Glycosaminoglycan concentration in synovium and other tissues of rabbit knee in relation to synovial hydraulic resistance

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1. The hydraulic resistance of the synovial lining of a joint, a key coupling coefficient in synovial fluid turnover, is thought to depend on the concentration of biopolymers (glycosaminoglycans (GAGs) and collagen) in the synovial intercellular spaces, because these polymers create hydraulic drag. The primary aim of this study was to obtain microscopically separated, milligram samples of the very thin synovium from eight rabbit knees, and to analyse these quantitatively for GAGs (chondroitin sulphate, heparan sulphate and hyaluronan) and collagen to allow comparison with published hydraulic resistance data. Synovial fluid and femoral cartilage were also studied.

2. Synovium comprised 73 ± 3% water by weight (mean ± S.E.M.). Of the 270 mg solid per gram of wet tissue, protein formed 136 mg (by automated amino acid analysis), and of this 94 mg was collagen by hydroxyproline analysis. From the collagen mass and fibril volume fraction (0·153 of tissue by morphometry), fibrillar specific volume was calculated to be 1·43 ml per gram of molecular collagen, and fibril water content 47% by volume.

3. The concentration of chondroitin 4-sulphate (C4S) plus chondroitin 6-sulphate (C6S), measured by capillary zone electrophoresis was 0·55 mg per gram of synovium – much greater than in synovial fluid (0·04 mg g^{-1}) and much less than in cartilage (27·8 mg g^{-1}). The C4S/C6S ratio in synovium (7·3) differed from that in cartilage (0·7), indicating that different proteoglycans predominated in synovium. The heparan sulphate concentration, assayed by radioactive Ruthenium Red binding, was 0·92 mg per gram of synovium (synovial fluid, 0·08 mg g^{-1}; cartilage, 0·72 mg g^{-1}).

4. In contrast to sulphated GAGs, the hyaluronan concentration was highest in synovial fluid (3·53 mg g^{-1}; biotinylated G1 domain binding assay). The concentration in synovial interstitium was only 0·56 mg g^{-1} (corrected for interstitial volume fraction, 0·66), even though there is open contact between synovial interstitium and synovial fluid. This may be due to exclusion or washout.

5. Total GAG mass was ~4 mg per gram of synovial interstitium. A model of trans-synovial flow indicated that a uniform GAG concentration of 4 mg g^{-1} is less than 1/3rd of that required to explain the experimental estimate of synovial hydraulic resistance. Errors in the resistance estimate do not appear to be large enough to resolve the problem. It is possible, therefore, that additional polymeric material in the interstitium, such as glycoproteins and proteoglycan core protein, may contribute to the hydraulic resistance.

Synovial joints are lined by a thin sheet of specialized mesenchymal tissue called synovium, synovial intima or synovial lining. This cellular, well-vascularized sheet is only 10–20 μm thick in the rabbit. Its primary roles are the production of the lubricating synovial fluid and the delivery of nutrients to the avascular articular cartilage. These roles require the transport of water, electrolytes, nutrients and plasma proteins into the joint cavity from capillaries just below the synovial surface, and the drainage of fluid, metabolic end-products, proteins, lubricants and cartilage degradation products from the joint cavity into the sub-synovium, which is a less cellular zone of connective tissue

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and lymph vessels. Transport of the lipophobic materials occurs chiefly by flow and diffusion through the extracellular matrix (ECM) between the synovial lining cells, which are ~67% specialized fibroblast-derived cells and ~33% tissue macrophages. The cells are almost devoid of intercellular junctions, being separated by irregular gaps 2 μm or more wide at the interface with the joint cavity. The ECM in these intercellular spaces is the final common pathway through which all lipophobic exchange with the joint cavity occurs. As well as conferring permeability, however, the ECM must also offer sufficient outflow resistance to retain the vital synovial fluid within the joint cavity. The biochemical composition of synovial ECM is thus physiologically important.

Theoretical and experimental work shows that in general the resistance of a unit cube of non-epithelial tissue (cells and interstitium) to transport depends on three factors (Levick, 1987): (a) the fraction of the tissue volume that is obstructed by cells (and other non-ECM structures like blood vessels); (b) the fraction of the ECM volume that is obstructed by collagen fibrils; and (c) the concentration of biopolymers in the ECM. The latter consist of proteoglycans (sulphated glycosaminoglycans (GAGs) bound to core protein), hyaluronan (a non-sulphated GAG) and the structural glycoproteins. Together these polymers finely subdivide the interstitial water space and generate considerable hydraulic drag. Regarding (a), cells and vessels together occupy 34% of tissue volume in rabbit synovium (ECM, 66%; Price, Mason & Levick, 1995). Regarding (b), the fibril-forming collagens, types I, III and V, are abundant in synovium and occupy 21·4% of the ECM volume (Price *et al.* 1995). 'Minor' collagens are also present, namely microfibril-forming type VI collagen superficially (Okada *et al.* 1990; Ashhurst, Bland & Levick, 1991) and network-forming type IV collagen around fibroblast-derived lining cells (Pollock, Lalor & Revell, 1990). Collagen raises hydraulic resistance by multiple mechanisms, namely creation of hydraulic drag, reduction of cross-sectional area available for transport, imposition of pathway tortuosity, and exclusion of other biopolymers from part of the synovial interstitial space. The last effect, a 21·4% exclusion in synovial ECM, raises the effective concentration of other matrix biopolymers (mass per unit *available* volume), causing a disproportionately large rise in transport resistance. Regarding (c), there is little quantitative information (see Discussion). Since points (a) and (b) were recently evaluated for rabbit synovium, estimation of GAG concentration as mass per unit interstitial extrafibrillar volume (the functionally important parameter) became feasible from biochemical analysis of whole tissue (mass per unit tissue volume).

This study had two objectives. The first was to provide quantitative biochemical data that might explain the resistance of normal synovium to fluid transport. Rabbit knee synovium was used because its hydraulic permeability and structure are known quantitatively. The second objective was to establish control values for biopolymer concentrations in normal synovium as a preliminary to comparison with saline-perfused synovium at raised, pathological intra-articular pressures. In the latter case the hydraulic resistance is markedly reduced (Levick, 1979, 1984). Although stretch undoubtedly contributes to this via increased surface area and reduced thickness, this does not provide a full explanation, which led to the inference that a fall in ECM biopolymer concentration also occurs (Levick, 1991).

Concerning the first objective, modelling work has indicated that to account quantitatively for normal synovial hydraulic resisitivity there should be an aggregate mass of around 13·9 mg of long-chain biopolymer per millilitre of interstitial extrafibrillar space (Levick, 1994). In interstitia such as cartilage and Wharton's jelly, the biopolymers principally responsible for hydraulic resistance are the GAGs chondroitin sulphate and hyaluronan. Immunohistochemical studies confirm the existence of hyaluronan, chondroitin 4- and 6-sulphate-bearing proteoglycans, and heparan sulphate in human synovium (e.g. Wells, Klareskog, Lindblad & Laurent, 1992; Fish & Revell, 1992; Worrall, Wilkinson, Bayliss & Edwards, 1994). These GAGs were therefore assayed quantitatively here, along with collagen. The results, corrected for volume of distribution, are related to synovial hydraulic resistivity in the Discussion. A study of synovial GAG concentration after intra-articular distension by pathological pressures is presented in the companion paper (Price, Levick & Mason, 1996). Some of the findings have been reported in abstract form (Price, Levick & Mason, 1994).

METHODS

Methods were developed to harvest selectively the extremely thin synovial lining and to assay its chondroitin 4-sulphate (C4S) and chondroitin 6-sulphate (C6S) content (by capillary zone electrophoresis), other sulphated GAGs (by radioactive Ruthenium Red assay), hyaluronan (by G1 domain binding assay), collagen (via hydroxyproline analysis), total amino acid (automated analyser), DNA (Hoechst 33258 assay), water content (via dry weight) and density (density gradient method). Samples of the other major joint tissues, namely cartilage, synovial fluid and subsynovium, were also taken to establish the concentration profiles within the joint.

Harvesting and weighing synovium

The microharvesting method was described in detail by Price *et al.* (1995). Briefly, synovium was microdissected from normal knees of eight freshly killed New Zealand White rabbits weighing 2–3 kg. Microdissection avoided contamination of the sample by non-synovial periarticular tissues, which can form most of a sample excised by gross dissection. Animals were killed by an intravenous overdose of sodium pentobarbitone (Euthatal; May & Baker Ltd, Dagenham, UK) via the marginal ear vein. Incisions parallel to and across the patellar ligament gave access to the anterior compartment of the joint cavity. After flushing the cavity gently with isotonic saline the animal was transferred into a polythene-draped chamber whose relative humidity was maintained at $\geqslant 95\%$ (measured by a wet–dry bulb thermohygrometer) by an utrasonic cool-vapour humidifier to prevent drying during dissection. The lateral and medial walls of the anterior compartment were then

retracted by weighted bulldog clips to expose the areolar synovium of the suprapatellar pouch (the largest synovial compartment in the knee). The surface was rinsed briefly with isotonic saline to remove synovial fluid, then gently blotted with fine filter paper (Whatman No. 50) under ×6 magnification (Zeiss OpMi-1 dissecting microscope) to remove surface liquid prior to dissection.

The synovial lining was gently microdissected from the underlying areolar subsynovium at ×16 magnification using ultrafine ophthalmic microdissection instruments (John Weiss Ltd, London, UK). A small incision was made in the synovium overlying the quadriceps muscle with a 1·5 mm goniotomy knife and the intima was separated from subsynovium (areolar connective tissue in the suprapatellar region) by blunt dissection with a Troutman cyclodialysis spatula or blunted Lang synechia knife. The sample was then excised with Roboz microdissection scissors. Each microdissection took roughly 10 min. Control studies showed that the inbuilt illuminating lamp of the dissecting microscope caused no detectable heating of the tissue at the working distance of ~20 cm. A total of ~1 cm^2 of synovial intima was obtained from the lateral and medial sides of one knee in each animal. The opposite knee was used to study the effects of raised intra-articular pressure, as described in the companion paper (Price *et al.* 1996).

Sampling other joint material

Synovial fluid was aspirated from the knees, ankles, shoulders and elbows of ten rabbits using the procedure of Knox, Levick & McDonald (1988). Because aspiratable volumes are small, aspirates were pooled to produce five 10 μl samples from the knee and four from each other category of joint. The fluid was weighed and stored at −80 °C. Synovial fluid density was taken to be 1·02 g ml^{-1} (Knox *et al.* 1988). Articular cartilage shavings (10–15 mg per animal) were taken from the medial and lateral femoral condyles by scalpel at the end of the experiment after opening up the humidity tent (to gain better access) and transecting the cruciate and other ligaments. Subsynovium, comprising a thin layer of areolar connective tissue, was difficult to collect, being formless, and for reasons that will emerge later it is thought that the mechanical forces involved in its collection by microdissection forceps may have reduced its water content (see Discussion).

Wet weight of sample

Tissue was placed in a sealed vial and immediately frozen in a Dewar vacuum flask packed with solid CO_2. The frozen sample was transferred to a pre-weighed vial and weighed on a Mettler AE 240 analytical balance (sensitivity, 0·01 mg; sample weight, ~1 mg).

Density

The density of synovium was measured so that weight and volume could be interconverted. A xylene–chlorobenzene density gradient of range 1·106–0·985 g ml^{-1} was prepared in a 500 ml measuring cylinder using a graded, vertical stirring protocol. The gradient was calibrated by measuring the equilibrium positions of drops of glucose solution of known density (concentration, 0–24 g (100 ml deionized water)$^{-1}$; density range, 1·00–1·10 g ml^{-1} at 20 °C, checked by specific gravity flask). The resolution of the gradient was 4 cm vertical distance per (10^{-3} g ml^{-1}). Tissue density was determined from the equilibrium position of the sample.

Wet/dry weight ratios and control for condensation

Synovium was harvested under 95% humidity to avoid dessication. This level of humidity produced a fine mist in the chamber, however, and microscopically visible deposition of mist droplets on the sample (and possibly incomplete removal of the initial synovial wash by blotting) increased the sample weight modestly. To measure this error, wet/dry weight ratios were determined on synovial samples harvested under two different conditions from knees of six rabbits. Samples from the right knee were harvested at 95% humidity (misting) as above. The humidifiers were then turned down, the mist allowed to clear, the left knee opened and synovium harvested without a saline flush (but with a gentle blot by Whatman No. 50 filter paper to remove endogenous synovial fluid). The mist-free chamber was maintained at ⩾ 85% humidity during work on the left knee. Samples from the left and right joints were immediately frozen and weighed as above, then freeze dried in an Edwards Super Modulo unit (Edwards High Vacuum International, Crawley, UK) for 64 h and reweighed.

Extraction prior to biochemical analysis

Sulphated GAGs were first split from proteoglycan by NaOH (100 μl 0·5 M NaOH per milligram of sample, agitated overnight at 4 °C), which cleaves the β-*O*-glycosidic bond linking sulphated GAGs to proteoglycan core protein ('β-elimination'). The NaOH was then removed by minidialysis against water in inverted Eppendorf tubes (48 h, 4 °C; dialysis membrane cut-off, 3500 Da; Spectrum Medical Industries, Los Angeles, CA, USA). After centrifugation the supernatant was freeze dried and redissolved in 25 μl deionized water for analysis of C4S, C6S, heparan sulphate and hyaluronan content (see below). The remaining tissue pellet was digested with papain solution, viz. 2 mg papain (Sigma) per millilitre of 0·1 M sodium acetate, using 90 μl solution per milligram of original tissue. After 24 h at 60 °C the papain was inactivated by 10 μl 100 mM iodoacetic acid and the digest analysed for hydroyproline and other amino acids, and for any residual GAGs. Control experiments confirmed that β-elimination had fully extracted chondroitin sulphate from the sample, leaving no measurable amount in synovial pellets, and leaving < 0·3% of the total mass in the cartilage pellets.

Synovial fluid was digested with papain (40 μl papain solution per 5 μl synovial fluid) before hyaluronan analysis in order to degrade any aggrecan (hyaluronan-binding proteoglycan from cartilage) that might otherwise interfere with the hyaluronan assay. After incubation the papain was inactivated with 5 μl 100 mM iodoacetic acid.

Capillary zone electrophoresis for chondroitin sulphate

Electrophoresis along very fine silica capillary tubes (i.d., 50 μm) under high potential differences enables rapid, well-resolved separation of charged solutes and highly sensitive, on-line detection using absorbance, which is measured continuously through the capillary wall. Only tiny (nanolitre) aliquots from microlitre samples are used, with vacuum-loaded volume variations of < 2%. The resolving power of the method is an order of magnitude greater than high-performance liquid chromatography. Standard solutions of disaccharides of C4S and C6S (Seikagaku, Tokyo, supplied by ICN Flow, High Wycombe, UK) were used to calibrate the Applied Biosystems 270A capillary electrophoresis system (Applied Biosystems, Risley, Warrington, UK). Although these disaccharides differ only in the position of the sulphate group on the *N*-acetyl galactosamine residue, they were readily separated.

Endogenous chondroitin sulphate was degraded to disaccharides by adding 5 μl chondroitinase ABC solution (0·05 mU μl^{-1} in 200 mM Tris base at pH 7·3) to 5 μl tissue supernatant (see earlier) and incubating at 37 °C for 1 h. Cartilage supernatant was diluted further because of its high C6S content. Solutions were filtered through a 0·2 μm Millipore filter (to prevent capillary blockage), loaded at the cathode for 10 s at 3·4 nl s^{-1} and electrophoresed at 15 kV and 40 °C, with 0·2 M orthophosphoric acid as the preloaded running buffer (pH 3; to eliminate electro-osmotic flow). Ultraviolet absorbance was monitored at 232 nm. The disaccharide peaks on

the electrophoretogram were identified by migration time, and identification was confirmed occasionally by adding standard to a sample already analysed and re-running the mixture (see Results). To calibrate the system, standard C4S and C6S disaccharide solutions containing the same concentration of chondroitinase ABC as the sample were loaded and the area under the peak (measured by an on-line integrator) was plotted as a function of applied mass or concentration. As little as 18 pg disaccharide could be measured.

The above identification procedure did not exclude the possibility that some of the material comprised the closely related dermatan sulphates; chromatographic evidence for the latter in synovial villi of canine knees, at ~1/5th the concentration of hyaluronan, was reported briefly by Myers & Fife (1989). Dermatan sulphate is very similar in structure to chondroitin sulphate except for the epimerization of some of the hexuronate (glucuronate) groups to iduronate. Some of the iduronate-bearing disaccharides, however, can carry two sulphate groups, and since no disulphated disaccharides were detected, the assayed material will be referred to as chrondroitin sulphate hereafter.

^{103}Ruthenium Red assay for heparan sulphate

Heparan sulphate is a known constituent of synovium (Fish & Revell, 1992). Here the quantitative dot-blot assay of Gaffen, Price, Bayliss & Mason (1994) was used. Selective enzymic digestion with chondroitinase ABC was used to prevent interference by chondroitin or dermatan sulphate; the enzyme degrades chondroitin and dermatan sulphates but not heparan or keratan sulphates (being specific for galactosaminoglycan chains). The disaccharide digestion products were removed by minidialysis. The retentate was freeze dried, redissolved to 5 μl in 0·2 M calcium acetate (pH 7·0) and three 1 μl spots applied to a calcium acetate membrane (dry, to limit spot diffusion), along with triplicate spots of standard solutions (below). The dried spots were stained with a radioactive cationic dye, ^{103}Ruthenium Red solution (see below). Ruthenium Red cations were displaced from non-sulphate anionic groups on DNA, RNA and hyaluronan (which otherwise interfere with the assay) by staining the spots in 0·04 M $MgCl_2$. Control studies showed that in the presence of 0·04 M $MgCl_2$ Ruthenium Red still binds to heparan sulphate, and also chondroitin and dermatan sulphates. In this study, the last two were removed by digestion and dialysis (see above and Fig. 1). Ruthenium Red binding to keratan sulphate is 1/20th of that to heparan sulphate and 1/10th of that to chondroitin sulphate, which may be in part because of the replacement of the charged uronic acid groups by neutral galactose in keratan sulphate.

^{103}Ruthenium, a high energy β- and γ-emitter (half-life, 39·6 days), was obtained as $^{103}RuCl_3$ (2 mCi mg^{-1}; Amersham, UK). The work space was protected by a 1 in lead shield. Ruthenium Red, $[(NH_3)_5\text{-Ru-}O\text{-Ru-}(NH_3)_4\text{-}O\text{-Ru}(NH_3)_5]^{6+}\text{-}6Cl^-$, was prepared from $RuCl_3$ as described by Carlson (1982). Four millilitres of the product (~120 μCi ml^{-1}) were made up to 8 ml in 0·04 M $MgCl_2$ and 12 ml 95% ethanol was added; the solution was then filtered. Loaded acetate membranes were immersed in the staining solution for 15 min at room temperature, washed in 0·04 M $MgCl_2$–ethanol (1/1) for 1·5 h with six to eight changes, rinsed in 95% ethanol and dried flat. Autoradiography film (Hyperfilm MP; Amersham) was exposed to the dry membrane at −70 °C for one or more days (according to radioisotope activity), developed and scanned in a Joyce Loebel Chromoscan 3 laser densitometer.

Attempts to confirm the identity of the stained non-chondroitin, non-dermatan GAG, by degradation using nitrous acid or heparinase, were inconclusive because these treatments failed to abolish completely the staining of either standard heparan sulphate or sample. The material stained at 0·125 M $MgCl_2$, however, and this is characteristic of heparan sulphate but not chondroitin/dermatan sulphate as shown in Fig. 1.

Selection of standard for heparan sulphate assay

Calibration curves were constructed by densitometry of stained spots of standard heparan sulphate solutions in doubling dilutions (2·5–40 μg ml^{-1}). The standards were prepared via the same steps as the samples, i.e. chrondoitinase treated, minidialysed, freeze dried and reconstituted. In selecting the standard, account was taken of the heterogeneity of heparan sulphates. Bovine renal and intestinal mucosal heparan sulphates (heterogeneous; Sigma) gave similar calibration curves, whereas a highly sulphated subfraction

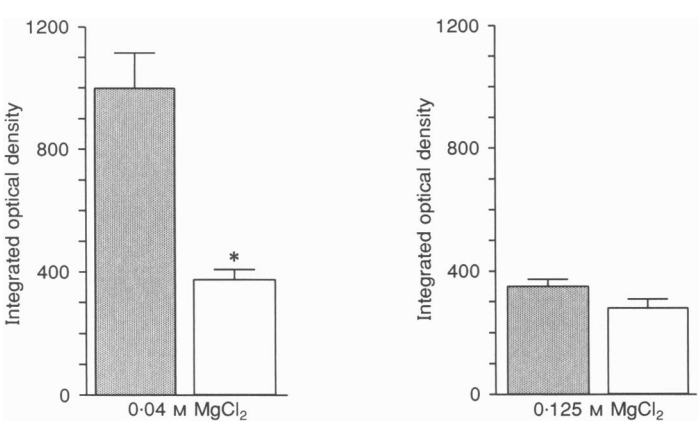

Figure 1. Ruthenium Red staining for sulphated GAGs using enzymic digestion and critical electrolyte concentration ($MgCl_2$)

Open columns represent tissue digested by chondroitinase ABC to remove chondroitin/dermatan sulphate; shaded columns represent untreated tissue (means with S.E.M. bars; $n = 5$). At 0·04 M $MgCl_2$ (left), chondroitinase reduces but does not eliminate staining (* $P = 0·01$, paired t test). Raising the electrolyte concentration (right; 0·125 M) is known to eliminate staining for chondroitin sulphate but not for heparan sulphate; the chondroitinase-treated and untreated samples no longer differ in staining density ($P = 0·36$), confirming the presence of heparan sulphate.

(type IIa; Williams & Mason, 1991) gave a steeper curve. As there was no reason to select a purified subfraction as standard, and taking into account the fact that native heparan sulphates are usually heterogeneous in charge and molecular weight, the bovine renal cortex heparan sulphate containing heparan sulphates from basement membrane, mesangium and cell membranes was adopted as standard. Adoption of this standard was supported by the finding that doubling dilutions of the non-chondroitin sulphated GAG from rabbit articular cartilage could be fitted to the standard curve and gave an estimate of 0·717 ± 0·110 mg heparan sulphate g^{-1}; this corresponded to a cartilage heparan/chondroitin sulphate ratio of 1/39 (see Results), which is similar to the ratio produced by cultured chondrocytes (R. M. Mason, unpublished results).

Hyaluronan

A sensitive absorption assay based on the binding of hyaluronan by the biotinylated G1 domain of aggrecan (a gift from Dr M. Bayliss, Kennedy Institute, London, UK) was used (Fosang, Hay, Carney & Hardingham, 1990). Both post-β-hydroxylation supernatant and papain-digested pellet were analysed but it was found that no detectable hyaluronan remained in the synovial (cf. cartilage) pellets after the β-elimination phase, perhaps because the alkaline pH disrupts protein structures involved in hyaluronan–ligand binding. Diluted material was applied to 96-well microtitre plates at 100 μl per well, each pre-coated with 5 μg human umbilical cord hyaluronan (Sigma); non-specific sites were blocked by 10 g l^{-1} bovine serum albumin. Hyaluronan standards of concentration 0·5–1000 ng (100 μl)$^{-1}$ were also applied. Biotinylated G1 domain (0·1 μg in 100 μl) was added to each well and after overnight incubation the plate was washed. The amount of biotinylated G1 remaining bound in the well was measured by the streptavidin–horseradish peroxidase reaction with 2,2-azino-bis(3-ethyl benzthiazoline-6-sulphonic acid) diammonium salt as peroxidase substrate. Well optical density was read at 405 nm using a Titertek Multiscan MCC/340 microplate reader (Life Sciences International, Basingstoke, UK). The standard curve for umbilical cord hyaluronan showed that the percentage inhibition of G1 binding to the well was a linear function of $\log_{10}$ hyaluronan concentration in the range 5–40 μg ml^{-1}.

Amino acid analysis

Collagen content was calculated from hydroxyproline content, and total tissue protein was estimated by summation of amino acids. Aliquots (10 μl) of the material digested by papain to polypeptides were acid hydrolysed to amino acids by 500 μl 6 N HCl for 24 h at 110 °C. The sample was vacuum dried and redissolved in loading buffer prior to application to the ion-exchange column of an amino acid analyser (LKB Biochrom 4151 Alpha Plus). Analysis was automatic, using standard amino acids from LKB Pharmacia. The imino acid hydroxyproline elutes before aspartic acid and is assayed by the yellow product formed on heating with ninhydrin (absorbance at 440 nm). The content of triple-helical collagen was calculated as hydroxyproline mass × 7·3 (Venn, Mehta & Mason, 1983). Ultrastructural work shows that the fibrillar (triple helix) collagens occupy 21·4% of interstitial volume (Price *et al.* 1995), whereas type VI microfibrils occupy only 1·7–3·4% of local interstitial volume, even near the surface where they are densest (Levick & McDonald, 1990). Thus, although the hydroxyproline ratio may differ for a minor collagen (in type VI collagen the triple helix has non-helix terminal domains), this is unlikely to create a substantial error in total collagen estimation.

Total protein mass was estimated by summing the amino acids in hydrolysates of five tissue samples digested by proteinase K, with correction for the amino acid content of the added proteinase K (analysed separately after acid hydrolysis).

DNA content was analysed to provide a biochemical measure of tissue cellularity. The method of Labarca & Paigen (1980) was used, based on enhanced fluorescence of Hoescht 33258 upon binding to DNA, which was liberated by digestion with proteinase K (Oegema, Carpenter & Thompson, 1984). DNA standard was supplied by Sigma (type 1, sodium salt). Fluorescence was measured in a Perkin-Elmer LS50 fluorimeter at an excitation wavelength of 356 nm and an emission wavelength of 45 nm.

Statistical analysis

Results are given as means ± S.E.M., unless otherwise indicated. The non-parametric Wilcoxon test was used for comparison of paired ratios or percentages, and Student's t test for other paired or unpaired results. Multiple comparison was by analysis of variance (ANOVA).

Terminology: hydraulic conductance, hydraulic conductivity and specific hydraulic conductivity

These three, different quantities are as defined previously (Levick, 1987). Briefly, 'conductance' is what is measured during a physiological experiment, namely change in flow of a stated liquid per unit change in pressure drop across the entire membrane; units are $length^3$ $time^{-1}$ $pressure^{-1}$. This is cited in the units actually measured (cm^3 min^{-1} cmH_2O^{-1}). Conductance depends on membrane area and thickness as well as the intrinsic permeability of the material and the viscosity of the permeating fluid. 'Hydraulic conductivity' is a more fundamental property, the conductance of unit area of membrane of unit thickness; its units are $length^4$ $time^{-1}$ $force^{-1}$ (here cm^4 s^{-1} dyn^{-1}). Its size depends only on the intrinsic permeability of the material and the viscosity of the fluid. It is the parameter calculated by combining the physiological estimate of conductance with anatomical measurements of membrane area and thickness. 'Specific hydraulic conductivity' (κ) is the most fundamental parameter, being hydraulic conductivity times viscosity. It represents the basic material property, independent of dimensions or perfusate viscosity, and its units are $length^2$ (cm^2). The terms resistance, hydraulic resistivity and specific hydraulic resistivity are the reciprocals of conductance, hydraulic conductivity and specific conductivity, respectively.

RESULTS

Histological examination of cross-sections of the microdissected joint lining showed that synovium and not subsynovium was harvested selectively by microdissection as intended (Fig. 2). In an ultrastructural study of eleven such microdissected synovia, the thickness of the dissected tissue (12·2 ± 0·9 μm; Price *et al.* 1995) was no greater than that of synovium *in situ* and much less than the thickness of areolar subsynovium (83 ± 6 μm; Levick & McDonald, 1989).

Density

The density of microdissected synovial samples was 1·073 ± 0·005 g ml^{-1} ($n = 4$).

Water content of synovium

Mean water content, calculated as 100 × (wet weight − dry weight)/wet weight, was 80·2 ± 2% (range, 76·0–90·2%) for synovium dissected under 95% (misting) humidity. The water content of tissue dissected under non-misting, ⩾ 85%

humidity from the opposite knee of the same animal was significantly lower, namely 72·7 ± 3% (range, 69·8–84·2%; $P = 0·03$, Wilcoxon test; $n = 6$). The latter value closely matches the dry weight of 28% indicated by the results of Balazs & Denlinger (1985). The higher water content under misting humidity was due to deposition of surface water; at ×16 magnification, microscopic mist droplets were seen to settle on the surface during dissection. This extraneous water accounted for 0·275 by weight of the harvested material, with synovial tissue forming 0·725 of the sample weight. Since all the synovial tissue for biochemical analysis was harvested under 95% humidity, concentrations are cited throughout the Results section first as actually measured and then corrected for extraneous water by the factor 1·38 (i.e. 1/0·725).

Chondroitin sulphates

Although seven different chondroitin sulphate disaccharides are known to exist, only two were found in synovium, namely the sulphated hexosamine disaccharides C4S and C6S (Fig. 3). C4S predominated in a ratio of 7·3 (±0·5) to 1 (range, 4·1–9·1). A small third peak on the electrophoretogram for synovium, subsynovium and some synovial fluids but not cartilage was not identified. No disulphated disaccharides such as are associated with dermatan sulphate were detected, although dermatan sulphate can be secreted by cultured periarticular cells (Fife & Myers, 1985) and has been reported in periarticular tissues (Akeson, Woo, Amiel, Coutts & Daniel, 1973; Myers & Fife, 1989).

The C4S concentration in *synovium* was 0·345 ± 0·031 mg (g sample)$^{-1}$ at 95% humidity ($n = 8$; range, 0·278–0·536 mg g^{-1}), while the C6S concentration was 15% of this, namely 0·053 ± 0·011 mg g^{-1}. There was a statistically significant correlation between the concentration of the two disaccharides (correlation coefficient, 0·90; $P = 0·003$), described by the linear regression equation:

$$[C6S] = (0·323 \pm 0·063)[C4S] - (0·059 \pm 0·022),$$

where the concentration is in milligrams per gram of sample (Fig. 4). The extrapolated intercept for C4S at zero C6S, namely 0·18 mg g^{-1}, was significantly different from zero ($P = 0·03$); this could indicate the presence of at least two proteoglycan species, one of which carries chiefly C4S, but it should be noted that the intercept is greatly influenced by the highest pair of values. Total chondroitin sulphate concentration averaged 0·397 ± 0·042 mg (g sample)$^{-1}$, with a wide range (0·315–0·665 mg g^{-1}; values shown in Fig. 4). This corresponded to a mean of 0·547 mg (g synovium)$^{-1}$ after correction for extraneous water (i.e. sample concentration × 1/0·725). Allowing for the tissue density of 1·073 g ml^{-1} and an interstitial volume fraction of 0·66

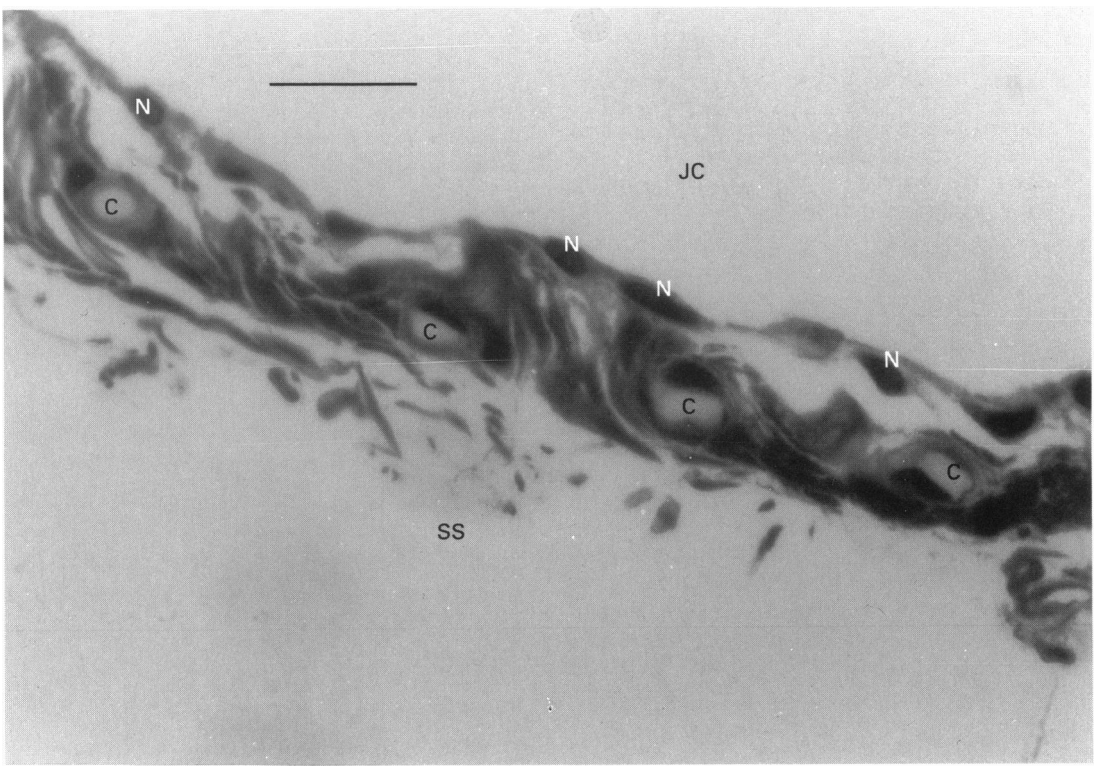

Figure 2. Photomicrograph of transverse section of suprapatellar synovial lining after separation from underlying areolar subsynovium (SS) by microdissection

A clean isolation of synovium was obtained, with only a few strands of subsynovial collagen remaining attached. Other features: C, capillary within synovium; JC, joint cavity; N, nucleus of synovial lining cell. Scale bar, 10 μm.

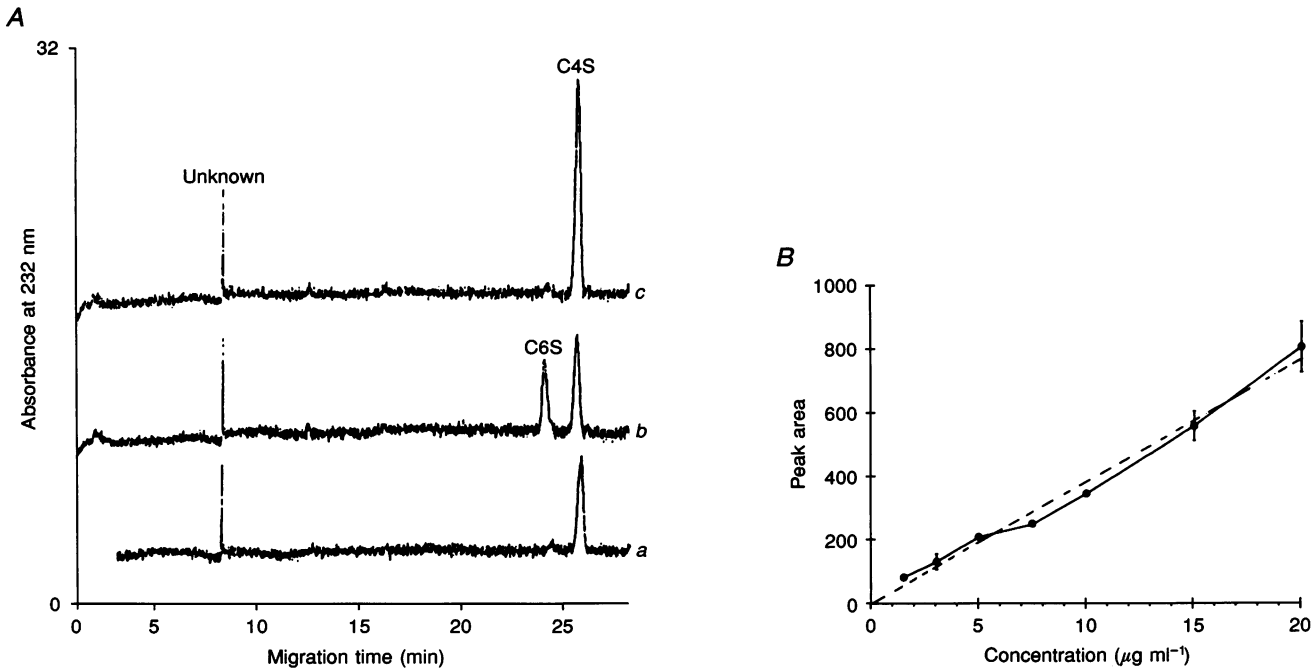

Figure 3

A, capillary electrophoretograms of chondroitin sulphate disaccharrides. Trace *a*, supernatant from β-eliminated synovium. The identity of the small and large peaks on the right was confirmed by overspiking with 5 μg ml^{-1} C6S (trace *b*) and 10 μg ml^{-1} C4S (trace *c*). The narrow spike marked 'Unknown' did not correspond with any of seven standard chondroitin sulphate disaccharides nor bovine kidney heparan sulphate, and could be related to the chondroitinase. The units on the ordinate axis are a percentage of full-scale deflection where 0–100% represents 1·0 absorbance units. *B*, electrophoretogram calibration for C4S disaccharide concentration; peak area in arbitrary units. Dashed line is regression relation, with a correlation coefficient of 0·99 and relative standard error of 5%.

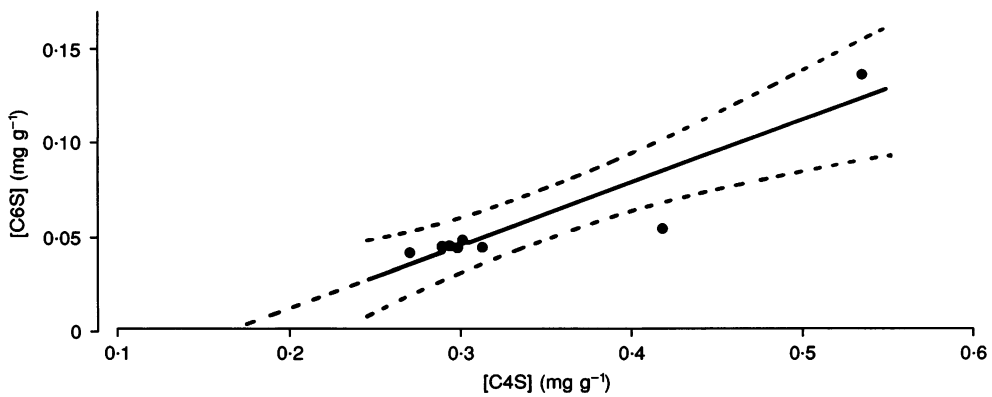

Figure 4. Relation between the concentration of synovial C4S and synovial C6S

Units are milligrams per gram of sample; correlation coefficient = 0·90; $P = 0·003$; $n = 8$. The regression equation of the fitted line is [C6S] = (0·323 ± 0·063)[C4S] − (0·059 ± 0·022); 95% confidence intervals are shown (dashed lines). The positive extrapolated intercept for C4S (0·18 mg g^{-1}) at zero C6S was significantly different from zero ($P = 0·03$), and may indicate the presence of two or more proteoglycan species, one of which carries chiefly C4S and little or no C6S.

(Price *et al.* 1995), the total chondroitin sulphate mass per millilitre of interstitium averaged 0·89 mg ml^{-1}.

The profiles for chondroitin sulphate concentration and C4S/C6S ratios across the whole joint are shown in Fig. 5. In *articular cartilage* the same two disaccharide peaks were found on the electrophoretogram but the C4S/C6S ratio, 0·67 ± 0·05, was the reverse of that in synovium (7·3 ± 0·5), indicating that the predominant proteoglycan differed markedly between the two tissues. Chondroitin sulphate concentrations in cartilage were much higher than in synovium, by ~30-fold for C4S (11·0 ± 2·0 mg g^{-1}) and 300-fold for C6S (16·8 ± 3·2 mg g^{-1}).

In *synovial fluid* the chondroitin sulphate concentration was 0·038 ± 0·004 mg ml^{-1}, which is a small fraction of the concentration in cartilage (⩽ 1/500th) and synovium (~1/35th for C4S; ~1/5th for C6S). C4S and C6S concentrations in knee synovial fluid were 0·022 ± 0·004 and 0·024 ± 0·007 mg ml^{-1}, respectively; in the shoulder, 0·023 ± 0·005 and 0·021 ± 0·005 mg ml^{-1}, respectively; and in the elbow, 0·020 ± 0·004 and 0·0179 ± 0·003 mg ml^{-1}, respectively. Lowest levels were found in the ankle (0·014 ± 0·002 and 0·015 ± 0·002 mg ml^{-1}, respectively) but the differences were not statistically significant ($P = 0·49$, one-way ANOVA). The C4S/C6S ratio in synovial fluid was close to unity (knee, 0·98 ± 0·12; ankle, 0·98 ± 0·16; shoulder, 1·11 ± 0·10; elbow, 1·27 ± 0·11), implying that much of the synovial fluid chondroitin sulphate derives from articular cartilage (ratio, 0·7) rather than synovium (ratio, 7·3).

In *subsynovium*, the C4S/C6S ratio was even bigger than in synovium, viz. 18·0 ± 2·7 in five samples, with [C6S] too low to measure in three other samples. Mean [C4S] was 0·313 ± 0·024 mg g^{-1} ($n = 8$) and [C6S], 0·020 ± 0·002 mg g^{-1} ($n = 5$). Since subsynovium is a relatively acellular region these values might, on the face of it, be compared with synovial interstitium, being 60% (C4S) to 16% (C6S) of the synovial interstitial concentrations ($P < 0·005$ in both cases, paired t test, with or without humidity correction); however, differences in collagen content and possibly hydration make meaningful comparison difficult (see Discussion).

Heparan sulphate

In *synovium*, the heparan sulphate concentration exceeded that of chondroitin sulphate 1·7 times, being 0·664 ± 0·071 mg (g sample)$^{-1}$ ($n = 6$; range, 0·454–0·982 mg g^{-1}), or 0·916 mg (g tissue)$^{-1}$ corrected for extraneous water.

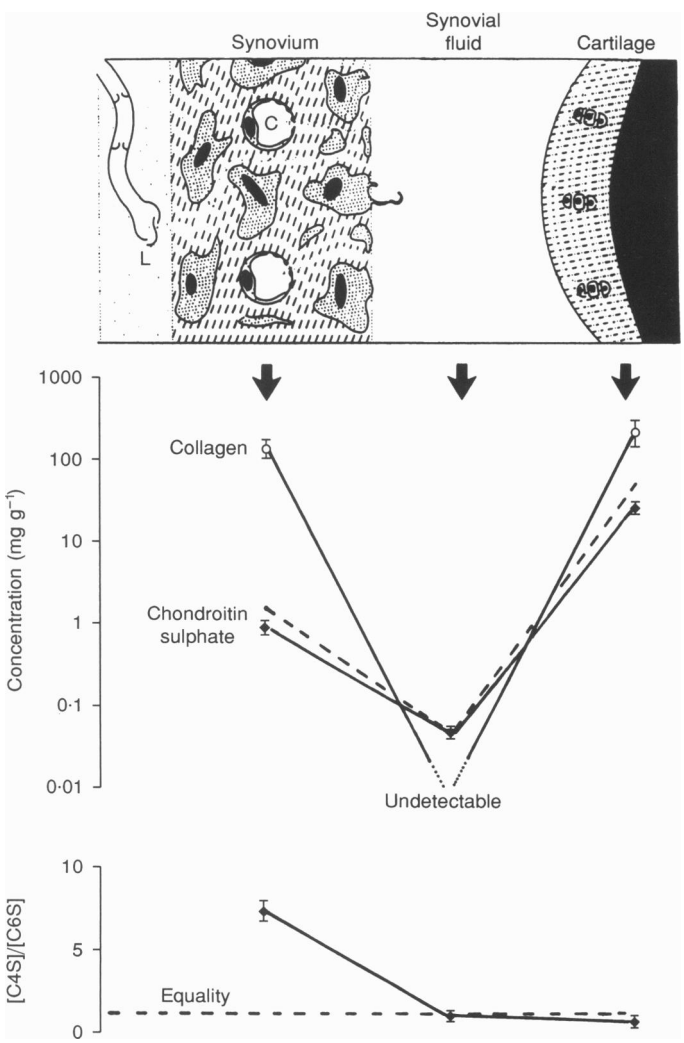

Figure 5

Upper graph, concentration profile, on a logarithmic scale, for total chondroitin sulphate (C4S + C6S; ◆) and collagen (○) across rabbit knee joint (top diagram; L, lymphatic; C, capillary.). Values are means ± S.D. Concentrations joined by continuous lines are mass per gram of interstitium, i.e. adjusted for cell volume (see text). Dashed lines are mass per millilitre extrafibrillar space, i.e. adjusted for collagen fibril volume as well as cellularity. Lower graph, change in ratio of C4S to C6S across the joint. Bars, S.D.

Heparan sulphate concentration correlated positively with chondroitin sulphate concentration (correlation coefficient, 0·89; $P = 0·02$) and was related by the regression equation:

$$[\text{Heparan sulphate}] = 1·162[\text{C4S} + \text{C6S}] + 0·205.$$

Adjusting for interstitial volume fraction, the mean interstitial concentration for heparan sulphate was $1·49 \pm 0·16$ mg ml^{-1} (1·39 mg g^{-1}).

In *articular cartilage* the heparan sulphate concentration was significantly lower than in synovial interstitium, namely $0·717 \pm 0·110$ mg g^{-1} ($n = 7$; $P < 0·01$, unpaired t test), and the ratio of heparan sulphate to chondroitin sulphate was only 0·026 (cf. 1·7 in synovium). The concentration profile for heparan sulphate across the joint was thus very different in pattern from that of chondroitin sulphate, as illustrated in Fig. 6.

In *synovial fluid*, as in synovium, heparan sulphate was more abundant than chondroitin sulphate, by a factor of 2·2. The ratio of heparan sulphate to chondroitin sulphate was almost 100 times higher in synovial fluid than in cartilage but was close to that in synovium, which may indicate that synovial fluid heparan sulphate derives mainly from synovium. The average concentration in all joint fluids was $0·083 \pm 0·01$ mg ml^{-1}. The concentration in knee fluid was $0·078 \pm 0·006$ mg ml^{-1} ($n = 5$); shoulder, $0·085 \pm 0·010$ mg ml^{-1} ($n = 5$); elbow, $0·106 \pm 0·011$ mg ml^{-1} ($n = 4$); and ankle, $0·062 \pm 0·009$ mg ml^{-1} ($n = 4$), with the concentration in ankle fluid again being the lowest ($P = 0·04$, one-way ANOVA). Total sulphated GAG in synovial fluid (heparan sulphate + chondroitin sulphate) was 0·124 mg ml^{-1} (knee), 0·128 mg ml^{-1} (shoulder), 0·1423 mg ml^{-1} (elbow) and 0·090 mg ml^{-1} (ankle).

In *subsynovium* the heparan sulphate/chondroitin sulphate ratio was 2·1, similar to synovium (1·7) and synovial fluid (2·2). The measured heparan sulphate concentration, $0·710 \pm 0·081$ mg g^{-1} ($n = 5$), was lower than in synovial interstitium ($P < 0·01$, irrespective of humidity correction; unpaired t test) but the same interpretational caution referred to for chondroitin sulphate applies here too.

Hyaluronan

In *synovium* less hyaluronan was present than either chondroitin sulphate or heparan sulphate, and the hyaluronan concentration did not correlate significantly with sulphated GAG concentration (e.g. correlation with chondroitin sulphate, 0·32; $P = 0·37$). The hyaluronan concentration was $0·265 \pm 0·039$ mg (g sample)$^{-1}$ (range, 0·130–0·420 mg g^{-1}; $n = 8$) or 0·366 mg (g tissue)$^{-1}$ corrected for extraneous

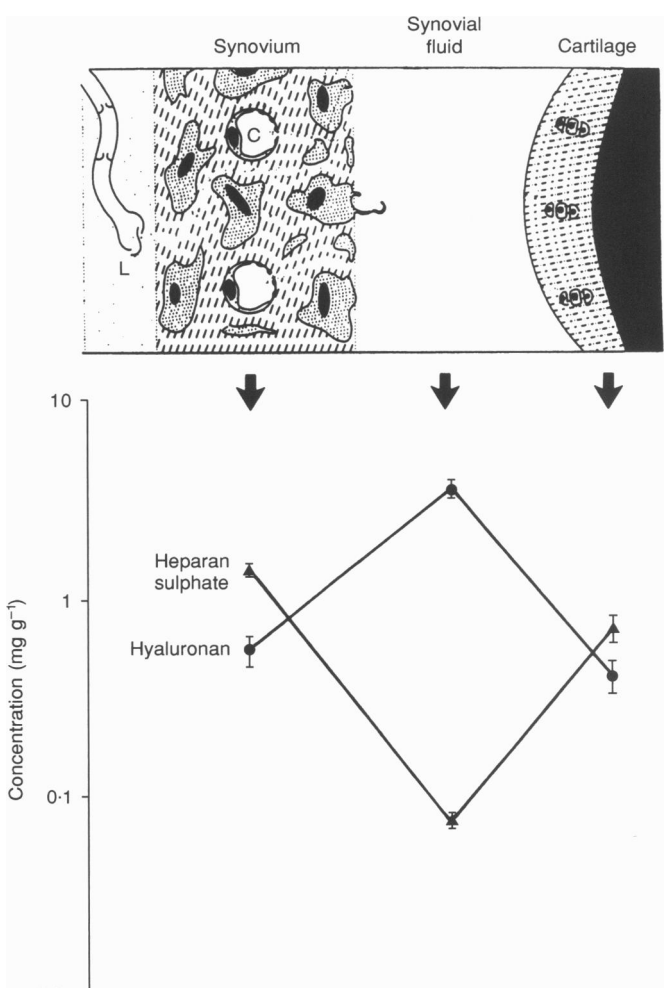

Figure 6. Contrasting concentration profiles for heparan sulphate and hyaluronan across rabbit knee joint

▲, heparan sulphate; ●, hyaluronan. Values are means ± S.D. The concentrations, on a logarithmic scale, represent mass per gram of interstitium, i.e. adjusted for the differing cell volume fractions of the tissues. A schematic diagram of the rabbit knee joint is shown at the top. L, lymphatic; C, capillary.

water. Allowing for interstitial volume fraction, the mean interstitial concentration was 0·595 mg ml^{-1} (0·555 mg g^{-1}).

In *articular cartilage* the hyaluronan concentration was similar to that in synovial interstitium, namely 0·415 ± 0·084 mg g^{-1} ($n = 8$). One difference, however, was that alkaline β-elimination extracted only 47% of cartilage hyaluronan (0·201 ± 0·053 mg g^{-1}), whereas extraction by β-elimination was complete for synovium. The 53% of the total hyaluronan that remained in cartilage could be extracted by papain digestion (0·224 ± 0·036 mg g^{-1}). The lower extractability of cartilage hyaluronan presumably reflects the greater diffusion barrier offered by cartilage and/or better anchorage of hyaluronan in cartilage by aggrecan, link protein and other binding agents.

In *synovial fluid* the hyaluronan concentration was 6–7 times higher than in synovial interstitium or articular cartilage. The concentration profile for hyaluronan across the joint was thus strikingly different from that of the sulphated GAGs, which attained their lowest concentratons in synovial fluid (Fig. 6). The hyaluronan concentration for all joint fluids averaged 3·34 ± 0·28 mg ml^{-1}. There was a wide range, from 1·74 mg ml^{-1} in one ankle fluid to 4·69 mg ml^{-1} in one shoulder fluid. The hyaluronan concentration varied significantly with the type of joint, being highest in the shoulder (3·95 ± 0·37 mg ml^{-1}) and knee (3·62 ± 0·19 mg ml^{-1}), 3·095 ± 0·36 mg ml^{-1} in the ankle, and least in the elbow (2·69 ± 0·07 mg ml^{-1}; $n = 5$ throughout). Differences between knee and elbow, and between shoulder and elbow were significant ($P = 0·02$, two-way ANOVA). Hyaluronan accounted for 96·5% of the total GAG in normal synovial fluid.

In *subsynovium* the hyaluronan concentration of 0·526 ± 0·105 mg g^{-1} ($n = 5$) was close to that in synovial interstitium and cartilage (difference not significant). Subsynovial hyaluronan was almost fully extracted by alkaline β-elimination, with only 6% remaining in the pellets.

Collagen

In *synovium* the collagen concentration was 72·53 ± 10·15 mg (g sample)$^{-1}$ ($n = 8$; range, 39·2–135·3 mg g^{-1}), or 100·0 mg (g tissue)$^{-1}$ corrected for extraneous water. Allowing for interstitial volume fraction, the mean interstitial concentration was 162·6 mg ml^{-1} (151·6 mg g^{-1}). Collagen concentration did not correlate significantly with GAG concentration (e.g. correlation with chondroitin sulphate, 0·48; $P = 0·23$), so the wide variation in GAG concentration between samples is unlikely to be an artefact arising from a variable degree of surface water deposition; the latter would affect the concentration of all constituents equally.

The high collagen content of synovium was consistent with the abundant fibrils seen ultrastructurally, and by combining the biochemical results obtained here with recent morphometric data on collagen fibril volume fraction, the proportion of a synovial collagen fibril that comprises molecular collagen was evaluated (leading to exclusion volume estimations, next section). From ultrastructural work the collagen fibrils occupy 0·153 ml per millilitre of rabbit knee synovium (whole tissue; Price *et al.* 1995). Dividing this by the molecular collagen content found here, 0·1073 g (ml synovium)$^{-1}$, the effective specific volume of a synovial fibril, v_{fibril}, was found to be 1·43 ml (g molecular collagen)$^{-1}$. Since the partial specific volume of molecular collagen, $v_{collagen}$, is only 0·75 ml g^{-1} (Meyer, Koblenz & Silberberg, 1977), the intrafibrillar non-collagen space (presumably mainly water) is 0·68 ml (g collagen)$^{-1}$. Thus the synovial fibril comprises approximately 47% water by volume (40% by weight). This is close to the value of 45% water by volume (0·61 ml H_2O (g collagen)$^{-1}$) found in rat tail tendon type I fibrils by Grynpas, Eyre & Kirschner (1980). Rabbit synovial fibrils contain type I, type III and probably type V collagen (Eyre & Muir, 1975; Ashhurst *et al.* 1991). The water content of fibrils generally depends on collagen type (type II collagen, found in cartilage, has 50–100% more water per gram than type I fibrils, i.e. 0·88–1·47 ml g^{-1}) and on the surrounding extrafibrillar osmotic pressure (Grynpas *et al.* 1980; Wachtel & Maroudas, 1990). Sources of error in the morphometric value have been discussed previously (Price *et al.* 1995)

In *articular cartilage* the collagen concentration was more than double that in synovial interstitum, namely 213·5 ± 41·3 mg g^{-1} ($n = 8$). The latter value is similar to the collagen concentration in superficial cartilage from the human femoral condyle (231 mg g^{-1}; Muir, Bullough & Maroudas, 1970). No collagen (as hydroxyproline) was detected in the normal synovial fluid. In subsynovium the measured collagen concentration was surprisingly high (122·6 ± 18·2 mg g^{-1}; $n = 6$; $P = 0·06$ compared with synovial interstitium, paired t test); see Discussion.

Extrafibrillar concentrations

The GAG concentration that is functionally important, governing hydraulic drag and protein exclusion, is the mass per unit volume of distribution. The distribution volume is tissue volume minus excluded volume. The excluded volume for GAG was calculated from the cell and vascular volume fractions and the collagen fibril volume fraction (Meyer *et al.* 1977). Fibril volume fraction in an individual synovial sample (Table 1) was calculated as measured concentration of molecular collagen multiplied by v_{fibril} (1·43 ml (g collagen)$^{-1}$; or for cartilage (type II collagen), 1·93 ml (g collagen)$^{-1}$, see above). Exclusion by the cell/vascular volume was important chiefly in synovium (combined cell and vascular volume fraction, 0·34), while in rabbit cartilage the chondrocyte volume fraction is only 0·07–0·08 (Paukonnen, Selkainaho, Jurvelin, Kiviranta & Helminen, 1985). In areolar subsynovium the cellularity, although unquantified, appears to be very low on microscopy.

Table 1 summarizes the interstitial extrafibrillar GAG concentrations calculated as above. Total GAG concentration in synovial interstitium averaged 4·1 mg (ml extrafibrillar

Table 1. Aggregate concentration of glycosaminoglycans (ΣGAG) in synovial interstitium

Rabbit	Collagen fibril volume as fraction of interstitial space*	ΣGAG (mg (ml interstitium)$^{-1}$)†	ΣGAG (mg (ml extrafibrillar space)$^{-1}$)‡
1	0·258	4·523	6·096
2	0·198	2·688	3·352
3	0·125	2·829	3·233
4	0·242	2·092	2·760
5	0·223	2·713	3·492
6	0·160	3·150	3·750
7	0·221	3·094	3·972
8	0·434	3·428	6·057
Mean	0·233	3·065	4·09
± S.D.	0·093	0·711	1·28

*(Grams collagen per millilitre interstitium) × v_{fibril} (1·43 ml g^{-1}); see text. †C4S + C6S + heparan sulphate + hyaluronan in milligrams per gram of sample × 1/0·725 (humidification correction) × 1·073 (density) × 1/0·66 (interstitial volume fraction); see text. ‡ Previous column divided by 1 − fibril volume fraction in interstitium (first column).

space)$^{-1}$, approximately double the tissue concentration. The GAG concentration in cartilage, 56·4 mg ml^{-1}, was roughly 14 times greater than in synovial interstitium. In view of the marked variation in synovial hydraulic resistance between joints, it is important to note that the net extrafibrillar GAG concentration in individual joints ranged from 33% below the mean (viz. 2·76 mg (ml extrafibrillar space)$^{-1}$) to 49% above it (6·10 mg (ml extrafibrillar space)$^{-1}$), i.e. the highest concentration was 2·2 times the lowest. Even the highest GAG concentration, however, was substantially smaller than the total extrafibrillar biopolymer concentration of ~13·9 mg (ml extrafibrillar space)$^{-1}$ predicted by a model of synovial hydraulic resistance (Levick, 1994). This problem is addressed in the Discussion.

Total protein

The synovial amino acid analysis is summarized in Table 2. The total protein content of five microdissected synovial samples, determined as the sum of the amino acids, was 98·4 ± 9·6 mg (g sample)$^{-1}$, or 135·7 mg (g tissue)$^{-1}$ corrected

Table 2. Concentration of individual amino acids in microdissected synovial samples from five rabbit knees

Amino acid	Concentration (mg (g synovium)$^{-1}$)
Glycine	25·647 ± 2·734
Alanine	3·232 ± 0·469
Serine	5·734 ± 0·619
Proline	5·925 ± 0·563
Valine	1·061 ± 0·135
Threonine	2·710 ± 0·180
Isoleucine	0·196 ± 0·050
Leucine	2·413 ± 0·405
Aspartate	5·895 ± 0·655
Lysine	3·534 ± 0·331
Glutamate	13·862 ± 1·602
Histidine	4·778 ± 0·504
Phenylalanine	3·140 ± 0·329
Arginine	6·819 ± 0·807
Tyrosine	5·384 ± 1·110
Hydroxyproline	8·096 ± 0·846

Values are means ± S.E.M. ($n = 5$). Of the five other amino acids normally found in tissue hydrolysates, asparagine and glutamine appear as aspartate and glutamate after HCl hydrolysis, and levels of cysteine, methionine and tryptophan are very low.

Table 3. Summary of biopolymer concentrations in rabbit knee (mg g^{-1})

	Synovium				Synovial fluid	Cartilage	
	Tissue		Interstitium †	Extrafibrillar space ‡		Tissue	Extrafibrillar space ‖
	Measured	Corrected *					
C4S	0·345 ± 0·031	0·547	0·89	1·160	0·022 ± 0·004	11·0 ± 2·0	54·94
C6S	0·053 ± 0·011				0·024 ± 0·007	16·8 ± 3·2	
Heparan sulphate	0·664 ± 0·071	0·916	1·49	1·943	0·078 ± 0·006	0·717 ± 0·110	1·30
Hyaluronan	0·265 ± 0·039	0·366	0·595	0·776	3·62 ± 0·19	0·415 ± 0·084	0·820
Collagen	72·53 ± 10·15	100·0	162·6	—	Undetectable	213·5 ± 41·3	—

Values are means ± S.E.M. * Corrected for mist deposition during high-humidity dissection; see text. † Adjusted for cell volume fraction (0·34) and tissue density (1·073 g ml^{-1}); units, mg ml^{-1}. ‡ Adjusted for collagen fibril volume fraction, 0·233. ‖ Adjusted for estimated collagen fibril volume fraction, 0·45.

for condensation. Of this, collagen in the same five samples accounted for 68·5 ± 7 mg (g sample)$^{-1}$ or 94·4 mg (g tissue)$^{-1}$. Collagen was thus the single most abundant protein in synovium (69·7 ± 2·3% of total protein; range, 63·8–73·5%). The concentration of non-collagen protein averaged 41·3 mg (g tissue)$^{-1}$. If most of the non-collagen protein were intracellular, cell protein content would be 133 mg (g synovial cell)$^{-1}$, or roughly 13% of the cell by weight (taking tissue volume fractions as 0·31 synovial cells, 0·66 ECM, 0·03 capillaries). This is not dissimilar to the textbook value for cells in general, ~18% (Alberts, Bray, Lewis, Raff, Roberts & Watson, 1994); values presumably vary with cell type.

DNA

The mean DNA content of six samples was 1·5 ± 0·4 mg g^{-1}, corresponding to 2·22 mg (ml tissue)$^{-1}$ (corrected for condensation). The variability of the assay precluded the intended use of DNA as an index of the cell content in individual samples; e.g. three repeat assays on the same sample gave values that varied more than 2-fold (1·08, 2·37 and 1·58 mg g^{-1}). The method is usually applied to cultured cells and highly cellular tissues, and the variability here might be related to the high interstial matrix/cell ratio, compounded by the minute amount of tissue available, which places the assay in the lower range of chromophore detection. Taking into account the mean cell volume fraction in synovium, the mean DNA content corresponded to 6·53 mg (ml synovial cell)$^{-1}$ (~0·65% of cell weight; cf. 0·25% for unspecified 'average' mammalian cell; Alberts *et al.* 1994); the percentage result will depend on the cell nucleus/cytoplasm ratio and on species chromosome mass.

The concentrations of ECM components in synovium, synovial fluid and cartilage are summarized in Table 3; tissue concentration, interstitial concentration and extracellular, extrafibrillar concentrations are tabulated to facilitate comparisons between tissues.

DISCUSSION

Comparison with literature: synovium

A few quantitative studies exist based on macroscopically dissected samples where the ratio of synovium (thin) to underlying tissue (thick) was unknown.

The sulphated GAGs. Synovial immunohistology has demonstrated the presence of decorin, biglycan, C4S and C6S, and heparan sulphate (Worrall *et al.* 1992, 1994). Akeson *et al.* (1973) reported 0·52 mg chondroitin/dermatan sulphate per gram of periarticular tissue in rabbit knees (63% chondroitin sulphate, 27% dermatan sulphate), while Denlinger (1982) found 0·45 mg chondroitin sulphate per millilitre of rabbit knee synovial tissue. Both results are close to our value of 0·55 mg (g synovium)$^{-1}$. Heparan sulphate concentration does not seem to have been reported previously. The striking reversal of the C4S/C6S ratio between synovium and cartilage implies the existence of different proteoglycans in cartilage (where aggrecan predominates) and synovium.

Hyaluronan. Synoviocytes possess enzymes such as uridine diphosphoglucose dehydrogenase (UDPGD) for the synthesis of hyaluronan and other GAGs (Edwards, 1994), hyaluronan has been demonstrated in the synovial lining immunohistologically (Wells *et al.* 1992), and synovial cells are rich in hyaluronan-binding CD44 receptors (Edwards, 1994). In macrodissected rabbit periarticular tissue Akeson *et al.* (1973) found 0·3 mg hyaluronan (g wet tissue)$^{-1}$ (cf. 0·37 mg (g synovium)$^{-1}$ in this study), while in canine synovial villi the concentration is similar, 0·27 ± 0·02 mg g^{-1} (wet; Dr S. L. Myers, Indiana University, personal communication). Higher values were reported in rabbit synovial tissue by Balazs & Denlinger (1985) (4·3 mg (g dry tissue)$^{-1}$, equivalent to 1·17 mg (g wet tissue)$^{-1}$) and in macrodissected human synovial tissue by Pitsillides, Worrall, Wilkinson, Bayliss & Edwards (1994) (1·07 mg g^{-1}). The

conditions of collection may be important; in our study a saline flush was used to remove the surface film of hyaluronan-rich synovial fluid (3·6 mg g^{-1}), which may contaminate unflushed samples. On the other hand, flushing may also leach out unbound intrasynovial hyaluronan, so our result (0·37 mg g^{-1}) seems best considered a minimum estimate, representing bound or well-entangled intrasynovial hyaluronan. There are at least two forms of hyaluronan-binding site in synovium, namely cell CD44 receptors and type VI collagen.

Collagen. Synovial lining cells possess prolyl hydroxylase and strongly express type III collagen mRNA. In macrodissected periarticular tissue, Akeson *et al.* (1973) found 198 mg collagen (g wet tissue)$^{-1}$ (result here, 100 mg (g synovium)$^{-1}$), while in macrodissected human synovial tissue Eyre & Muir (1975) found that collagen comprised 20–50% of the dry weight. For a tissue dry weight of 27·3%, this corresponds to 55–137 mg collagen (g wet tissue)$^{-1}$, similar to the range observed here (54–187 mg g^{-1}).

Subsynovium

The sampling of areolar subsynovium was considered unsatisfactory; the dense, sticky consistency of the sample indicated that water may have been expelled from this loose, formless tissue by the modest mechanical stresses involved in its collection. The finding that collagen and hyaluronan concentrations in the subsynovial sample were as high as in synovium could reflect this, since immunohistochemistry reveals more intense staining for hyaluronan, hyaluronan synthase and the hyaluronan-binding CD44 receptor in synovium than subsynovium, and the concentration of UDPGD in synovium is 6–9 times greater than in subsynovium (normal human knee; Edwards, 1994). The issue is important because if the subsynovial biopolymer concentration were similar to that in synovium, this would imply a comparable subsynovial hydraulic resistance. In contrast, negligible subsynovial hydraulic resistance was assumed in predicting the synovial biopolymer concentration via the trans-synovial flow model of Levick (1994). If this assumption were incorrect, a lower synovial GAG concentration would be predicted (see 'Uncertainties in estimation of resistivity', below).

Synovial fluid

The hyaluronan concentration (3·6 mg ml^{-1}; knee) was close to that reported by Sunblad (1953) (3·9 mg ml^{-1}). Hyaluronan accounted for 96·5% of synovial fluid GAG. The C4S/C6S ratio in the rabbit fluids (1/1) was higher than in human fluids (1/2 to 1/5).

Articular cartilage

The chondroitin sulphate concentration in rabbit articular cartilage (27·8 mg g^{-1}) was close to that reported by Denlinger (1982) (25·7 mg g^{-1}). Similarly in man the total GAG concentration (including keratan sulphate) ranges from 17 mg g^{-1} superficially to 59 mg g^{-1} in deeper femoral condylar cartilage (Muir *et al.* 1970), while in mouse tibial cartilage the chondroitin sulphate concentration is lower, 2·6–4·6 mg g^{-1} (Gaffen *et al.* 1994). The hyaluronan concentration in rabbit cartilage (0·42 mg g^{-1}) is at the lower end of the range found in human cartilage (0·5–2·5 mg g^{-1}; Holmes, Bayliss & Muir, 1988).

Concentration profile across joint

Large differences in extracellular, extrafibrillar biopolymer concentration existed between the three closely contiguous joint tissues (synovium, synovial fluid and articular cartilage; Figs 5 and 6). Sulphated GAGs were present at much higher concentrations in the 'solid' tissues than in synovial fluid, because they form part of tissue-bound proteoglycans. Heparan sulphate proteoglycans are associated with cell membranes, so a greater concentration in synovium than cartilage probably reflects the greater cellularity of synovium. The 20- to 200-fold greater extrafibrillar concentration of chondroitin sulphates in cartilage than synovium reflects the abundance of the chondroitin sulphate proteoglycan aggrecan, and endows cartilage with its extremely high hydraulic resistance and load-bearing ability. The reversal of the C4S/C6S ratio in synovium implies that non-aggrecan species predominate there.

The hyaluronan concentration profile contrasted sharply with that for sulphated GAG, hyaluronan being 4·5 times more concentrated in synovial fluid than in synovial or cartilage interstitium despite open contact between synovial fluid and each interstitial matrix (Table 3). In cartilage, the apparent disequilibrium may arise from steric and electrostatic exclusion of synovial fluid hyaluronan from much of the cartilage water space by the densely packed proteoglycan, as with other large solutes. In synovium, partial exclusion of synovial fluid hyaluronan by proteoglycan may likewise contribute to the difference but is unlikely to explain it fully, because the proteoglycan concentration is much lower than in cartilage. Leaching of unbound hyaluronan from the very thin synovial layer into the flushing solution may have contributed to the difference.

A third factor may be a dynamic steady state (cf. equilibrium) in which synovial fluid hyaluronan escapes from the joint cavity less easily than water during fluid absorption, producing molecular sieving. There is indirect evidence for this from studies of trans-synovial flow in the presence of hyaluronan (McDonald & Levick, 1995), and from experimental measurements of the removal rate of intra-articular hyaluronan, which is much slower than that of albumin (for a review see Levick, Price & Mason, 1996). The hyaluronan secretion data also support this view, as follows. Hyaluronan is secreted into rabbit joint cavities at a rate dm_{hy}/dt of only 4–8 $\mu g\ h^{-1}$ (rabbit shoulder and elbow, Knox *et al.* 1988; rabbit knee, Denlinger, 1982; P. Coleman, J. R. Levick & R. M. Mason, unpublished observations). If intra-articular hyaluronan were to drain freely through synovial matrix along with water (flow, dV/dt), its steady-state concentration in the cavity would be simply the ratio $(dm_{hy}/dt)/(dV/dt)$, and therefore the intra-articular

concentration of 3·62 μg μl^{-1} (Table 3) would imply a bulk volume turnover rate of only 1·1–2·2 μl h^{-1} (joint)$^{-1}$. Several lines of evidence indicate, however, that volume turnover is actually an order of magnitude greater than this (20–40 μl h^{-1} (rabbit knee)$^{-1}$, or 1·3–3·5 μl h^{-1} (cm synovium)$^{-2}$; Levick, 1979, 1984). This implies selective retention of hyaluronan within the joint cavity and a concentration difference in the direction observed here.

Physiological implications: relation to hydraulic resistance of joint lining

Interstitial hydraulic resistance is in general governed by the concentration of GAG, associated proteoglycan core protein, glycoproteins and collagen (Levick, 1987). The hydraulic resistance of the normal rabbit knee lining is very variable, spanning an order of magnitude. The wide, 2·3-fold variation in extrafibrillar GAG concentration found in this study helps to explain this, since the relation between resistance and concentration is non-linear. For example, a 2·3-fold change in chondroitin sulphate proteoglycan concentration causes a 7·1-fold change in its permeability, as given by:

$$\kappa = 1{\cdot}615 \times 10^{-18}(0{\cdot}49C)^{-2{\cdot}354} = 1/R, \quad (1)$$

where κ is specific hydraulic conductivity (cm^2; reciprocal of specific hydraulic resistivity R) and C is concentration in grams per millilitre (data of Comper & Zamparo, 1989; summarized in Levick, 1994). The conductivity of other GAGs is similar.

For a rabbit knee with an observed lining conductance of $1{\cdot}8 \times 10^{-3}$ cm^3 min^{-1} cmH$_2$O^{-1} (see Methods, Terminology), modelling predicts that the net concentration of all biopolymer chains (excluding collagen) should be 13·9 mg (ml interstitial extrafibrillar space)$^{-1}$ for a uniform GAG distribution (Levick, 1994). In the present study, however, the total extrafibrillar GAG concentration was found to be only 4·1 mg ml^{-1} (maximum, 6·1 mg ml^{-1}). Although this approaches the predicted order of magnitude, it is nevertheless only 30% of the predicted total biopolymer concentration.

Conversely, the conductance of a synovial lining whose matrix contained solely the material found in this study can be predicted. A uniform GAG concentration of 4·1 mg ml^{-1} generates an extrafibrillar specific hydraulic conductivity (κ) of $3{\cdot}48 \times 10^{-12}$ cm^2 (eqn (1)). This is the property of a unit cube of material, however. To translate this into a predicted conductance for the whole joint lining, account must be taken of the area and thickness of the lining, the fraction of tissue volume occupied by ECM, the extracellular pathway tortuosity and the ECM collagen content. To do this the 1994 computer model was re-run using the new biochemical results and latest morphometric values (Price *et al.* 1995). The predicted trans-synovial flows, e.g. 79×10^{-3} cm^3 min^{-1} at 6 cmH$_2$O intra-articular pressure, were many times greater than those observed experimentally at the given pressure, and the predicted synovial lining conductance of $13{\cdot}3 \times 10^{-3}$ cm^3 min^{-1} cmH$_2$O^{-1} was 7·4 times bigger than the experimental value.

The mismatch problem discussed above is based on the assumption that GAG is distributed relatively uniformly along the flow axis at the low pressures used to measure control conductance. If there were marked non-uniformity along the flow axis (layering) or a systematic [GAG] gradient normal to flow even at low pressures, a set of resistances in series would exist, and this would reduce the mean concentration of GAG needed to acount for the control conductance values. Inspection of immunohistology preparations provides no clear evidence for steep matrix gradients along the flow axis, although quantitative information is lacking.

The present work therefore points to two main questions. (a) Might there be additional interstitial biopolymers present in significant quantity, besides GAG and collagen, to account for the apparent deficit of biopolymer mass? (b) Alternatively, was interstitial conductivity underestimated by Levick (1994), leading to an overprediction of interstitial biopolymer concentration? These two possibilites are considered in turn.

Do additional interstitial biopolymers exist in quantity?

Although GAGs are major contributors to interstitial hydraulic drag, they actually fail to explain fully the low interstitial conductivity in many other tissues too (Levick, 1987). Thus the problem highlighted above for synovium is by no means unique, as Fig. 7 shows. In Fig. 7*A* the logarithm of measured conductivity is plotted as a function of $\log_{10}$[GAG] for a wide range of interstitia, and also for a pure GAG matrix. Interstitial conductivities mostly fall well below the pure GAG relation and synovium shows a similar deviation to that of many other tissues. In Fig. 7*B*, the concentration values have been adjusted for exclusion by collagen fibrils, but even then the measured interstitial conductivities remain substantially smaller than conductivities predicted from the extrafibrillar GAG concentrations, namely by factors of 1/2 to 1/10th (Fig. 7*B*). (Notable exceptions are vitreous humor and Wharton's jelly, which are relatively simple hyaluronan gels.) Synovium, whose physiologically derived interstitial conductivities are only 1/4·6 to 1/8·9 times the conductivity predicted from the GAG and collagen results, is similar to many other tissues in this respect.

The discrepancy between interstitial GAG concentration and conductivity was resolved in many tissues by taking into account additional fixed interstitial components, namely the proteoglycan core protein and structural glycoproteins (Levick, 1987). The interstitial proteoglycan mass (GAG + core protein) is greater than the sulphated GAG mass by a factor that depends on proteoglycan species. In synovium these include biglycan and decorin (Worrall *et al.* 1992), which typically have core protein/GAG ratios of 38/100 to

38/250. Also, the concentration of glycoproteins can be as high as the proteoglycan concentration in some interstitia. As a result, the total biopolymer concentration in a tissue can be more than double the GAG concentration.

By analogy, then, it seems likely that non-collagen fibrous proteins may contribute at least partially to the resolution of the conductivity puzzle in synovium. Glycoproteins were first reported in synovium by Sarget & Rebeyrotte (1976) and later immunohistological identification of the specific glycoproteins fibronectin, laminin, entactin and tenascin is reviewed elsewhere (Levick *et al.* 1996). Fibronectin concentration may be around 2 mg (g synovial tissue)$^{-1}$ (human osteoarthritic and rheumatoid joints; Dr S. Carson, Winthrop-University Hospital, personal communication); and Okada, Nakanishi & Kajikawa (1981) suggested on the basis of ultrastructural changes after selective enzymic digestion that glycoprotein may be more abundant than GAGs in mature synovium. Proteoglycan core proteins carrying heparan sulphate and chondroitin sulphates are also present (e.g. decorin, biglycan) and possibly also a keratan sulphate-carrying protein (Price *et al.* 1994; perhaps fibromodulin). Further sources of hydraulic drag include the fine, superficial microfibrils of type VI collagen (Levick & McDonald, 1990) and the type IV collagen around synoviocytes (Pollock *et al.* 1990). The small fibre radius of these collagens offers a greater specific surface area for hydraulic drag than do fibrillar collagens. It is thus likely that structural proteins contribute at least some of the 'missing' resistance in synovium.

The total protein and dry weight results enable us to address the limited question of whether there is actually enough organic material in synovium potentially to resolve the puzzle. (1) The non-collagen protein content of synovium was 44·3 mg (ml tissue)$^{-1}$, whereas the estimated biopolymer deficit is only ~4·96 mg (ml tissue)$^{-1}$ (9·8 mg (ml extrafibrillar space)$^{-1}$). Thus only a relatively small proportion of the measured protein (11% or less) would need to be extracellular to raise interstitial resistance substantially, leaving $\geqslant$ 89% intracellular at a cell protein concentration of $\geqslant$ 127 mg (ml cell)$^{-1}$. (2) One gram of wet synovium contained 273 mg dry material. Identified constituents amounted to 155 mg (100 mg collagen, 41 mg non-collagen protein, 2 mg DNA, 2 mg GAG, ~10 mg electrolytes), leaving 118 mg of unidentified material. The

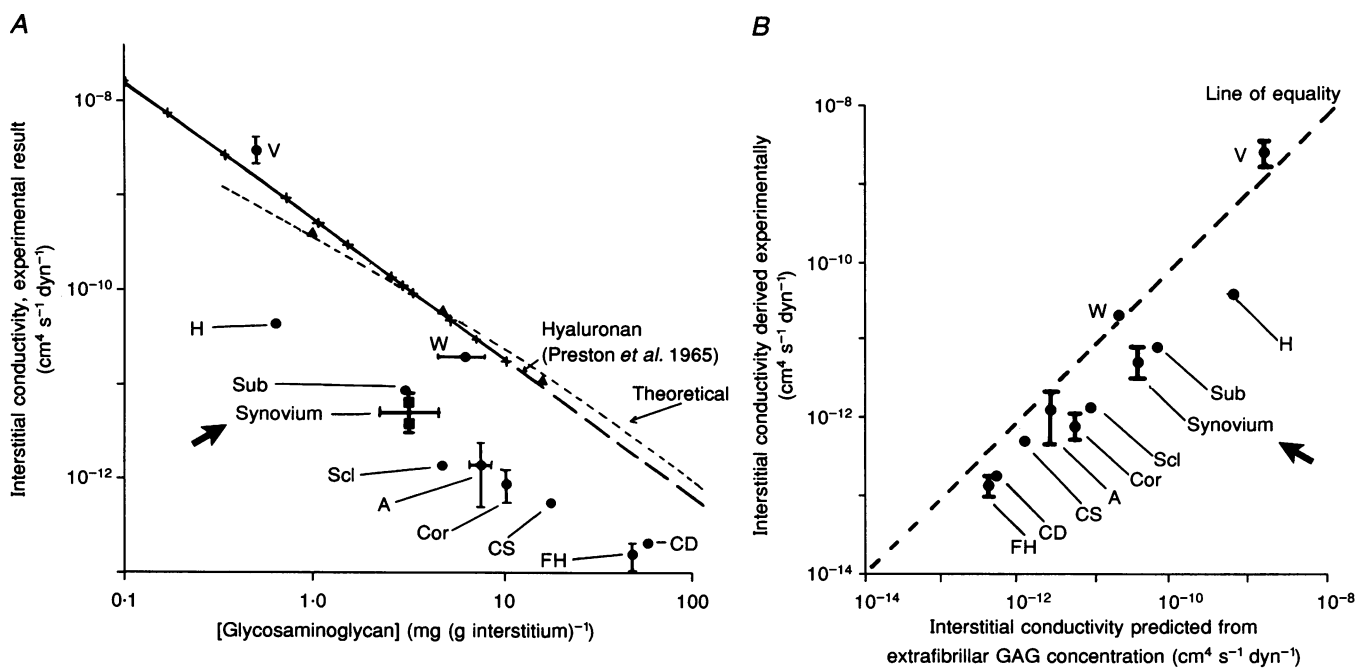

Figure 7. Relation between GAG concentration and hydraulic permeability

A, relation between hydraulic conductivity of interstitium from various tissues and the GAG concentration. Labels: V, vitreous body; H, hepatoma; W, Wharton's jelly; Sub, subcutaneous tissue; Scl, sclera; Cor, corneal stroma; A, aorta; CS and CD, cartilage of femoral condyle, superficial and deep, respectively; FH, femoral head cartilage. Data as summarized in Levick (1987). For synovium, the upper and lower squares show mean values of synovial interstitial conductivity from two series (McDonald & Levick, 1993, upper point; F. M. Price, J. R. Levick & R. M. Mason, unpublished, lower point; ± S.E.M.), plotted against the current result for total GAG mass per gram of interstitium (mean ± S.E.M.). The crosses (continuous line) are results for ox synovial hyaluronan *in vitro* (Preston, Davies & Ogston, 1965) and the triangles are results for chondroitin sulphate *in vitro*, showing that different GAGs create similar hydraulic drag. Dashed line is a theoretical relation for hyaluronan (see Levick, 1987). *B*, comparison of experimental estimate of interstitial conductivity and that calculated from GAG and collagen content, taking into account fibril volume and area fractions and tortuosity (after Levick, 1987). Labels as in *A*.

interstitial 'deficit' of biopolymer, ~4·96 mg (ml tissue)$^{-1}$, represents only 4% of the uncharacterized dry weight, again demonstrating that there is potentially enough material to account for the 'missing' resistance if a small percentage is extracellular filamentous material. Much of the unidentified material could be membrane lipids and other cellular components (e.g. RNA, carbohydrate, other metabolites, occasional adipocytes, haem in red cells) plus small amounts of unidentified extracellular carbohydrate associated with glycoproteins, fibromodulin etc.

Uncertainties in estimation of resistivity

If interstitial resistivity were overestimated by the model of Levick (1991, 1994), the predicted interstitial biopolymer concentration would likewise be an overstimate. Several difficulties and uncertainties in translating the relation between trans-synovial flow and intra-articular pressure into interstitial resistivity were noted (Levick 1991, 1994) and their significance is evaluated next.

(1) It was argued that hydraulic drag in synovium arises chiefly from interstitial biopolymers rather than from cell surfaces (Levick, 1987). This view is strongly reinforced by the new results for biochemical composition and cell volume fraction (0·34; Price *et al.* 1995). For a tube of radius r filled with matrix of specific hydraulic conductivity κ_{matrix}, the hydraulic drag exerted by the confining walls becomes negligible relative to the drag created by the matrix when the dimensionless ratio κ_{matrix}/r^2 falls below 10^{-2} (Ethier & Kamm, 1989). At a GAG concentration of 4·1 mg (ml extrafibrillar space)$^{-1}$ (present results), κ_{matrix} is $3{\cdot}6 \times 10^{-12}$ cm^2 (eqn (1); less if additional biopolymer is present). Concerning r, although the channels between synovial cells are not regular cylinders their characteristic dimension is of the order of 10^{-4} cm or more. Thus κ_{matrix}/r^2 is of the order of $\leqslant 3{\cdot}6 \times 10^{-4}$, far lower than the critical value of 10^{-2}, showing that interstitial matrix overwhelmingly dominates hydraulic drag in synovium. Considering a diffent geometry, namely parallel slits of width h filled with matrix, an analogous calculation for overall conductivity, κ_{net}, is:

$$\kappa_{net}/\kappa_{matrix} = 1 - \{[\tan h(h/\sqrt{\kappa_{matrix}})]/(h/\sqrt{\kappa_{matrix}})\} \quad (2)$$

(Ethier & Kamm, 1989). This evaluates as $\kappa_{net}/\kappa_{matrix} = 0{\cdot}98$ for synovium; in other words, the hydraulic drag of the confining cell walls reduce the net slit conductivity, κ_{net}, by only ~2%. The main effects of the cells are thus simply to reduce the cross-sectional area available for flow and to increase the tortuosity of the flow pathway; neglect of their hydraulic drag does not lead to a significant overestimation of the specific hydraulic resistivity of ECM.

(2) Pressure in the subsynovial space (P_{ss}) is a source of uncertainty when calculating the conductance of the synovial lining from physiological experiments. Synovial lining conductance is calculated as the increase in net trans-synovial flow ($\Delta\dot{Q}_s$) per unit increase in intra-articular pressure (ΔP_j) on the assumption that the pressure gradient is confined to synovium rather than extra-synovial tissues, i.e. it is assumed that synovial resistance greatly outweighs subsynovial resistance and that in consequence P_{ss} does not increase significantly above atmospheric pressure when P_j is raised in experiments. Several lines of evidence bearing on this crucial assumption are assessed below.

(i) Perforation of the synovial lining by a needle greatly increases $\dot{Q}_s$. In two recent experiments, $\dot{Q}_s$ increased from intitial values of $15{\cdot}3 \times 10^{-3}$ and $30{\cdot}0 \times 10^{-3}$ cm^3 min^{-1} ($P_j = 16$–17 cmH$_2$O) to 216×10^{-3} and 146×10^{-3} cm^3 min^{-1}, respectively, after deliberately perforating the suprapatellar lining with a 21 gauge needle (P. Coleman & J. R. Levick, unpublished data; see also Levick, 1980). The resistance of the synovial lining is thus much greater than that of the surrounding tissue. The experiment does not, however, totally exclude the existence of a significant resistance in the immediate subsynovial space, because the perforation must have extended through subsynovium (80–170 μm thick) as well as through synovium on account of the macroscopic bevel dimensions.

(ii) When periarticular pressure was measured by gradually advancing a 23 gauge hypodermic needle probe through the quadriceps, with P_j clamped at 21 cmH$_2$O, interstitial pressures rose to only 1 cmH$_2$O as the joint cavity was approached, and jumped abruptly to 21 cmH$_2$O as the cavity was entered (McDonald & Levick, 1993). Thus the lining tissue supports a very steep pressure gradient. Again, however, the macroscopic size of the needle bevel probably precludes detection of any pressure gradient in the relatively narrow subsynovial space.

(iii) Much of the subsynovium is areolar connective tissue. The compliance of hydrated areolar connective tissue is very high, and as a result its fluid pressure plateaus at 1 cmH$_2$O or so above atmospheric pressure with increasing hydrations (Aukland & Reed, 1993).

(iv) Dissection of infused rabbit knees post mortem shows that dyed intra-articular infusate tracks away from the subsynovium along connecting areolar tissue planes, in keeping with the high conductivity of hydrated connective tissue (Aukland & Reed, 1993). This, along with a high compliance, is thought to prevent any substantial build-up of subsynovial pressure.

(v) In order to 'clamp' peri-articular pressure at atmospheric level and so reduce the uncertainty in P_{ss}, synovium was divested of the surrounding muscle and tissue layers by dissection to expose subsynovium to the atmosphere (Levick, 1980; 2 joints). The observed trans-synovial flows (e.g. $14{\cdot}6 \times 10^{-3}$ cm^3 min^{-1} at 6 cmH$_2$O) and hydraulic conductance $d\dot{Q}_s/dP_j$ (mean, $2{\cdot}65 \times 10^{-3}$ cm^3 min^{-1} cmH$_2$O^{-1}), though somewhat higher than usual, were nevertheless only 18 and 20%, respectively, of the values predicted using the present GAG and collagen results (predicted $\dot{Q}_s$, 79×10^{-3} cm^3 min^{-1} at 6 cmH$_2$O; predicted $d\dot{Q}_s/dP_j$, $13{\cdot}3 \times 10^{-3}$ cm^3 min^{-1} cmH$_2$O^{-1}; see earlier). Like observations

(i)–(iv) above, this again indicates that the discrepancy between the physiological results and the biochemical results is not an artefact arising from misestimation of the pressure gradient across the lining in the physiological experiment.

(vi) An observation suggestive of a modest rise in P_{ss} during a trans-synovial flow experiment is 'hysteresis' of the $\dot{Q}_s$–P_j relation. That is to say, $\dot{Q}_s$ at a given P_j is smaller when P_j is approached by reduction from a higher value than when the same P_j is approached from a lower value (Levick, 1979). The hysteresis loop is typically about 3 cmH_2O wide, indicating that P_{ss} may rise by ~3 cmH_2O during the measurement of the pressure–flow relation. Taking a worst-case scenario where all the rise in P_{ss} occurred while raising P_j from 3 to 9 cmH_2O (the range over which normal conductance is measured), the true conductance $d\dot{Q}_s/d(P_j - P_{ss})$ would be underestimated by 50% and resistance overestimated 2-fold. Even given an error of this magnitude in the physiological result, however, the GAG concentration reported here is far too low to account for the resistance of the tissue (see earlier).

(3) Owing to the complex geometry of the knee joint, it is difficult to measure the synovial surface area with any great accuracy at a given P_j. Even so, the measured surface area (which has been derived from joint geometry, resin casts and excised tissue) would have to be overestimated by an improbable 600% to reconcile the physiological and biochemical results.

To summarize, although there is some uncertainty in the physiological estimate of synovial conductivity, due to difficulties in ascertaining subsynovial pressure and in measuring synovial area precisely, any underestimation of conductance is unlikely to be sufficient (namely 7-fold) to resolve the apparent discrepancy between the GAG concentration and conductance. Work with an isolated synovial membrane preparation would be of value in addressing the problem, but no such preparation exists as yet. On balance it seems likely that the GAG and collagen concentrations reported here account only partially for the total biopolymer concentration in synovial ECM, and that substantial quantities of other biopolymers may await measurement. The issue of the homogeneity or heterogeneity of GAG distribution also requires more detailed study.

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Acknowledgements

We thank Dr John Gaffen for collaboration in the development of the Ruthenium Red method, and Dr Doreen Ashhurst and Ms Yvette Bland for histological collaboration. The biotinylated G1 domain of aggrecan was a generous gift from Dr M. Bayliss. We apologize to authors that we are unable to cite because of journal citation restrictions. The research was funded by The Wellcome Trust grants 031333/Z and 039033/Z.

Received 11 December 1995; accepted 22 May 1996.