Histochemistry (1985) 82:249–255

Histochemistry

Microspectrophotometric quantitation of glycosaminoglycans in articular cartilage sections stained with Safranin O

I. Kiviranta *, J. Jurvelin, M. Tammi, A.-M. Säämänen, and H.J. Helminen
Department of Anatomy, University of Kuopio, P.O. Box 6, SF-70211 Kuopio, Finland

Accepted November 24, 1984

Summary. A new microspectrophotometric method was developed for quantitation of glycosaminoglycans with Safranin O dye in articular cartilage matrix. From histological sections molar extinction coefficient of Safranin O was determined and used to measure the dye content of the sections. The amount of glycosaminoglycans was determined with depth of bovine articular cartilage by both gas chromatography and thin layer chromatography to calculate the fixed negative charge content. Comparison between the results revealed that binding of Safranin O to glycosaminoglycan polyanions was stoichiometric and showed minimal nonspecific staining. The method provides an accurate technique for quantitation and localization of fixed negative charge content of glycosaminoglycans in the articular cartilage matrix. Specific enzyme digestions enable detection of separate glycosaminoglycans.

Introduction

Proteoglycans are major components of the intercellular matrix of articular cartilage and are responsible for the elasticity and resilience of the tissue (Kempson et al. 1971; Scott 1975). Proteoglycans of articular cartilage consist of a central protein core to which negatively charged glycosaminoglycans as well as oligosaccharides are covalently attached, so that carbohydrate comprises more than 90 per cent of the weight of the molecule (Muir 1982).

The histochemical demonstration of carbohydrates may be carried out by using cationic dyes, chemical methods, methods involving use of lectins or by using immunohistochemical techniques (see, e.g., Pearse 1968; Kiernan 1982). Most of these methods can also be used in conjunction with chemical blocking procedures for the reactive groups or with specific enzyme digestions of carbohydrate components. For the demonstration of cartilage proteoglycans the use of cationic dyes is a simple procedure and has traditionally been the most common method.

Schaffer (1930) and Hirsch (1944) observed that the basophilia and metachromasia of cationic dyes were less intense in the superficial zone of human articular cartilage than in the deeper zones. The qualitative analysis of metachromasia has so far been widely used as a marker of glycosaminoglycan concentration.

* To whom offprint requests should be sent

The loss of metachromatic staining during cartilage matrix breakdown has been investigated by using Toluidine Blue (Lucy et al. 1961) and Azure A (Ali 1964). In these studies correlation was found between loss of staining and decrease in the content of hexosamine and hexuronic acid of cartilage matrix. Studies on the interaction of purified glycosaminoglycans with cationic dye Azure A in solution revealed that there is a quantitative relationship between the number of dye molecules bound and the number of anionic groups per disaccharide unit (Szirmai 1963). Using microspectrophotometric method, Poole (1970) showed that linear relationship between the density of Toluidine Blue staining and the hexuronic acid content of cartilage matrix existed. However, there was not specificity between binding of the cationic dye and carbohydrate content. The staining was lost, when the hexuronic acid content of cartilage was 42% of control value.

Stockwell and Scott (1967) were the first to use a combination of histochemical and biochemical methods to show that glycosaminoglycan concentration in the human articular cartilage varied through the depth of the tissue. Since then many investigators have confirmed biochemically these results. It has been shown that the glycosaminoglycan content is highest in the deep zone of the cartilage and that there is a differential distribution of chondroitin sulphate and keratan sulphate in human articular cartilage (Bayliss et al. 1983). Consistent results have been obtained also from adult bovine (Lemperg et al. 1974) and pig cartilage (Ratcliffe et al. 1984).

For quantitation of proteoglycans the dye must be bound specifically to the substrate. If the cationic dye is used it should bind only to tissue polyanions and not to other components of the cartilage matrix. The dye molecule must also be small enough to penetrate freely into the tissue. For this reason cationic dyes of large molecular size such as Alcian Blue are not suitable for quantitation of cartilage polyanions (Goldstein and Horobin 1974). The binding of the dye should also be stoichiometric, so that concentration of the dye in the matrix would be proportional to the concentration of proteoglycans. To fulfil the last criteria the dye must be bound to polyanions in the orthochromatic form. The capacity of various glycosaminoglycans to induce metachromasia is different due to different intercharge distances in glycosaminoglycans. For this reason metachromatic staining is not proportional to the concentration of proteoglycans (Rosenberg 1971; Horobin 1982).

Safranin O is a cationic dye composed of a mixture of dimethyl phenosafranin (mw. 351) and trimethyl phenosafranin (mw. 365) (Lillie 1977). Rosenberg (1971) has shown that the interaction of Safranin O with chondroitin 6-sulphate and keratan sulphate in solution is stoichiometric. Electron microscopic studies have also revealed the high specificity of Safranin O to glycosaminoglycans (Shepard and Mitchell 1976). The aim of the present investigation was to find out whether stoichiometric dye binding occurs also in tissue sections. A test model from bovine articular cartilage was constructed to compare the results of quantitative histochemistry with the biochemical proteoglycan determinations.

We were able to show that standardized microspectrophotometric technique makes possible to quantitate Safranin O dye concentration in the cartilage sections. The content of dye molecules in the histological sections was in direct relation to the fixed negative ion content of glycosaminoglycans determined biochemically. The result justifies the use of Safranin O in quantitative determination of glycosaminoglycans in cartilage matrix.

Materials and methods

Procedure for making sections. Articular cartilage from distal femoral condyle of 1.5-year old bulls were used. From the medial condyle small slices were sawn, perpendicular to articular surface by a Leitz 1600 saw microtome (Leitz Wetzlar GmbH, Wetzlar, FRG). During sawing the tissue was kept moist by spurting cooled 0.9% (w/v) sodium chloride on the cartilage surface. The slices were fixed in 4% (w/v) formaldehyde in 0.07 mol/l sodium phosphate buffer, pH 7.0, for 48 h at 4 °C. After fixation the slices were decalcified with 10% (w/v) EDTA (ethylene diamine tetraacetic acid) and 4% (w/v) formaldehyde in 0.1 mol/l sodium phosphate buffer, pH 7.4, for 10 days at 4 °C (Kiviranta et al. 1984).

Dehydration and paraffin infiltration was carried out in a series of ethanol solutions (70%, 80%, 96% and five absolute ethanols), in two xylenes and three times (the last in vacuum) in melted Paraplast plus wax (Lancer Division of Sherwood Medical, Kildare, Ireland) for two hours in each. The sections were cut at 3 µm perpendicular to articular surface with an LKB 2218 HistoRange microtome (LKB-Produkter Ab, Bromma, Sweden) using glass-knives, and dried overnight at 37 °C.

Characterization of Safranin O staining conditions. The effect of different stain concentrations were studied by staining four series of 5 sections with Safranin O with concentrations 0.005%, 0.05%, 0.5% and 1.0%. For another four series of 5 sections the staining time with 0.5% Safranin O varied between 1 min and 2 h. The other staining conditions were as described below.

A special series of sections with thicknesses 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 µm were cut to study the relationship between the section thickness and absorbance of the stained sections.

To evaluate the effect of pH on the staining pattern, 0.5% (w/v) Safranin O solutions were prepared in sodium acetate buffer (Walpole) at pH 1.0, 2.6, 4.0, 5.2 and 7.4 (Drury and Wallington 1980). For each pH value five sections were analysed.

Standard staining procedure. The sections were deparaffinized in xylene and hydrated in a graded series of ethanol solutions to distilled water. The staining was carried out for 10 min with 0.5% (w/v) Safranin O (Lot 713466; Fisher Scientific Co., Fair Lawn, NJ, USA) prepared in 0.1 mol/l sodium acetate buffer at pH 4.6. The sections were dehydrated twice in 95% ethanol solution and once in absolute ethanol, cleared in xylene and mounted in D.P.X. mounting medium (Difco, West Molesey, Surrey, UK).

Digestions with chondroitinase ABC. Prior to the staining procedures some sections were subjected to digestion with chondroitinase ABC (Seikagaku Kogyo Co., Tokyo, Japan) using the method described in detail by Yamada et al. (1982). The incubation of the sections was carried out for 4 h at 37 °C in a solution prepared in 0.1 mol/l Tris-HCl buffer pH adjusted to 8.0 containing enzyme activity of 1.5 units/ml, 100 µg bovine serum albumin and 4 proteinase inhibitors (10 mmol/l N-ethylmaleimide, 5 mmol/l phenylmethanesulphonyl fluoride (PMSF) and 0.36 mmol/l pepstatin (Sigma Chemical Co., St. Louis, MO, USA) and 10 mmol/l EDTA (Merck, Darmstadt, FRG)).

Microspectrophotometric analysis. The analysis system consisted of a Leitz MPV 3 microspectrophotometer interfaced to a Hewlett-Packard 85 A microcomputer (Hewlett-Packard, Corvallis, OR, USA). The data processing was performed in a VAX 11/780 computer (Digital Equipment Corporation, Maynard, MA, USA). The light source XBO 150 W high pressure Xenon lamp (Osram, FRG) was used with a grating monochromator in the illuminating side with spectral bandwidth of 6.6 nm. During analysis the Orthoplan microscope was equipped with an EF 63 × /0.85 objective and an L40 × /0.65 condenser. The diameters of the field diaphragm and the measuring diaphragm were 34 µm and 0.8 µm, respectively. Spot measurements in two scan lines were performed from articular surface to bone. The distance between individual spot measurements and the two scan lines was 12.5 µm. The background values were determined outside the section preceeding each analysis. During the scan the readings equal to the background values were rejected. The transmittance values were fed into the microcomputer, corrected for the dark signal of the photomultiplier and converted into absorbance values. All the measurements were made twice from exactly the same points with different amplification of the photomultiplier during each analysis. This procedure widened the linear measuring range of the photometer up to 3.6 extinction units. The calibration curve of the photometer system with neutral density filters is shown in Fig. 1. The filters were purchased either from Leitz or from Schott (Mainz, FRG). From the desired number of measurements along with the scan line the mean value of absorbance was determined to represent each layer. It was also possible to sum up the values of the successive layers. This integrated absorbance characterized the staining properties of the whole section.

The measurement of Safranin O in tissue sections. 2.5 mm × 4.0 mm sections were cut parallel to the cartilage surface at microtome settings of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 µm. Conditions of tissue processing and the staining was the same as described above. Some sections were stained with 0.001% Safranin O to obtain weaker staining. The mean absorbance was calculated from 600 measurements/section. After photometric analysis the section area was measured with a computer-coupled graphical analyzer (Summagraphics ID, Summagraphics Corp., Fairfield, CT, USA). Subsequently the coverslip was removed and the part of the slide containing the section was cut away and placed in a test tube. The dye was eluted from the section by shaking it in a known volume (0.6 ml) of 2 mol/l HCl prepared in 50% (v/v) ethanol. After centrifugation (2,000 × *g*, 10 min) absorbance of the solution was measured in a Perkin-Elmer Spectrophotometer 139 (Hitachi Ltd, Tokyo, Japan) at absorption maximum 535 nm. Appropriate standards were used to calculate the amount of Safranin O/mm^2 of the section. Safranin O solution obeys the Beer-Lambert law. The measurement must be performed at once because the dye loses its colour within 24 h.

Combined histochemical and biochemical analyses. Perpendicular slices with dimensions of 1 mm × 4 mm and 2.5 mm × 4 mm were consecutively sawn from femoral condyles (Fig. 2). For gas chromatographic analysis, 6 tissue blocks were frozen with isopentane cooled to −160 °C with liquid nitrogen and stored at −80 °C. 100 µm thick cartilage strips were cut from the blocks with a PMV Cryo-Microtome type 450 MP (PMV, Stockholm, Sweden) at

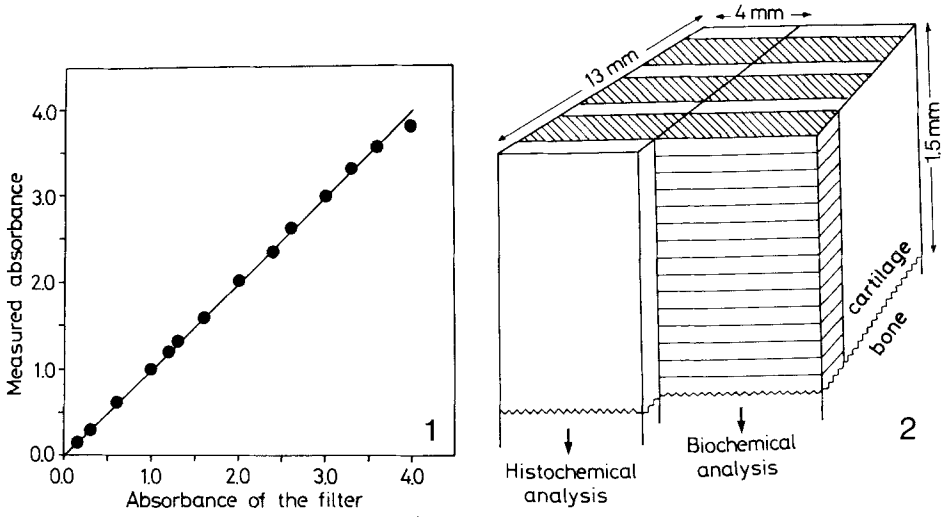


Fig. 1. The relationship between the measured absorbance at 500 nm wavelength and the known absorbance of the neutral density filter

Fig. 2. Cartilage preparation. Slices from bovine articular cartilage were sawn alternately for histology and biochemical proteoglycan analyses. For biochemical analyses 100 µm thick cartilage strips were cut parallel to the articular surface

−20 °C parallel to the articular surface. For thin layer chromatographic analysis an equal set of tissue blocks was prepared.

The strips were digested in a solution containing 5 mmol/l cysteine-HCl, 5 mmol/l EDTA and 25 µg/ml papain (2× crystallized, Sigma) at pH 6.5 for 6 h at 60 °C.

For gas chromatographic analysis of monosaccharides, glycosaminoglycans were precipitated in 1 ml of ethanol and 20 µl of 25% (w/v) sodium acetate at 4 °C overnight. The pellet was collected by centrifugation (2,500× *g*, 20 min) and dissolved in water. An aliquot of the solution was hydrolyzed in 2 mol/l HCl for 17 h at 103 °C. The acid was evaporated by air stream. Myoinositol (1 µg) and alfa-methylmannoside (0.5 µg) were added as internal standards before derivatization into alditol acetates (Torrello et al. 1980). The separation and quantitation of galactosamine and glucosamine was achieved in a silica capillary column (0.32 mm × 25 m) coated with OV-1701 (Orion Analytical, Mankkaa, Finland) using a Carlo Erba 4160 chromatograph (Carlo Erba Strumentazione, Milan, Italy) with the on column injection system.

For thin layer chromatographic separation and quantitation of the disaccharides derived from different chondroitin sulphate isomers and hyaluronic acid, papain digested samples were precipitated in 1% (w/v) cetylpyridinium chloride containing 0.02 mol/l sodium chloride at 20 °C for 3 h. The sample was centrifugated (2,500× *g*, 20 min) and the pellet washed with water, dissolved in a small volume of n-propanol and reprecipitated in ethanol containing 0.5% (w/v) sodium acetate. The precipitate was digested with chondroitinase AC II. The resulting disaccharides were determined by thin layer chromatography as described in detail earlier (Säämänen and Tammi 1984).

For histochemical quantitation one scan analysis was performed/section. During analysis 16 measurements (8 steps and 2 scan lines) were performed from articular surface to bone to get absorbance values for each of the 100 µm thick cartilage layers. Three sections from each of the 8 tissue blocks were analysed. Using the molar extinction coefficient Safranin O content in each layer was calculated for comparison with the biochemical analyses.

Results

Characterization of cartilage staining with Safranin O

It appeared that staining times from 1 min to 2 h gave almost similar staining. Safranin O concentration 0.5% (w/v) was sufficient for maximum staining of the sections (Table 1). Absorption spectrum of Safranin O in cartilage sections is shown in Fig. 3. The single-peaked absorption maximum is at 500 nm, which means that Safranin O dye occurs in its orthochromatic form in the cartilage sections. Within

Table 1. Characteristics of Safranin O staining of articular cartilage matrix. The effect of different stain concentrations and staining times

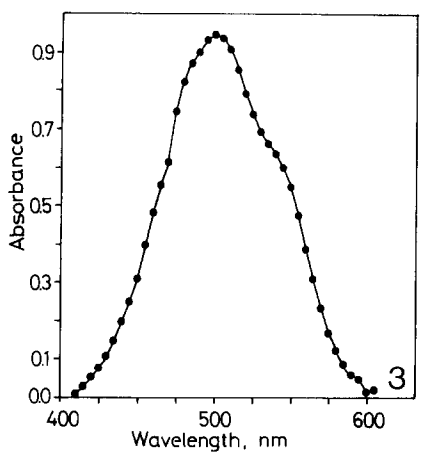
Safranin O conc.	Staining time	Integrated absorbance at 500 nm
% (w/v)	min	mean ± SEM ($N=5$)
0.005	10	48.21 ± 1.42
0.05	10	764.99 ± 84.22
0.5	10	871.52 ± 35.47
1.0	10	834.07 ± 65.65
0.5	1	958.70 ± 53.62
0.5	10	957.39 ± 14.89
0.5	30	1,033.20 ± 47.22
0.5	120	1,006.03 ± 56.22

a period of one month the microspectrophotometric analysis showed no fading of the dye or change in the absorption spectrum.

The pH values of the staining solution was altered and its effect on the staining intensity was tested. Dye binding of the cartilage matrix enhanced as the pH of the staining solution increased from 1.0 to 5.2. Thereafter the stainability was about constant (Fig. 4). This kind of a response was probably due to the electrostatic nature of the staining mechanism (Horobin 1982).

Reproducibility of the quantitation system was evaluated by preparing two series of 24 cartilage sections from the same 8 tissue blocks. The sections were cut, stained and analysed independently. Starting from the surface 13 comparable absorbance values were determined from the superficial cartilage layers to the deep cartilage zone. The difference between the values was 4.4% ± 1.9% (mean ± SD). The correlation between the analyses of the two series was highly significant ($r=1.0$, $P<0.001$, $N=13$).

A linear relationship was obtained between the mean absorbance of the section and the microtome setting (Fig. 5). To evaluate the range of the section thickness cut at a certain microtome setting 17 pairs of sections were photometrically analysed. The difference in absorbances of two adjacent sections was thought to reflect the change in the section thickness. The range of absorbances in pairs of sections was 5.0% ± 0.6% (mean ± SEM, $N=17$).



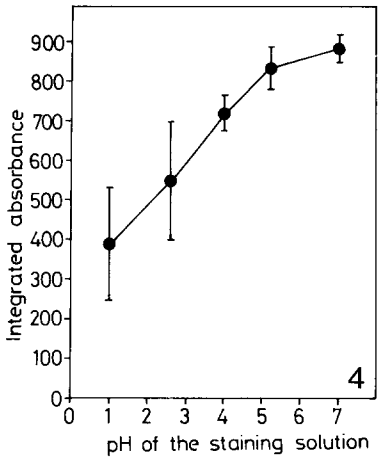
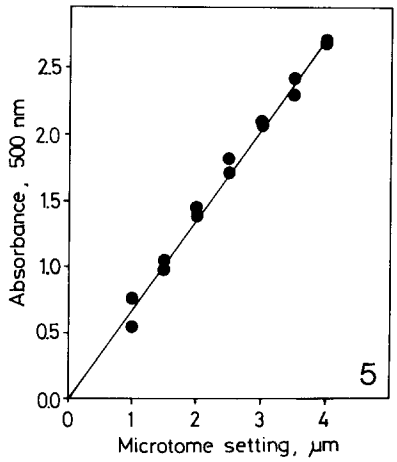


Fig. 3. Spectral analysis of Safranin O stained cartilage section

Fig. 4. The effect of pH on the staining properties of articular cartilage matrix. Absorbance was measured at 500 nm. Mean ± SEM, $N=5$



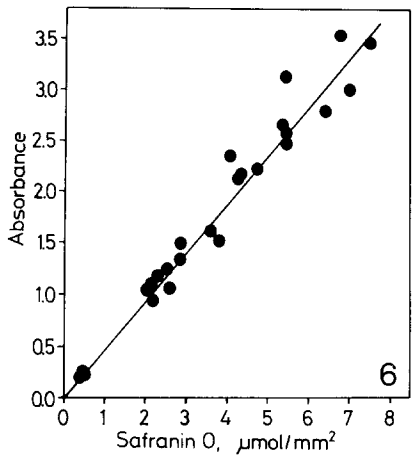


Fig. 5. The mean absorbance of the Safranin O stained sections at 500 nm plotted against the microtome setting (thickness of the sections)

Fig. 6. The correlation between the mean absorbance value at 500 nm of the Safranin O stained sections and the eluted dye content per mm^2 of the same sections

The microspectrophotometric determination of Safranin O

The basis of spectrophotometry is the Beer-Lambert law (see, e.g., Slayter 1970), which relates concentration (c) of the chromophore to the measured extinction (E):

$$E=kcl, \quad (1)$$

where k is a molar extinction coefficient for the chromophore at particular wavelength and l is the path-length of the light through the specimen. In microspectrophotometry the mass of the chromophore per unit area of the section can be determined (see, e.g., Butcher 1972; Bitensky 1980). Since concentration equals mass (m) divided by volume (V) and volume is equal to surface area (A) multiplied by path-length, the Eq. (1) can be written as follows:

$$E=k\frac{m}{A}. \quad (2)$$

Equation (2) was used to calculate molar extinction coefficient of Safranin O. Linear relationship was found between the mean absorbance of the section and the dye content per unit area (Fig. 6). In two separate experiments molar extinction coefficients of 0.442 and 0.475 were obtained. The mean of these values 0.459 was then used to determine the mass of Safranin O/mm^2 of the section. Knowing the section thickness the result was converted to amount of the dye/mm^3 of the section. This made comparison between histological and biochemical analyses possible.

Combined histochemical and biochemical analyses

The galactosamine and glucosamine contents of cartilage reflect the relative proportions of chondroitin and keratan sulphate chains, respectively. Hyaluronic acid also contributes to the glucosamine content, but it is a minor component as compared with other glycosaminoglycans of cartilage as shown in Table 2. The mean proportion of hyaluronic acid of total disaccharides was 2.9%. The slight amount of mannose in gas chromatographic determinations (result not shown) revealed that a negligible amount of glucosamine was derived from N-glycosidic oligosaccharides (cf. Hascall and Hascall 1980). The distribution of galactosamine and glucosamine through depth of the cartilage is shown in Table 3. Galactosamine content increased to an 8-fold value from superficial to deep zone. The glucosamine content was more constant through the cartilage thickness. The total fixed negative ion content of articular cartilage was calculated from chondroitin sulphate and keratan sulphate amount of the 100 µm thick cartilage strips. Chondroitin sulphates carry two negatively charged groups per repeating disaccharide unit, whereas keratan sulphate, hyaluronic acid and nonsulphated chondroitin have only one negatively charged group per disaccharide.

Histochemical quantitation of Safranin O was made from areas corresponding the biochemical determinations. Safranin O distribution in cartilage sections was compara-

Table 2. Relative proportions of unsulphated chondroitin (Δ Di-0S) and hyaluronic acid (HA) in 100 μm thick cartilage strips of bovine articular cartilage from surface (1) to bone junction (15). (Δ Di-4S = chondroitin 4-sulphate, Δ Di-6S = chondroitin 6-sulphate, CS = Δ Di-0S + Δ Di-4S + Δ Di-6S). Mean of 5–6 measurements

Fraction number	$\frac{\Delta \text{ Di-0S}}{\text{CS+HA}}$	$\frac{\text{HA}}{\text{CS+HA}}$
1	0.21	0.03
2	0.24	0.06
3	0.19	0.03
4	0.13	0.02
5	0.12	0.03
6	0.12	0.03
7	0.06	0.07
8	0.07	0.04
9	0.07	0.01
10	0.08	0.02
11	0.09	0.02
12	0.08	0.01
13	0.03	0.03
14	0.05	0.02
15	0.17	0.02

Table 3. Distribution of galactosamine and glucosamine in bovine articular cartilage in successive 100 μm thick layers from surface to bone. Mean of 5–6 measurements ± SEM

Fraction number	Galactosamine [nmol/mm³]	Glucosamine [nmol/mm³]
1	23.5± 4.2	12.2±2.0
2	35.7± 9.6	15.7±4.5
3	68.5± 9.7	21.3±3.3
4	88.8± 8.9	28.7±2.5
5	94.4± 8.9	26.3±4.9
6	104.1±11.9	32.2±3.3
7	81.7± 6.3	24.6±3.1
8	112.5±15.2	32.9±4.4
9	132.9± 8.2	34.2±2.5
10	122.2±12.7	42.0±8.7
11	143.3±12.3	23.5±3.0
12	167.4±19.4	29.0±2.4
13	197.4±20.2	27.1±4.2
14	199.8±19.4	27.9±4.4
15	141.1±28.8	21.6±3.6

ble to the biochemically determined fixed negative ion content (Fig. 7). The total fixed charge content increased from 90 nmol/mm³ in the surface to 400 nmol/mm³ near the bone. The coefficient of correlation between biochemical and histochemical determinations was $r=0.96$ ($P<0.001$, $N=14$).

The binding specificity of Safranin O dye to articular cartilage proteoglycans was tested digesting the sections with chondroitinase ABC and staining with Safranin O at the same time with adjacent control sections. The sections incubated in the digestion medium without enzyme had the same dye binding properties as the control sections. The superficial 1 mm thick cartilage zone was analysed. The loss of stain from the sections due to chondroitinase ABC digestion was equal to the amount of fixed negative ions calculated on the basis of the corresponding chondroitin

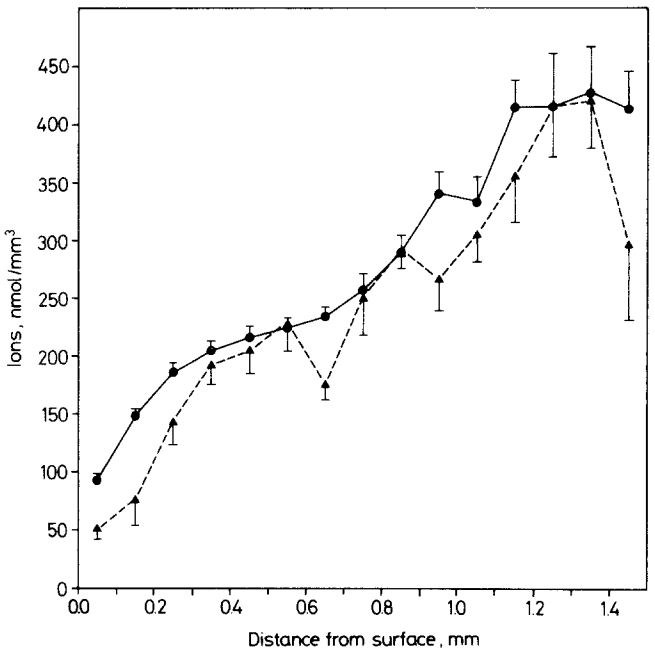


Fig. 7. Distribution of fixed negative ions of glycosaminoglycans (▲) in cartilage and the photometrically measured Safranin O content (●) of cartilage sections. Mean ± SEM, $N=24$

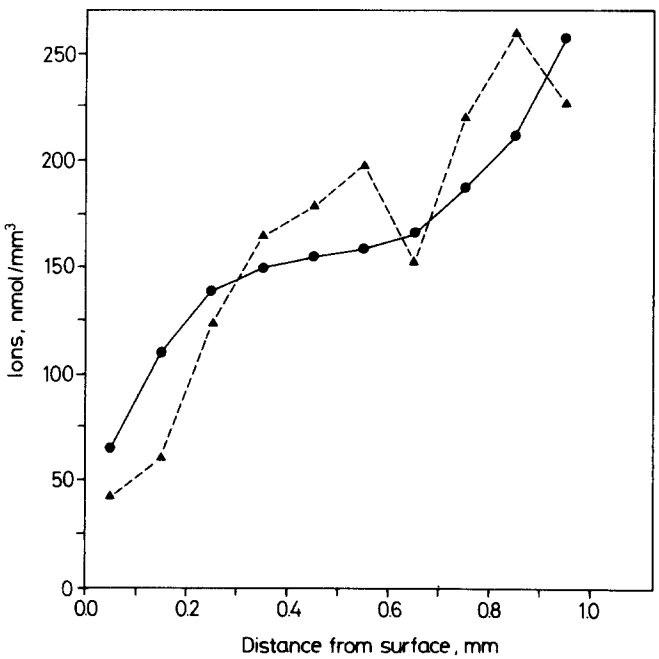


Fig. 8. Distribution of fixed negative ions calculated from the biochemically analysed chondroitin sulphate content in articular cartilage (▲) and the reduction of Safranin O stain content due to chondroitinase ABC digestion (●)

sulphate determinations (Fig. 8). These results (Figs. 7 and 8) confirm that Safranin O is bound in a stoichiometric way to fixed negative ions of glycosaminoglycans.

The amount of non-specific staining could be estimated by calculating the surplus of Safranin O in the sections after chondroitinase ABC digestion as compared to glucosamine content (representing keratan sulphate, Table 3). The proportion of surplus staining of the total dye binding was

11.2% ± 1.3% (mean of 10 determinations ± SEM). This surplus may exist due to dye binding to other substrates than fixed negative ions of glycosaminoglycans or it expresses oversulphation of keratan sulphate (Muir 1980, p 35).

Discussion

Succesful histochemical quantitation of proteoglycans implies that during fixation and tissue processing loss of carbohydrate components is minimal. For this reason some of the conventionally used fixation and tissue processing methods are not suitable. Methods described in a previous study (Kiviranta et al. 1984) were used to preserve cartilage during histological tissue processing.

Since electrostatic forces bind dye molecules to anionic sites of disaccharides, the amount of the ionized charge groups in disaccharides depends on the pH of the staining solution. Intrinsic pK values for carboxyl groups of hyaluronic acid and chondroitin sulphate have been between 2.8 and 3.3 (Grodzinsky 1983). The differences are ascribed to electrostatic interactions. The pK of sulphate groups is lower, being about 1.0 (Horobin 1982). Szirmai (1963) has observed that electrostatic dye binding occurs when cationic dye is mixed with glycosaminoglycans in solution. The Azure A binding to chondroitin sulphate steeply increased to a two-fold value as the pH of the solution increased from 1.0 to 5.0. On the basis of the known pK values it can be calculated that at pH 1.0 half of the sulphate groups of glycosaminoglycans are ionized whereas at pH 4.6 practically all sulphate and carboxyl groups are ionized and can bind the dye. The effects of pH of the staining solution on cartilage staining confirmed that the dye binding occurs due to electrostatic forces also in tissue sections (Fig. 4). The observations are in accordance with the investigations using a tracer cation method to study the effects of pH on the fixed charge density of cartilage (Maroudas 1980).

The dye molecule must be small enough to penetrate into the cartilage tissue and to reach the negatively charged groups. The intercharge distances of chondroitin sulphate and keratan sulphate are about 0.5 nm and 1.0 nm, respectively (Chakrabarti and Park 1980). The short intercharge distances make a requirement on the molecular size of the dye, when one dye molecule is wanted to get bound to each negatively charged group of disaccharides.

When dye molecules are packed close to each other by electrostatic forces, dye-dye interaction occurs, which may induce some dyes to show metachromatic staining. Metachromasia of some cationic dyes disables their use for histochemical quantitation of glycosaminoglycans, since the dye cannot be quantitated reliably if it has two absorption maxima (Rosenberg 1971). The absorption spectrum of the dye in section must be single-peaked to enable the quantitation of the dye.

The molar extinction coefficient of Safranin O was determined in order to quantitate the dye content from the sections. For this reason sections of bovine joint cartilage cut parallel to articular surface were used. The dye is evenly distributed in the matrix of the whole section. The matrix is the predominant tissue component, only 1–10% of the volume of cartilage is occupied by cells (Stockwell 1979). The absorbance of Safranin O, measured by spectrophotometer was correlated with content of the dye in the same section as measured by elution and spectrophotometry. This comparison gave a linear relationship (Fig. 6), i.e., the dye obeys the Beer-Lambert law and the extinction coefficient could be determined.

Proteoglycan analysis of sequential slices starting from cartilage surface to bone junction enabled us to compare the local Safranin O content with the amount of fixed negative ions. The dye content was practically equal to the fixed negative charge content of cartilage matrix. As the fixed negative ion concentration increased along with depth of the cartilage, the Safranin O concentration increased as well (Fig. 7). The binding of Safranin O to glycosaminoglycan polyanions of articular cartilage is stoichiometric; one molecule of the dye is bound to each of the negatively charged groups of glycosaminoglycans.

Digestions with specific enzymes, i.e., chondroitinases and keratanase (Yamada et al. 1982), provide therefore possibilities to quantitate local changes of chondroitin sulphate and keratan sulphate in cartilage. Chondroitinase ABC digestion was found to reduce the dye concentration of the sections about by the amount of fixed negative ions occurring in chondroitin sulphate disaccharides (Fig. 8). The enzyme makes possible to quantitate local changes of chondroitin sulphate in cartilage matrix. Technical difficulties with our keratanase preparation prevented us from using that enzyme to quantitate directly keratan sulphate content. The staining after chondroitinase ABC digestion should reflect the amount of keratan sulphate. The sections were, however, observed to contain more stain than one sulphate group/keratan sulphate disaccharide could bind. The reasons for this surplus staining might be that Safranin O as a cationic dye is bound also to other negatively charged groups than those of glycosaminoglycans. Glutamic (pK 4.3) and aspartic (pK 3.7) acids of collagen are partly ionized in the pH of the staining solution (pH 4.6), and possibly bind the dye. On the other hand keratan sulphate disaccharides may themselves bind more than one dye molecule since keratan sulphate disaccharides containing more than one sulphate group are known to occur in articular cartilage (Hjertquist and Lemperg 1972).

Acknowledgements. The authors wish to thank Mrs. Eija Voutilainen, Mrs. Elma Sorsa, Ms. Arja Venäläinen and Mrs. Arja Voutilainen for skilful technical assistance. The financial support of the Emil Aaltonen Foundation; The Duodecim Foundation; The Yrjö Jahnsson Foundation; The North Savo Fund of The Finnish Cultural Foundation; The Finnish Research Council for Physical Education and Sports, Ministry of Education; and The Medical Research Council of the Finnish Academy is acknowledged.

References

Ali SY (1964) The degradation of cartilage matrix by an intracellular protease. Biochem J 93:611–618

Bayliss MT, Venn M, Maroudas A, Ali SY (1983) Structure of proteoglycans from different layers of human articular cartilage. Biochem J 209:387–400

Bitensky L (1980) Microdensitometry. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation symposium 73 (new series). Excerpta Medica, Amsterdam Oxford New York, pp 181–202

Butcher RG (1972) Precise cytochemical measurement of neotetrazolium formazan by scanning and integrating microdensitometry. Histochemie 32:171–190

Chakrabarti B, Park JW (1980) Glycosaminoglycans: structure and interactions. CRC Crit Rev Biochem 8:255–313

Drury RAB, Wallington EA (1980) Carleton's histological tech-

nique. Oxford University Press, Oxford New York Toronto, pp 496–497
Goldstein DJ, Horobin RW (1974) Surface staining of cartilage by Alcian Blue, with reference to the role of microscopic dye aggregates in histological staining. Histochem J 6:175–184
Grodzinsky AJ (1983) Electromechanical and physicochemical properties of connective tissue. CRC Crit Rev Biomed Eng 9:133–199
Hascall VC, Hascall GK (1980) Proteoglycans. In: Hay ED (ed) Cell biology of extracellular matrix. Plenum Press, New York London, pp 39–63
Hirsch C (1944) A contribution to the pathogenesis of chondromalacia of the patella. Acta Chir Scand (Suppl) 83:1–106
Hjertquist SO, Lemperg R (1972) Identification and concentration of the glycosaminoglycans of human articular cartilage in relation to age and osteoarthrosis. Calcif Tissue Res 10:223–237
Horobin RW (1982) Histochemistry. An explanatory outline of histochemistry and biophysical staining. Gustav Fisher, Stuttgart New York; Butterworths, London
Kempson GE, Spivey CJ, Swanson SAV, Freeman MAR (1971) Patterns of cartilage stiffness on normal and degenerate human femoral heads. J Biomech 4:597–609
Kiernan JA (1982) Histological and histochemical methods: Theory and practice. Pergamon Press, Oxford New York
Kiviranta I, Tammi M, Jurvelin J, Säämänen A-M, Helminen HJ (1984) Fixation, decalcification, and tissue processing effects on articular cartilage proteoglycans. Histochemistry 80:569–573
Lemperg R, Larsson S-E, Hjertquist S-O (1974) The glycosaminoglycans of bovine articular cartilage. I. Concentration and distribution in different layers in relation to age. Calcif Tissue Res 15:237–251
Lillie RD (1977) Biological stains. Williams and Wilkins, Baltimore
Lucy JA, Dingle JT, Fell HB (1961) Studies on the mode of action of excess of vitamin A. 2. A possible role of intracellular proteases in the degradation of cartilage matrix. Biochem J 79:500–508
Maroudas A (1980) Physical chemistry of articular cartilage and the intervertebral disc. In: Sokoloff L (ed) The joints and synovial fluid, vol II. Academic Press, New York London, pp 239–291
Muir H (1980) The chemistry of the ground substance of joint cartilage. In: Sokoloff L (ed) The joints and synovial fluid, vol II. Academic Press, New York London, pp 27–94
Muir H (1982) Proteoglycans as organizers of the intercellular matrix. Biochem Soc Trans 11:613–622
Pearse AGE (1968) Histochemistry. Theoretical and applied, vol 1. Churchill, London, pp 295–380
Poole AR (1970) The relationship between Toluidine Blue staining and hexuronic acid content of cartilage matrix. Histochem J 2:425–430
Ratcliffe A, Fryer PR, Hardingham TE (1984) The distribution of aggregating proteoglycans in articular cartilage: Comparison of quantitative immunoelectron microscopy with radioimmunoassay and biochemical analysis. J Histochem Cytochem 32:193–201
Rosenberg L (1971) Chemical basis for the histological use of Safranin O in the study of articular cartilage. J Bone Joint Surg 53-A:69–82
Schaffer J (1930) Die Stützgewebe. In: von Möllendorff W (ed) Handbuch der Microskopischen Anatomie des Menschen, vol 2. Menschen. Springer, Berlin
Scott JE (1975) Physiological function and chemical composition of pericellular proteoglycan (an evolutionary view). Philos Trans R Soc B 271:235–242
Shepard N, Mitchell N (1976) The localization of proteoglycan by light and electron microscopy using Safranin O. J Ultrastruct Res 54:451–460
Slayter EM(1970) Optical methods in biology. Whiley-Interscience, New York London, pp 528–551
Stockwell RA (1979) Biology of cartilage cells. Cambridge University Press, Cambridge London New York, p 74
Stockwell RA, Scott JE (1967) Distribution of acid glycosaminoglycans in human articular cartilage. Nature 215:1376–1378
Szirmai JA (1963) Quantitative approaches in the histochemistry of mucopolysaccharides. J Histochem Cytochem 11:24–34
Säämänen A-M, Tammi M (1984) Determination of unsaturated glycosaminoglycan disaccharides by spectrophotometry on thin-layer chromatographic plates. Anal Biochem 140:354–359
Torrello LA, Yates AJ, Thompson DK (1980) Critical study of the alditol acetate method for quantitating small quantities of hexoses and hexosamines in gangliosides. J Chromatogr 202:195–209
Yamada K, Fujita Y, Shimizu S (1982) The effect of digestion with keratanase (Pseudomonas sp.) on certain histochemical reactions for glycosaminoglycans in cartilaginous and corneal tissues. Histochem J 14:897–910