applied.
Test - Alginate and hyaluronate stained intensely but CSA and heparin did not stain.

0.10M Na_2CO_3

Cellulose acetate:

Test - Spots of alginate and hyaluronate were intensely stained. CSA did not stain and heparin only stained faintly.

0.50M Na_2CO_3

Whatman 54:

All 4 spots stained moderately intensely.

Cellulose acetate:

Only spots for hyaluronate and alginate stained and their staining was intense.

Effect of CPC upon PAS reactivity of glycosaminoglycuronan spots on Sartorius cellulose acetate paper

Materials

Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Bromo-thymol blue pH 6.0 – 7.6 (British Drug Houses)
Anhydrous sodium carbonate Na_2CO_3 (Merck)
Sodium hyaluronate and sodium chondroitin-4-sulphate were prepared from human mesothelioma fluid and bovine nasal septum respectively, by the method of Scott (1960)
Mucous heparin, 5,000 I.U. in 5ml. Without preservative (Evans Medical Ltd.)
Sodium alginate (British Drug Houses)
Sartorius cellulose acetate paper
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
Lillie's 'Cold Schiff' (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses)
A stock solution was stored at 4°C.
Bisulphite rinses were according to Lillie (1965)

Procedure

Sheets of cellulose acetate paper were labelled using a minimal amount of pencil labelling. The sheets were soaked in distilled water for 30 minutes then excess water was blotted off, with paper tissues.

The sheets were spread over pipettes on the bench. Spots of 10g/l polysaccharide solutions were applied from melting-point tubes taking care to support the paper by pipettes so that the spot was not in contact with any surface. Then the sheets were cut into strips.

Half of the total number of strips were left in a glass jar, with a lid, containing damp tissues to maintain a moist atmosphere.

The remainder of the strips were immersed in CPC solution for ½ hour at 30°C. Upon being removed from CPC solution the strips were washed with 3 changes of distilled water then placed in a glass jar, with a lid, containing damp tissues.

Strips had been prepared to be oxidised in 20g/l sodium periodate solution at 30°C for respectively 1, 2, 4, 8 hours and overnight. The strips to be oxidised overnight were placed in sodium periodate solution straight away. The remainder were placed in this solution next day at staggered intervals so that they were all removed together.

Upon removal from periodate solution, strips were washed with 3 changes of distilled water and blotted between paper tissues before being stained with Schiff reagent or Bromo-thymol blue in the procedures described previously.

Results

Bromo-thymol blue:

All strips (1 hour - overnight oxidation) exhibited staining for each of the four polysaccharide spots.

Schiff reagent:

There was no Schiff staining of any spot. On all the CPC treated papers the four spots showed up as white discs. Background of strips was pink of increasing intensity for increasing duration of exposure to periodate solution. CPC treated strips were less pink than those not so treated.

Comment

Bromo-thymol blue staining showed that all the CPC-polysaccharide complexes stayed on the paper when exposed to periodate solution but they were not reactive to Schiff reagent following this exposure.

Reaction of Schiff reagent with pre-oxidised polygalacturonate, in solution and spotted on Sartorius cellulose acetate paper

Materials

Pre-oxidised sodium polygalacturonate prepared by R. Harbinson (Scott & Harbinson, 1969)
Sartorius cellulose acetate paper
Lillie's 'Cold Schiff' (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses)
A stock solution was stored at 4°C.
Bisulphite rinses were according to Lillie (1965)

Procedure and Results

In solution

To approximately 2ml of distilled water, in a small test-tube, were added 5 drops of 10g/l solution of oxidised polygalacturonate plus 1 drop of Schiff reagent. A rubber stopper was inserted in the test-tube and the contents mixed. A deep-red coloured solution was obtained.

As a control, 1 drop of Schiff reagent was added to 2ml distilled water in a test-tube. After insertion of a rubber stopper the contents were mixed. No colour developed apart from a very slight pink.

It was apparent that oxidised polygalacturonate, in solution, reacted with Schiff reagent giving a red-coloured product.

Cellulose acetate paper

3 crosses were pencilled on a strip of Sartorius cellulose acetate paper. The strip was placed on the surface of distilled water in a beaker. The paper wetted immediately and was pushed below the surface. It was removed 15 minutes later and excess water blotted off.

A spot of 10g/l oxidised polygalacturonate was placed on each of the three crosses using a melting-point tube.

The strip was immersed in Schiff reagent in a Coplin jar. Three pink spots appeared on the strip in this solution. The strip was removed from Schiff reagent after ½ hour and put through bisulphite rinses and the usual 10 minute tap water wash. The 3 spots became more intensely stained in tap water. The paper itself was not stained.

It was apparent that oxidised polygalacturonate could react with Schiff reagent when spotted on cellulose acetate paper.

Effect of cetylpyridinium upon reaction of Schiff reagent with pre-oxidised alginate and polygalacturonate spotted on Sartorius cellulose acetate paper

Materials

Pre-oxidised sodium polygalacturonate and pre-oxidised sodium alginate prepared by R. Harbinson (Scott & Harbinson, 1969)
Sartorius cellulose acetate paper
Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Lillie's 'Cold Schiff' (Lillie, 1965) was prepared from Basic Fuchsin and A.R. chemicals (British Drug Houses)
A stock solution was stored at 4°C.
Bisulphite rinses were according to Lillie (1965)

Procedure

Crosses were pencilled on a sheet of cellulose acetate paper before soaking it in distilled water for 15 minutes. Spots of 10g/l oxidised polysaccharide solutions were applied from melting-point tubes. The sheet was cut into two strips.

One strip was kept in a glass jar with a lid while the other strip was immersed in CPC solution for ½ hour at 30°C before being washed with 3 changes of distilled water.

The two strips were placed together in the Coplin jar of Schiff reagent, separating them by means of a glass slide. After ½ hour in Schiff reagent the strips were given bisulphite rinses and a 10 minute tap water wash before being examined.

Results

Both oxidised polysaccharides stained on the CPC treated strip. On the non-CPC treated strip only oxidised polygalacturonate stained and the spot was more diffuse than on the CPC treated strip.

The Schiff staining of CPC treated spots was a little blue compared with the true red of the non-treated spots.

Effect of CPC treatment upon PAS reactivity of acidic polysaccharides spotted on glass-fibre paper

Materials

Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Bromo-thymol blue pH 6.0 – 7.6 (British Drug Houses)
Anhydrous sodium carbonate Na_2CO_3 (Merck)
Sodium hyaluronate and sodium chondroitin-4-sulphate were prepared from human mesothelioma fluid and bovine nasal septum respectively, by the method of Scott (1960)
Mucous heparin, 5,000 I.U. in 5ml. Without preservative (Evans Medical Ltd.)
Sodium alginate (British Drug Houses)
Sodium polygalacturonate prepared by neutralising polygalacturonic acid 85% (Pract., Koch-Light) with 1.0M NaOH
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
GF/C Glass fibre paper (Whatman 15 cm circles)

Procedure

Spots of 10g/l polysaccharide solutions were applied to a sheet of glass-fibre paper, from melting-point tubes. The sheet was cut in half and one half immersed in 25g/l CPC for ½ hour at 30°C. It was then washed with 3 changes of distilled water.

The CPC and non-CPC treated sheets were cut into strips with a scalpel. Control strips were left in a glass jar with a lid while the others were immersed in 20g/l $NaIO_4$ solution at 30°C overnight. The CPC and non-CPC treated strips were kept in separate jars of $NaIO_4$ solution. Next morning, strips were removed from periodate solution, washed with 3 changes of distilled water and blotted between paper tissues. Strips were stained with Schiff reagent or Bromo-thymol blue in 0.5OM Na_2CO_3.

Results

No spots stained (Schiff or Bromo-thymol blue) on any strip (CPC or non-CPC treated) exposed to $NaIO_4$ solution.

Comparison of $NaIO_4$ and $K.NaIO_3$ solutions in respect of causing loss of CPC-polysaccharide complexes from glass-fibre paper

Materials

Cetylpyridinium chloride. 1 H_2O (Pharm.) (F.W. Berk & Co.)
Bromo-thymol blue pH 6.0–7.6 (British Drug Houses)
Anhydrous sodium carbonate Na_2CO_3 (Merck)
Sodium hyaluronate and sodium chondroitin-4-sulphate were prepared from human mesothelioma fluid and bovine nasal septum respectively, by the method of Scott (1960)
Mucous heparin, 5,000 I.U. in 5ml. Without preservative (Evans Medical Ltd.)
Sodium alginate (British Drug Houses)
Sodium polygalacturonate prepared by neutralising polygalacturonic acid 85% (Pract., Koch-Light) with 1.0M NaOH
Sodium metaperiodate $NaIO_4$ (Analar, British Drug Houses)
0.10M $K.NaIO_3$ prepared by dissolving KIO_3HIO_3 in water, adjusting to pH 5.4 with 1.0M NaOH solution using an electrode and making up to a known total volume of solution with water
GF/C Glass-fibre paper (Whatman 15 cm circles)

Procedure

Spots of 10g/l polysaccharide solutions were applied to a sheet of glass-fibre paper, from melting-point tubes. The sheet was cut into strips then immersed in 25g/l CPC for ½ hour at 30°C. Washed with 3 changes of distilled water.

The 1st strip was put aside in a glass jar with a lid. The 2nd strip was exposed to 20g/l $NaIO_4$ for 2 hours, at 30°C, and the 3rd strip to 0.10M $K.NaIO_3$ for the same period of time. Upon removal from their respective solutions, the strips were washed with 3 changes of distilled water.

Finally, the 3 strips were stained with Bromo-thymol blue in 0.5OM Na_2CO_3.

Results

Polysaccharide	Treatment before staining with Bromo-thymol blue	Intensity of staining (Bromo-thymol blue)
Chondroitin-4-sulphate	Untreated Control	4+
	2 hr in 20g/l $NaIO_4$ solution	No stain
	2 hr in 0.1M $K.NaIO_3$ solution	4+
Alginate	Untreated Control	4+
	2 hr in 20g/l $NaIO_4$ solution	½+
	2 hr in 0.1M $K.NaIO_3$ solution	4+
Heparin	Untreated Control	4+
	2 hr in 20g/l $NaIO_4$ solution	No stain
	2 hr in 0.1M $K.NaIO_3$ solution	4+
Hyaluronate	Untreated Control	4+
	2 hr in 20g/l $NaIO_4$ solution	No stain
	2 hr in 0.1M $K.NaIO_3$ solution	4+
Polygalacturonate	Untreated Control	4+
	2 hr in 20g/l $NaIO_4$ solution	No stain
	2 hr in 0.1M $K.NaIO_3$ solution	4+

Comment

Loss of polysaccharide occurred when strips were exposed to sodium periodate solution but no loss occurred when they were exposed to potassium-sodium iodate solution.

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