Changes in glycosaminoglycan concentration and synovial permeability at raised intra-articular pressure in rabbit knees

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1. When intra-articular pressure is raised to pathological values (> 9 cmH_2O) by saline, the hydraulic conductance of the synovial lining increases manyfold. The increase at 25 cmH_2O is only partially accounted for by stretching of the tissue and has been ascribed to washout and/or dilution of interstitial matrix biopolymers. This suggestion was tested in this study by sampling synovium from control joints (rabbit knees) and from joints perfused with saline to 25 cmH_2O, and analysing them quantitatively for collagen, chondroitin sulphate, heparan sulphate and hyaluronan.

2. Pressure and trans-synovial flow measurements showed that in samples taken at 25 cmH_2O the conductance of the synovial lining had increased by a factor of $5{\cdot}23 \pm 1{\cdot}5$ (mean ± S.E.M.) over the conductance at low pressures (just above atmospheric pressure).

3. The tissue concentrations of collagen and the sulphated glycosaminoglycans (GAGs) were reduced by similar amounts after perfusion to 25 cmH_2O, namely to 62·8–70·4% of control. The hyaluronan concentration by contrast was not significantly reduced (106% of control).

4. The reduction in collagen concentration (fixed material) indicated increased interstitial hydration. The closely similar reduction in sulphated GAGs indicated that dilution rather than washout of these components was occurring. The hyaluronan results could be explained by synthesis *in vivo* at a rate of $\geqslant 91$ $\mu g\ h^{-1}$ (ml synovium)$^{-1}$ (possibly a non-basal rate under the conditions of the experiment, i.e. raised pressure and a stretched hydrated membrane).

5. Because interstitial hydraulic drag is related to biopolymer concentration by a power function, the overall matrix dilution observed here was more than sufficient to explain the rise in synovial lining hydraulic conductance at 25 cmH_2O when taken in conjunction with stretching of the synovial lining (increased area, reduced thickness).

The hydraulic conductance of the synovial lining couples trans-synovial fluid movement to intra-articular pressure and is thus an important factor in synovial fluid dynamics. Measurements of trans-synovial flow in the rabbit knee, both *in vivo* and post mortem, show that the conductance of the synovial lining increases when intra-articular pressure is raised to pathological levels (above ~9 cmH_2O), such as occurs in joint effusions (Edlund, 1949). This causes a marked steepening of the pressure–flow relation and faster trans-synovial flows ('yield' phenomenon). Above ~9 cmH_2O each successive, applied step in pressure causes an increment in conductance to a new level, i.e. the pressure–conductance relation is graded above ~9 cmH_2O (Levick, 1980). A monotonic relation between conductance and intra-articular pressure above ~9 cmH_2O has been confirmed by measurements of blood-to-joint cavity conductance, i.e. the conductance of the capillary wall and synovial lining in series (Knight & Levick, 1985; Knight, Levick & McDonald, 1988).

The trans-synovial movement of fluid takes place through wide interstitium-filled spaces between the lining cells, which stem from fibroblasts and macrophages, not epi- or mesothelium. At high intra-articular pressures this interstitial pathway becomes wider and shorter, due to stretching of the synovial lining (Levick & McDonald, 1989*a*, *b*), and this contributes towards the increase in conductance. Nevertheless, the geometrical changes are not sufficiently large to account fully for the conductance increases (Levick, 1991). It has therefore been inferred that the specific hydraulic conductivity of the interstitial matrix (i.e. the conductance of a unit cube of matrix) also increases at raised intra-articular pressures. Since interstitial

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conductivity is in general governed by the concentration of collagen and fixed biopolymers (glycosaminoglycans (GAGs), proteoglycan and glycoproteins) in the interstitial matrix, it was also inferred that matrix concentration should decrease at high intra-articular pressures. A decrease by about 28% was predicted from known, non-linear relations between GAG concentration and hydraulic resistivity (1/conductivity) *in vitro*. A decrease in concentration might be brought about by washout of poorly tethered components as fluid velocity increases, or by expansion of the synovial interstitial space by increased hydration.

The aims of the present study were, therefore, to discover by direct quantitative biochemical analysis whether the raised synovial lining conductance at pathological intra-articular pressures is associated with a decrease in synovial matrix concentration, and to discover whether the mechanism involved is a process of GAG washout or increased tissue hydration. In addition, the results provided an unexpected bonus in the form of an estimate of the rate of matrix hyaluronan synthesis by synovium *in vivo*.

METHODS

Overview

One knee of an anaesthetized rabbit received controlled intra-articular infusions of Krebs solution at a series of increasingly higher, constant pressures in order to chart out the relation between intra-articular pressure and trans-synovial flow and to increase the permeability of the lining. The opposite knee received no intervention and served as a control. Synovium was then microdissected from each knee and its biochemical composition was analysed quantitatively. Eight pairs of knees were studied in this way.

Intra-articular pressure and trans-synovial flow measurement

The pressure–flow relation was measured as described previously (McDonald & Levick, 1993). Briefly, New Zealand White rabbits weighing 2–3 kg were anaesthetized via the marginal ear vein (sodium pentobarbitone (30 mg kg^{-1}) plus urethane (500 mg kg^{-1}), maintained half-hourly), tracheostomized and two 21-gauge hypodermic cannulae inserted into the suprapatellar region of the test knee. One cannula was connected to a Gould-Statham pressure transducer calibrated by water column, to record intra-articular pressure (P_j). The other was connected to an infusion reservoir of Krebs solution (for composition see Knight *et al.* 1988), whose height above the joint controlled P_j. An intervening drop counter recorded flow into the joint, and trans-synovial flow ($\dot{Q}_s$) was calculated as measured inflow in the steady state after 15–20 min at constant pressure minus the wall's residual rate of viscous creep (Levick, 1979). Prior to determining the P_j–$\dot{Q}_s$ relation the joint was flushed with Krebs solution for 2 min to wash out endogenous synovial fluid, then P_j was raised in steps of 2–3 cmH_2O at 15–20 min intervals, beginning at the lowest pressure at which a net trans-synovial flow could be recorded (typically around 2 cmH_2O) and ending at 25 cmH_2O, a pathological pressure at which permeability is increased substantially. Flows were measured in the quasi-steady state 15–20 min after each pressure step.

The protocol of multiple pressure steps took 3 h and was the same as that used in previous work on trans-synovial flow and conductance. Samples were thus harvested after being subjected to conditions that matched those used previously.

Harvesting tissue

The microdissection method decribed in the companion paper (Price, Levick & Mason, 1996) was used to obtain a sample of synovial lining after completion of the pressure–flow study. Briefly, the animal was killed by an intravenous overdose of sodium pentobarbitone (Euthatal; May & Baker Ltd, Dagenham, UK), transferred into a polythene-draped chamber where relative humidity was maintained at $\geqslant 95\%$ (measured by wet–dry bulb thermohygrometer) and the joint cavity was cut open. After flushing the surface gently with isotonic saline to remove synovial fluid, the synovial surface was gently blotted with fine filter paper (Whatman No. 50) to remove surface liquid. The synovial lining was then microdissected from the underlying areolar subsynovium under ×16 magnification (Zeiss OpMi-1 dissecting microscope) using ultrafine ophthalmic microdissection instruments (John Weiss Ltd, London, UK). Approximately 1 cm^2 (variable) of synovial intima overlying the quadriceps was obtained from the lateral and medial sides of the perfused suprapatellar zone. The total time between loss of pressurization and freezing the excised sample was about 30–45 min. The opposite knee, which had received no experimental intervention at all, was then opened and microdissected to obtain control samples of suprapatellar synovium from the same anatomical sites. Samples of subsynovial connective tissue were also taken for analysis from each side.

Biochemical analyses

Excised synovium 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). Quantitative biochemical analyses were carried out using the protocol described in the companion paper (Price *et al.* 1996). Briefly, chondroitin 4 sulphate (C4S) and chondroitin 6 sulphate (C6S) were assayed by capillary zone electrophoresis, heparan sulphate by radioactive Ruthenium Red assay, hyaluronan by the G1-domain binding assay, and collagen by hydroxyproline analysis. For interconversion of weight and volume, a tissue density of $1{\cdot}073 \pm 0{\cdot}005$ g ml^{-1} was used (Price *et al.* 1996). Measured synovial concentrations were corrected for vapour condensation during microdissection under misting ($\geqslant 95\%$) humidity using the factor 1/0·725 (Price *et al.* 1996).

Statistical analysis

Results are given as means ± S.E.M. The problem of how best to summarize and compare P_j–$\dot{Q}_s$ relations was discussed by McDonald & Levick (1993) and the customary expedient of fitting two linear regression lines was again adopted (because the relation characteristically changes slope rapidly at around 9 cmH_2O and is relatively linear at lower and higher pressures). 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).

RESULTS

Pressure–flow relation

As Edlund (1949) first reported, the P_j–$\dot{Q}_s$ relation usually steepens markedly at pressures around 9 cmH_2O (Fig. 1). In the current series, the mean pressure at which this occurred, determined by inspection, was $7{\cdot}8 \pm 0{\cdot}7$ cmH_2O ($n = 8$). At pressures below this, the slope $d\dot{Q}_s/dP_j$ determined by linear

Table 1. Pressure–trans-synovial flow relation in present and previous studies of rabbit knee

Source	n	$(d\dot{Q}_s/dP_j)_{<P_y}$* (μl min^{-1} cmH$_2$O^{-1})	$(d\dot{Q}_s/dP_j)_{>P_y}$* (μl min^{-1} cmH$_2$O^{-1})	P_y† (cmH$_2$O)
Present series	8	0·93 ± 0·27 (0·2–2·2)	3·30 ± 0·29 (2·1–4·4)	7·8 ± 0·7 (5·8–10·4)
Edlund (1949)	20	0·71 ± 0·10‡	4·21 ± 0·49‡	9·6 ± 0·6
Levick (1979)	16	0·49 ± 0·09 (0·14–0·92)	2·81 ± 0·30 (1·3–4·6)	9·2 ± 0·6 (6·1–15·3)
McDonald & Levick (1993)	43	1·84 ± 0·17	3·82 ± 0·35	7·4 ± 0·4

Values are means ± S.E.M., with the range, where indicated, in parentheses. * Change in trans-synovial flow $\dot{Q}_s$ per unit rise in intra-articular pressure P_j, below yield pressure ($<P_y$) or above it ($>P_y$). Slope by regression analysis. † Pressure at which slope steepens, P_y, determined by inspection. ‡ Values were not corrected for viscous creep of cavity walls and were measured at 4–12 min.

regression analysis averaged $0{\cdot}93 \times 10^{-3} \pm 0{\cdot}27 \times 10^{-3}$ cm^3 min^{-1} cmH$_2$O^{-1}. In the higher pressure range, $d\dot{Q}_s/dP_j$ was $3{\cdot}30 \times 10^{-3} \pm 0{\cdot}29 \times 10^{-3}$ cm^3 min^{-1} cmH$_2$O^{-1}. The ratio of the two slopes averaged 6·9 ± 2·3 (range, 1·54–17·6), showing that a substantial increase in the hydraulic conductance was produced as expected. The increase in slope was similar to that in an earlier series (Levick, 1979; mean slope increase, 5·7 times; range, 2–12) and the present results were broadly similar in other respects to earlier series as summarized in Table 1.

The slope $d\dot{Q}_s/dP_j$ at low pressures is a measure of the composite conductance of the synovial lining (K_l in Fig. 1), whereas the observed slope $d\dot{Q}_s/dP_j$ above yield pressure depends on the rate of increase of conductance with pressure and is not itself the conductance of the lining (eqn (4A) of Knight & Levick, 1985). The conductance of the lining at 25 cmH$_2$O (K_{25}) was estimated as the slope of a straight isoconductive line constructed between the flow at 25 cmH$_2$O and zero flow (see Fig. 1) as described previously (Levick, 1980). The increase in hydraulic conductance of the tissue taken for biochemical analysis, K_l/K_{25}, averaged 5·23 ± 1·5, with a range of 1·3–11·8.

Effect of perfusion on tissue concentration of GAGs and collagen

Chondroitin sulphates. Two different chondroitin sulphates, namely C4S and C6S, were detected in the electrophoretogram from perfused synovium, as in control tissue. In control synovium, C4S predominated in a ratio of 7·3 (±0·5) C4S to 1 C6S. In the perfused synovium the ratio of C4S to C6S was slightly greater, namely 9·6 (±1·2) to 1 ($0{\cdot}10 > P > 0{\cdot}05$, Wilcoxon test; $P = 0{\cdot}052$, paired t test). Evidence for the existence of separate proteoglycan species, one carrying chiefly C4S side-chains and the other C6S, is described in the companion paper (Price *et al.* 1996).

The concentration of both species of chondroitin sulphate was markedly reduced in perfused synovium in every case (Fig. 2*A*). The total chondroitin sulphate concentration in control synovium was 0·588 ± 0·046 mg (ml synovium)$^{-1}$, while in perfused synovium the total chondroitin sulphate concentration was reduced to 0·414 ± 0·040 mg (ml synovium)$^{-1}$, or 70·4% of the control value ($P = 0{\cdot}03$, t test).

Heparan sulphate. The concentration of heparan sulphate was likewise reduced in every pair of joints (Fig. 2*B*),

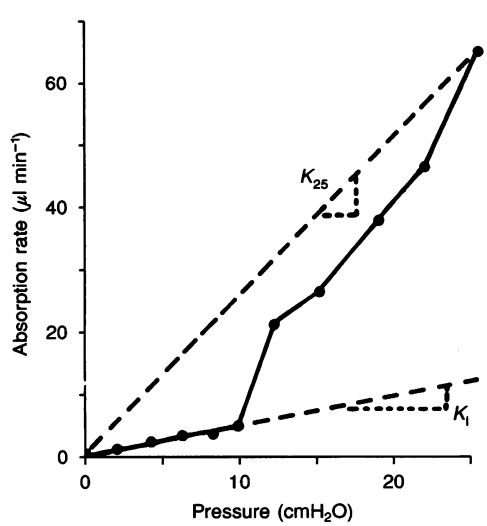

Figure 1. Effect of intra-articular pressure on rate of absorption of Krebs solution by rabbit knee

Values were obtained after 15–20 min infusion at constant pressure and show a steepening above 10 cmH$_2$O. Slopes of dashed lines represent hydraulic conductances of the synovial lining (absorption pathway) at low pressure (K_l) and at 25 cmH$_2$O (K_{25}).

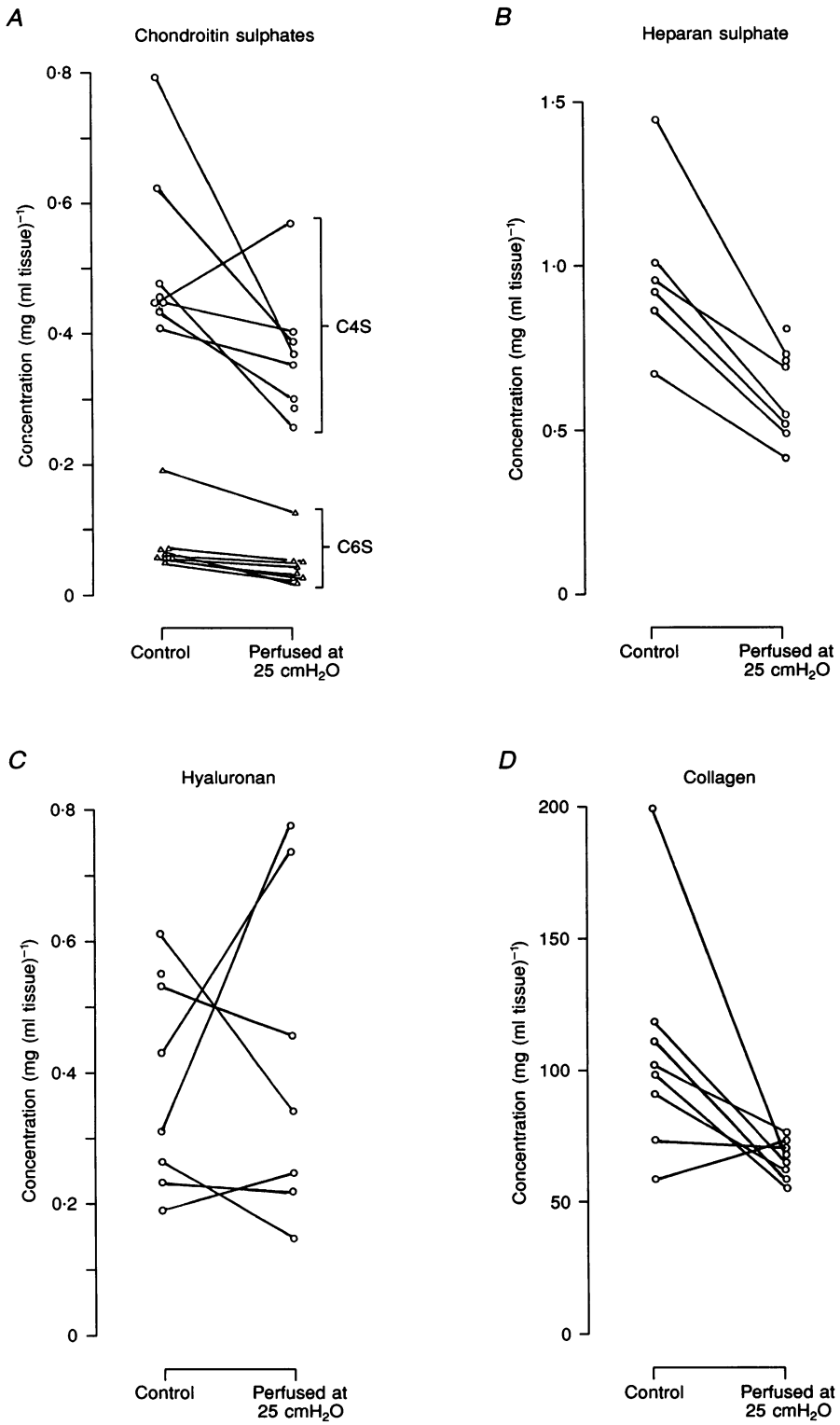

Figure 2. Comparison of C4S, C6S, heparan sulphate, hyaluronan and molecular collagen concentrations in control synovium *versus* synovium perfused with Krebs solution at 25 cmH_2O

A, chondroitin sulphates (C4S, C6S); *B*, heparan sulphate; *C*, hyaluronan; *D*, collagen. Lines join values obtained from opposite knees of same rabbit. The same animal accounts for the largest difference in chondroitin sulphate and heparan sulphate concentration in *A* and *B* and the second largest collagen difference in *D*.

falling from 0·983 ± 0·105 mg (ml control synovium)$^{-1}$ to 0·626 ± 0·050 mg (ml perfused synovium)$^{-1}$ (63·7% of the control value; $P < 0·01$, t test).

Hyaluronan. In marked contrast to the effect on sulphated GAGs, perfusion had no consistent effect on hyaluronan concentration, as illustrated in Fig. 2*C*. The mean concentration in control synovium was 0·392 ± 0·058 mg ml^{-1} while that in perfused synovium was 0·417 ± 0·095 mg ml^{-1} ($n = 7$); the 6% rise was not statistically significant ($P = 0·64$, t test). The possible implication of this unexpected finding regarding hyaluronan synthesis is discussed later.

Total glycosaminoglycan. The total GAG concentration (sulphated plus hyaluronan) was 26·2% lower in perfused synovium than control synovium. In interpreting this observation, it is important to consider whether interstitial volume changed. The results for collagen, described in the next section, indicate that interstitial volume did indeed increase.

Collagen. As with the sulphated GAGs, perfusion caused a significant reduction in the concentration of collagen in synovium, from 107·3 ± 15·0 mg ml^{-1} in control synovium to 67·3 ± 2·4 mg ml^{-1} in perfused synovium or 62·8% of control. Collagen concentration fell in 7/8 cases (Fig. 2*D*; $P = 0·04$, t test). Since collagen fibrils cannot be washed out of the tissue, this finding indicated an increase in tissue hydration. The change in interstitial volume implied by the change in tissue hydration was evaluated as follows.

Change in interstitial volume fraction

The volume fraction occupied by interstitium in control suprapatellar synovium is 0·66 ± 0·03, and the fraction of the interstitial volume that is occupied by synovial collagen fibrils (estimated specific volume, v_{fibril}, 1·43 ml (g collagen)$^{-1}$) is 0·20–0·23 in non-perfused tissue (Price, Mason & Levick, 1995; Price *et al.* 1996). Collagen fibrils are a fixed structural component of tissues, so the fall in collagen concentration in perfused synovium here (mean, 0·628-fold) indicated an increase in tissue volume of 1·594-fold. Since the trans-synovial flow passes through interstitium, and cell volume is not affected by isotonic Krebs solution, the increase in tissue hydration indicated an expansion of the interstitial space. From the fall in collagen concentration, mean interstitial volume fraction was calculated to increase from 0·66 to 0·787, collagen concentration being 162·6 mg (ml interstitial space)$^{-1}$ in control synovium (i.e. 107·3 mg (ml tissue)$^{-1}$/0·66) and 85·5 mg (ml interstitial space)$^{-1}$ in perfused synovium (i.e. 67·3 mg (ml tissue)$^{-1}$/0·787). The changes in perfused tissue volume, interstitial volume fraction and collagen concentration are summarized in Fig. 3, along with the changes in extrafibrillar GAG concentration described in the next section.

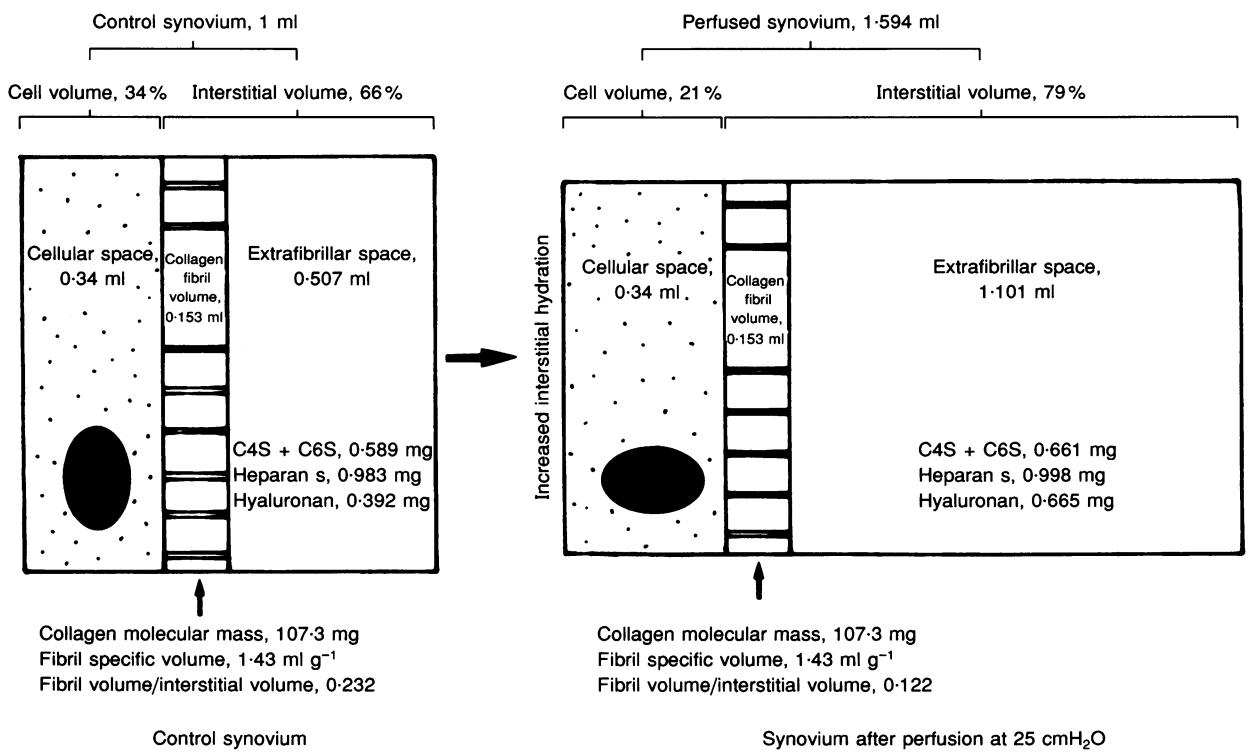

Figure 3. Average change in composition of 1 ml synovium upon raising intra-articular pressure to 25 cmH_2O by infusion of Krebs solution as indicated by results of biochemical analysis

Volume fraction of 0·66 for extracellular compartment in control tissue is taken from previous morphometric work (Price *et al.* 1995). Heparan s, heparan sulphate.

Table 2. Concentration of GAGs and collagen in synovium from control knee and from opposite joint perfused with saline to 25 cmH_2O

	Collagen	C4S	C6S	Heparan sulphate	Hyaluronan
Whole tissue					
Control (mg ml^{-1})	107·3 ± 15·0 (8)	0·511 ± 0·046 (8)	0·078 ± 0·017 (8)	0·983 ± 0·105 (6)	0·392 ± 0·058 (8)
Perfused at 25 cmH_2O (mg ml^{-1})	67·3 ± 2·4 (8)**	0·367 ± 0·034 (8)*	0·048 ± 0·012 (8)**	0·626 ± 0·050 (8)**	0·417 ± 0·095 (7)†
Interstitium ‡					
Control (mg ml^{-1})	162·6	0·774	0·118	1·489	0·594
Perfused at 25 cmH_2O (mg ml^{-1})	85·5	0·466	0·061	0·795	0·530
Perfused/control (%)	52·6	60·2	51·7	53·4	89·2
Extrafibrillar space §					
Control (mg ml^{-1})	—	1·009	0·154	1·941	0·774
Perfused at 25 cmH_2O (mg ml^{-1})	—	0·531	0·069	0·906	0·603
Perfused/control (%) ‖	—	52·6	44·8	46·7	78·0

Values are means ± S.E.M., with n values in parentheses. * $P < 0·05$, ** $P < 0·01$, † $P = 0·64$; paired t test of control *versus* perfused tissue. Units of concentration are: for whole tissue, mg (ml synovium)$^{-1}$; for interstitium, mg (ml interstitium)$^{-1}$; for extrafibrillar space, mg (ml interstitial extrafibrillar space)$^{-1}$. ‡ Interstitial volume = 0·66 times tissue volume (control) or 0·787 times (perfused). § Fibril volume = 0·232 times interstitial volume (control) or 0·122 times (perfused). ‖ Total GAG perfused/control = 0·544, i.e. perfusion reduced total GAG concentration by 45·6%.

Effect of perfusion on extrafibrillar GAG concentration

Synovial hydraulic drag is dominated to a considerable degree by the concentration of biopolymers in the extrafibrillar compartment (cf. in whole tissue), so the change in extrafibrillar GAG concentration was calculated. For this purpose, the fibril volume fraction in each synovial sample was calculated as the measured molecular collagen concentration times v_{fibril} (1·43 ml (g collagen)$^{-1}$ – taken as unaltered in perfused tissue, since fibril radius is unchanged; Levick & McDonald, 1989*b*). The fibril volume fraction, 0·232 ± 0·033 of the interstitial space in control tissue, fell to 0·122 ± 0·011 in perfused tissue owing to the interstitial expansion. GAG concentrations adjusted for these changes in non-fibrillar interstitial space are summarized in Table 2. The extrafibrillar, thermodynamically effective GAG concentrations were reduced by a substantially greater factor than would appear from whole-tissue concentrations, the net GAG concentration falling by 45·6% in the perfused extrafibrillar space (cf. by 26% taking tissue concentrations). Results for individual joints are plotted in Fig. 4.

Mechanism: washout of sulphated GAGs or increased hydration?

Given that synovial collagen fibrils are unlikely to be washed out of the tissue in these experiments, and that collagen turnover time is of the order of weeks to months rather than hours, the percentage fall in interstitial collagen concentration allowed assessment of whether the change in other interstitial components was due entirely to increased hydration, or whether it required washout of poorly tethered material from the lining. The percentage fall in total chondroitin sulphate mass per millilitre interstitial space, namely by 41% (C4S, 40%; C6S, 48%), was similar to the fall in collagen mass per millilitre interstitial space, namely by 47% (differences not significant; $P = 0·75$ for C4S, $P = 0·39$ for C6S, paired t test). This indicated that increased tissue hydration rather than washout of weakly anchored chondroitin sulphate accounted for most of the observed reduction in concentration. Interstitial heparan sulphate concentration fell by the same amount as collagen, viz. by 47%, so increased tissue hydration rather than washout could again account fully for the observed change. The possibility that, in addition to dilution, washout plus resynthesis occurred is not excluded by these results but it seems unlikely, because this would require rapid proteoglycan core protein synthesis too. An analogous study after metabolic inhibition would be needed to assess this.

Maintained concentration of matrix hyaluronan; estimation of synthesis rate

The results for hyaluronan were suggestive of significant hyaluronan synthesis over the course of the perfusion, because the mean hyaluronan concentration did not fall significantly in perfused synovium, unlike other components. The hyaluronan concentration within interstitium after perfusion changed by between +4% (calculated as the mean of individual percentage changes) and −10·8% (calculated as the percentage difference between mean concentrations), in contrast to the 40–48% reductions in interstitial collagen and sulphated GAG concentrations. The probability of the hyaluronan results being attributable to matrix dilution without fresh synthesis was low, between 0·11 (comparison of hyaluronan changes with C6S dilution results; paired t test) and 0·21 (hyaluronan changes *versus* collagen dilution

results), though the conventional rejection level of 0·05 was not reached.

Since other work has indicated that the whole sheet of synovium in a rabbit limb joint secretes around 6–8 μg hyaluronan h^{-1} into the cavity (Denlinger, 1982; Knox, Levick & McDonald, 1988), it seemed of value to ask what rate of synthesis of matrix hyaluronan would account for the present results. The hyaluronan mass in 1 ml control synovium was 0·392 ± 0·058 mg. Perfusion increased the volume of each millilitre of tissue to 1·594 ml (calculated from collagen dilution), and the hyaluronan concentration became 0·417 ± 0·095 mg (ml tissue)$^{-1}$. The mass of hyaluronan present in 1·594 ml perfused tissue was therefore 0·665 ± 0·151 mg (Fig. 3). This was an average increase of 0·273 mg hyaluronan (70%) over and above the mass of hyaluronan originally present in 1 ml control synovium. Since the perfusion experiment took on average 3 h, the minimum hyaluronan synthesis rate was therefore of the order of 91 μg h^{-1} (ml synovium)$^{-1}$ under the prevailing conditions (raised pressure, stretched hydrated tissue). The estimate is considered a minimum because no account is taken of any putative hyaluronan washout over the 3 h. The result is similar to the previous estimates of 6–8 μg hyaluronan per whole joint when joint lining volume is taken into account (see Discussion: Comparison with hyaluronan secretion into synovial fluid).

Relation between change in synovial conductance and change in extrafibrillar GAG concentration

The relations observed experimentally in individual animals are shown in Fig. 4, along with relations for chrondroitin sulphate proteoglycan and hyaluronan *in vitro* from the literature. For the control points, the extrafibrillar GAG concentration in the control joint is plotted against the synovial lining conductance, K_l, measured on the opposite, perfused side at low pressures during the early part of the experiment; there is evidence that K_l is the same in a pair of joints from the same animal, although it varies substantially between animals (Knight & Levick, 1985). GAG concentration at the end of the perfusion experiment is plotted against K_{25} in the same joint at the end of the experiment. The two points from the same animal (control and perfused) are connected by an arrowed line in the

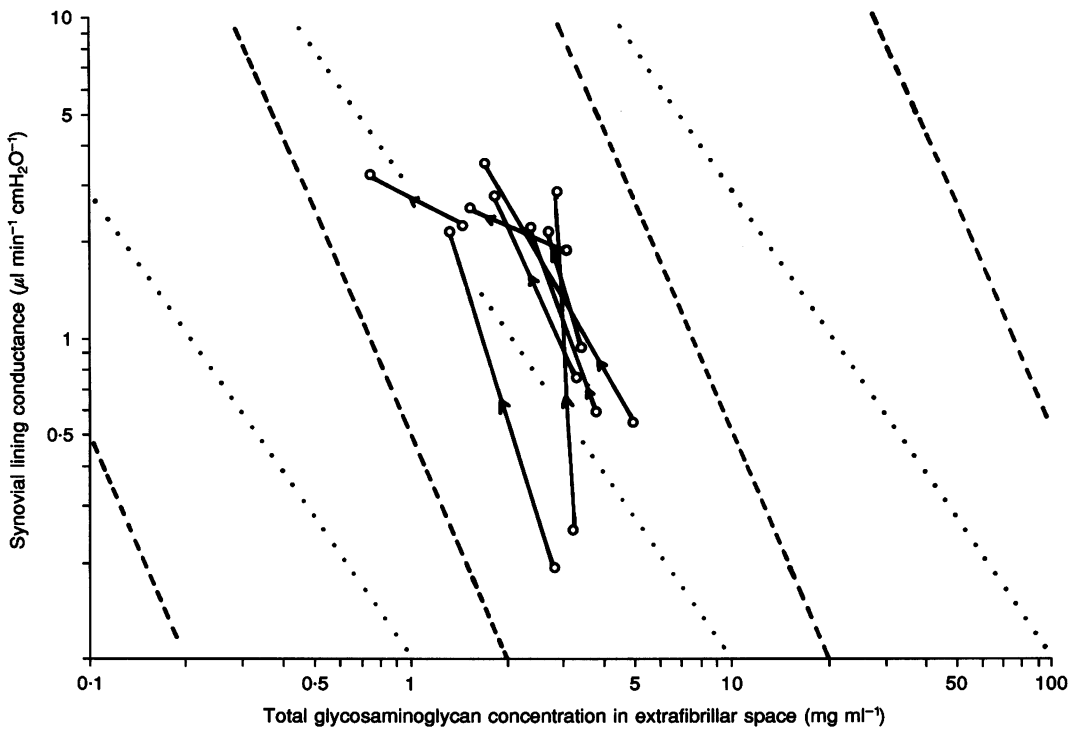

Figure 4. Relation between hydraulic conductance and GAG concentration on a log–log plot

Each continuous line joins values obtained from opposite knees of the same rabbit. The lower value in each case is synovial lining hydraulic conductance below yield pressure and control biochemical value from the opposite joint (sum of C4S, C6S, heparan sulphate and hyaluronan concentrations, corrected for cell and collagen fibril volumes), while the upper value is synovial lining conductance at 25 cmH_2O and corresponding biochemical value. The slopes of the parallel sets of background lines show the rate of change of conductance of a pure GAG network with concentration *in vitro*; dashed lines refer to chondroitin sulphate proteoglycan (eqn (1); slope, −2·354) and dotted lines to hyaluronan (data from Preston, Davies & Ogston, 1965; slope, −1·485). The absolute position (cf. slope) of the *in vitro* lines depends on additional factors such as area and path length, because the ordinate here is conductance rather than specific hydraulic conductivity.

figure. Increases in conductance were clearly related to decreases in GAG concentration, although the slope of the experimental line showed considerable scatter. An unpaired scattergram of all conductance values *versus* all [GAG] values did not achieve a statistically significant correlation because of the substantial inter-animal variation.

In Fig. 4, the results are plotted on a log–log scale because the relation between the logarithm of conductivity (conductance of a unit cube of material) and the logarithm of biopolymer concentration *in vitro* is linear, with a slope of −1·485 (hyaluronan; data of Preston *et al.* 1965) through −1·7 (tissue and synthetic polymers; Bert & Fatt, 1970) to −2·354 (C4S, C6S and large chondroitin sulphate proteoglycan; Zamparo & Comper, 1989). These slopes *in vitro* are indicated by the guidelines in Fig. 4. In interpreting the relation *in vivo*, however, it is necessary to take account not only of matrix dilution but also of stretching of the lining, as considered in the Discussion.

Changes in subsynovium

The possibility of hydrational changes during the sampling of the formless areolar subsynovium was discussed by Price *et al.* (1996), but, because the same sampling procedure was used for both control and perfused subsynovium, a comparison of the two states will be made.

Mean collagen concentration in subsynovium fell by 33%, from 122·6 ± 18·2 mg g^{-1} in control samples to 82·38 ± 18 mg g^{-1} after trans-synovial perfusion ($P < 0·01$, paired t test; $n = 6$). This indicated increased hydration of the subsynovium. In support of this, morphometry has shown that mean subsynovial thickness in the suprapatellar areolar region expands from 100 ± 7 μm in saline-perfused joints at 5 cmH_2O to 169 ± 13 μm after perfusion to 25 cmH_2O (Levick & McDonald, 1989*a*), which in itself would produce a fall in matrix concentration to 59·2% of control (cf. observed fall in collagen concentration to 63·6 ± 6·5% of control). Similarly, capillary density in suprapatellar subsynovium at 25 cmH_2O was reduced to 51% of the control value.

In control subsynovium, the ratio of C4S to C6S was 18·0 (± 2·7) to 1 and the total chondroitin sulphate concentration was 0·333 ± 0·024 mg (g sample)$^{-1}$. In subsynovium collected after perfusion the concentration was reduced by 32% to 0·226 ± 0·019 mg (g sample)$^{-1}$ ($P < 0·01$, t test; $n = 8$) and C6S was detectable in only one sample. The net fall in concentration was very close to the 33% fall in mean collagen concentration. Heparan sulphate concentration was also reduced substantially, from 0·710 ± 0·081 mg g^{-1} (control) to 0·542 ± 0·063 mg g^{-1} (perfused; $n = 5$).

Hyaluronan, in contrast to sulphated GAG and collagen, showed no significant reduction in concentration after trans-synovial perfusion. Taking the five animals where both control and perfused sample results were available from the same animal, the hyaluronan concentration in the perfused subsynovium was 1·04 ± 0·22 times that in control subsynovium ($P = 0·55$, paired t test). Taking all cases, the hyaluronan concentration in control subsynovium was 0·526 ± 0·105 mg g^{-1} ($n = 5$) and that in perfused subsynovium was 0·451 ± 0·045 mg g^{-1} ($n = 7$; 14% difference not significant, $P = 0·54$, unpaired t test). This contrasted with the significant 33% fall in collagen concentration. The probability of the hyaluronan results being explicable solely by matrix dilution (cf. addition of new material) was low, namely 0·11 (paired comparison of percentage fall in hyaluronan concentration with percentage fall in collagen concentration; $n = 5$). The maintained hyaluronan concentration in subsynovium could be due partly to addition of hyaluronan secreted by synovium and washed through into the subsynovium, and/or to subsynovial fibroblasts increasing their hyaluronan synthesis rate to the level required to maintain the normal hyaluronan concentration.

DISCUSSION

Interstitial volume expansion

The fall in concentration of the fixed interstitial component, collagen, indicated a 1·90-fold increase in the absolute interstitial volume within synovium (Fig. 3), raising the fractional interstitial volume ($V_{V,int}$; full thickness) from 0·66 to 0·787. Structural evidence on this point is equivocal. By ultrastructural morphometry, $V_{V,int}$ between the surface and a parallel plane 5 μm below the surface (cf. full thickness) increased from 0·54 ± 0·05 in control synovium and 0·61 ± 0·05 in synovium at 5 cmH_2O to 0·67 ± 0·02 at 25 cmH_2O intra-articular pressure (Levick & McDonald, 1989*b*); the $V_{V,int}$ values at 0–5 μm are smaller than full-thickness values because of a gradient in cellularity across the lining (Price *et al.* 1995). Although the rise in $V_{V,int}$ with perfusion did not achieve significance in that study upon analysis of variance ($n = 5$), an increase in superficial $V_{V,int}$ from 0·54 (control) to 0·67 at 25 cmH_2O would entail a 1·73-fold increase in superficial interstitial volume, which is similar to the full-thickness increase indicated by the collagen results (1·90-fold). Also, there was other, statistically significant structural evidence for interstitial expansion after perfusion of suprapatellar synovium, namely a significant increase in the proportion of the surface plane occupied by interstitium (interstitial area fraction), from 0·27 ± 0·02 (control) and 0·34 ± 0·02 (intra-articular pressure, 5 cmH_2O) to 0·53 ± 0·01 after perfusion to 25 cmH_2O intra-articular pressure (McDonald, 1988; McDonald & Levick, 1988). The biochemical and morphometric results are thus broadly compatible.

One apparently contradictory structural result was that synovial capillary density fell by only 3% after perfusion to 25 cmH_2O ($n = 5$; not significant; Levick & McDonald, 1989*a*). The wide coefficient of variation (33% at 25 cmH_2O), however, may have impaired detection of increased hydration. A power calculation for a presumptive difference in tissue capillary density of 37% (inferred from

Fig. 3) shows that an n value of 18 is required to demonstrate such a difference at a P value of 0·05 and power of 0·9, given a coefficient of variation of 33% – whereas n in the study was only 5. It is also possible that tissue processing caused greater extraction of water from the overhydrated tissue at 25 cmH_2O (and hence greater shrinkage) than from normally hydrated tissue.

Synovial interstitial compliance

The present results suggest a relatively tight (low) compliance for synovial interstitium in comparison with areolar connective tissue. From published compliance curves (pressure–volume relation) for the areolar connective tissue of dog subcutis, interstitial volume increases by around 640% when interstitial pressure is raised acutely to 25 cmH_2O (Guyton, Taylor & Granger, 1975, their Figs 6 and 7), giving an overall compliance of 25·6 ml dl^{-1} cmH_2O^{-1} (i.e. 640 ml (100 ml)$^{-1}$ (25 cmH_2O)$^{-1}$). Here, by contrast, interstitial volume increased by only 90% when pressure on one side of the tissue was raised from control pressure (−5·7 cmH_2O; Knox *et al.* 1988) to 25 cmH_2O. Even if mean pressure within the lining increased only by 12·5 cmH_2O (pressure being approximately atmospheric on the hydrated subsynovial side; Price *et al.* 1996), the average synovial interstitial compliance *in situ* was only 4·9 ml dl^{-1} cmH_2O^{-1} (i.e. 90%/(12·5 + 5·7)), which is almost an order of magnitude smaller than average areolar interstitial compliance over this pressure range (41·6 ml dl^{-1} cmH_2O^{-1} at 0–12·5 cmH_2O; Guyton *et al.* 1975). Both the biochemical and morphometric results thus indicate that synovial interstitium *in situ* has a substantially tighter knit, less expandable structure than areolar connective tissue.

The hydration of a stressed, deformable hydrated sheet is set by the interplay of mechanical factors (the hoop stress set up by a transmural pressure difference, which in itself should tend to wring water out of the matrix) and hydraulic factors (flow into and through the matrix, which in itself should tend to hydrate the matrix). This was demonstrated by the aortic wall studies of Tedgui & Lever (1987). When the aortic lumen was pressurized with air, the hoop stress of the wall compacted the tissue and reduced interstitial hydration; similarly, raising transmural pressure across an isolated lens capsule stretches the membrane but reduces its hydration (Fisher, 1982). When the aorta was pressurized with water, however, the concomittant hydraulic flow through the interstitium partially prevented compaction; and when the aorta was de-endothelialized (a state closer to that of the synovial lining), the transmural flow even increased the hydration of the stressed wall, as seen in synovium here.

Did the perfused sample lose part of its raised hydration over the 30–45 min depressurized period between opening the joint and freezing the excised sample? Physiological experiments have been conducted in which the joint lining was subjected to high pressures (e.g. 25 cmH_2O) and then the pressure was lowered to just above atmospheric; the lining conductance was found to remain supranormal over the time course of its subsequent determination at low pressure (an hour or so; Edlund, 1949; Levick, 1979). This finding indicates that there is no rapid, complete return to normality. If there were a partial loss of the 'excess' fluid from the perfused interstitium upon depressurization (harvesting), the true fall in interstitial matrix concentration at 25 cmH_2O would have been even more pronounced than found here, reinforcing the main conclusion.

Hyaluronan synthesis rate *in vivo*

Hyaluronan has been identified in the synovial lining by immunohistology, and at least some of this hyaluronan must be bound there, because the tissue is rich in hyaluronan-binding CD44 receptors (Henderson, Edwards & Worrall, 1994) and type VI collagen, which also binds hyaluronan (Kielty, Whittaker, Grant & Shuttleworth, 1992). The hyaluronan is secreted chiefly by synovial fibroblast-related B cells ('synoviocytes'), which possess the necessary enzymic machinery (Edwards, 1994) and secrete hyaluronan *in vitro* when grown out from excised periarticular tissue (Daireaux, Redini, Loyau & Pujol, 1990; Haubeck, Kock, Fischer, Leur, Hoffmeister & Greiling, 1995).

The calculated hyaluronan synthesis rate *in vivo* in this study was of the order of $\geqslant 91$ $\mu g\ h^{-1}$ (ml synovium)$^{-1}$, which corresponds to replacement of the entire hyaluronan mass within synovium in 3·6 h or less (though the rate here may have been elevated by the prevailing conditions). From the data now available we can evaluate the hyaluronan synthesis rate per synoviocyte *in vivo* under the conditions prevailing (raised pressure, stretched cells). Synovial cell volume fraction is 0·31 and the fibroblast-related synoviocytes form 67% of the cell population (Levick & McDonald, 1989*b*). The interstitial hyaluronan secretion rate was therefore $\geqslant 438$ $\mu g\ h^{-1}$ (ml synoviocyte)$^{-1}$, or $\geqslant 0{\cdot}04\%$ of the cell's own mass per hour. In the next two sections this estimate is compared with the synthesis rate by cultured cells *in vitro*, and with the rate of secretion of hyaluronan into synovial fluid *in vivo*.

Comparison with cultured cells

Skin fibroblasts *in vitro* secrete hyaluronan, estimated as uronic acid, at 0·264–0·267 $\mu g\ h^{-1}$ (10^6 cells)$^{-1}$ (Hopwood & Dorfman, 1977; simian virus transformed cells) to 0·06 $\mu g\ h^{-1}$ (10^6 cells)$^{-1}$ (normal skin fibroblasts). It is interesting to note, in light of the failure of synovium to maintain the sulphated GAG concentration, that sulphated GAG was secreted at only 6% (transformed cells) to 26% (normal cells) of the hyaluronan rate. Cultured fibroblasts from synovium likewise secrete predominantly hyaluronan (Clarris, Fraser, Muirden, Malcolm, Holmes & Rogers, 1984; Daireaux *et al.* 1990).

To allow comparison with the results in this present study, cultured cell uronic acid secretion rates were converted to hyaluronan secretion rate per gram of cell, on the basis that

cultured fibroblasts contain 0·5 mg protein $(10^6$ cells$)^{-1}$ (P. Prehm, personal communication) and that protein forms between 18% of the cell mass (Alberts, Bray, Lewis, Raff, Roberts & Watson, 1994) and 13% (rabbit synovial cells; Price *et al.* 1996). From this, the transformed fibroblasts of Hopwood & Dorfman (1977) secreted 69–96 μg uronic acid h^{-1}(g cells)$^{-1}$. Multiplying by 2·279 for the acetylglucosamine content of hyaluronan (polymer of *N*-acetylglucosamine-glucuronic acid; Cleland, 1984), the hyaluronan synthesis rate *in vitro* was 0·013–0·018% of the cell mass per hour. For non-transformed skin fibroblasts the rate was about a quarter of this. For fibroblasts cultured from human synovium/subsynovium, a somewhat higher initial hyaluronan secretion rate of ~1·67 mg h^{-1} (g cell protein)$^{-1}$ was reported by Nagata, Matsumura, Motoyoshi, Yamasaki, Fukuda & Tanaka (1992), which tallies with a report that synovial but not dermal fibroblasts can maintain high uridine diphosphoglucose dehydrogenase activity in culture (Edwards, 1995). For a cell protein content of 13–18% (w/w), the data of Nagata *et al.* (1992) indicate a hyaluronan secretion rate *in vitro* of 0·022–0·030% of cell mass per hour. A broader range, equivalent to 0·01–0·11% h^{-1}, was reported by Sisson, Castor & Klarons (1980) for cultured human synoviocytes stimulated by various activating factors. The estimate here of synovial matrix hyaluronan synthesis during a perfusion experiment *in vivo*, 0·04% of the cell mass per hour, is thus of similar magnitude to that for synoviocytes *in vitro*.

The restoration of a normal interstitial hyaluronan concentration after interstitial volume expansion implies not only a faster secretion of hyaluronan than proteoglycan but also a feedback system promoting homeostasis of interstitial composition. Hopwood & Dorfman (1977) noted that the hyaluronan (but not dermatan sulphate) secretion rate per cell was inversely proportional to cell density; reducing cell density increased the hyaluronan synthesis rate, again implying control by negative feedback. Similarly, Nagata *et al.* (1992) noted that the hyaluronan synthesis rate decreased progressively as the hyaluronan concentration approached a plateau. Both sets of observations imply that our estimate of the hyaluronan synthesis rate *in vivo* could represent a stimulated rather than basal rate. Our results, coupled with the results *in vitro*, imply an ability of synoviocytes to detect changes in interstitial hyaluronan concentration (presumably via a cell-surface receptor system; Underhill, 1989; Mason, Crossman & Sweeney, 1989) and to respond by regulating the activity of hyaluronan synthase. Hyaluronan synthase is a cell membrane enzyme whose activity is regulated both via induction of transcription and by kinase-induced phosphorylation (Prehm, 1989). The cell-surface RHAMM receptor for hyaluronan appears to be a possible signal transducer because it influences the intracellular second messenger level and also, within a minute, the activity of the phosphorylating enzyme tyrosine kinase (Hall, Wang, Lange & Turley, 1994). There is similar evidence that a fast-responding hyaluronan homeostatic mechanism exists for other tissues too; in lung, skin and small intestine, raising interstitial flow increases the rate of washout of hyaluronan, yet tissue hyaluronan mass is preserved (Fraser & Laurent, 1989; Reed, Townsley, Zhao, Ishibashi & Laurent, 1994; Østgaard & Reed, 1994).

Comparison with hyaluronan secretion into synovial fluid

The rate of secretion of hyaluronan into the joint cavity, as opposed to synovial interstitium, is of the order of 8 μg h^{-1} for the entire lining of the rabbit knee (Denlinger, 1982) and 6 μg h^{-1} for the rabbit elbow and shoulder (Knox *et al.* 1988) – the latter work involving no intra-articular distension. The secretion rate is equivalent to a turnover time for synovial fluid hyaluronan of 20–28 h. To compare this with the above estimate of secretion into interstitium ($\geqslant$ 91 μg h^{-1} (ml synovium)$^{-1}$; interstitial hyaluronan turnover time, 3·6 h), the volume of the synovial lining of the entire joint is required. The synovial area in a rabbit knee is estimated as 13·5–18·5 cm^2 (Levick, 1979) and 11·8–20·4 cm^2 (Knight & Levick, 1983), while the average thickness, weighted for sites, is $17{\cdot}8 \times 10^{-4}$ cm (Knight & Levick, 1983). The volume of synovium in a rabbit knee is thus 0·021–0·036 cm^3 and the mass is 23–39 mg (density, 1·073 g ml^{-1}). From these values and the present biochemical results, the interstitial secretion rate for the entire synovial lining of the knee under the prevailing conditions (raised pressure, stretched hydrated tissue) is between $\geqslant$ 1·91 and $\geqslant$ 3·28 μg h^{-1}, i.e. the same order of magnitude as previous estimates of secretion rate into synovial fluid, namely 6–8 μg h^{-1}. As noted earlier, this estimate of interstitial secretion rate must be considered a minimum for the prevailing conditions, because washout of hyaluronan into subsynovium and lymphatics may have been occurring.

The possibility of a contribution by hyaluronan from synovial fluid (cf. fresh synthesis) to the rise in interstitial hyaluronan mass over the course of the experiment needs to be considered. As described in Methods, the cavity was flushed with Krebs solution for 2 min to wash out endogenous synovial fluid prior to commencement of the pressure–flow study and thus reduce such a possibility. Also, if most of the hyaluronan had come from the cavity, it would be a surprising coincidence to find that the rate of gain was very similar in magnitude to the rate of secretion of hyaluronan by cultured synoviocytes, and by synovial lining cells into the joint cavity in the studies of Denlinger (1982) and Knox *et al.* (1988).

Reduction in sulphated GAG concentration

A fall in synovial interstitial biopolymer concentration upon perfusion to 25 cmH_2O was predicted by a model of trans-synovial flow (Levick, 1991) and was confirmed here for sulphated GAGs. Synovial sulphated GAGs are normally secreted as part of proteoglycans, as indicated by their large exclusion volumes on gel chromatrography (Castor, Roberts, Hossler & Bignall, 1983; molecular mass >750 000 Da). Smaller proteoglycans found in synovium include decorin

and biglycan (Worrall, Wilkinson, Bayliss & Edwards, 1992), which are bound by type VI collagen (Bidanset, Guidry, Rosenberg, Choi, Timpl & Hook, 1992), amongst other things. The sulphated GAG synthesis rate was evidently not fast enough to maintain a normal concentration over the 3 h period. The rate-limiting factor is probably the rate of synthesis of the proteoglycan core protein rather than the GAG side-chains (Castor *et al.* 1983).

Relation between reduced biopolymer concentration and increased hydraulic permeability

The net fall in interstitial GAG concentration (inclusive of hyaluronan) was associated with a large rise in synovial hydraulic conductance, and must have contributed to the latter because interstitial conductance is inversely related to matrix concentration (Bert & Fatt, 1970). Another contributory factor, however, is the effect of joint distension on membrane geometry, namely increased synovial surface area and reduced thickness. These geometrical changes are generally insufficient to explain fully the increased conductance (Levick, 1991), so the question arises as to whether the fall in interstitial biopolymer concentration observed here is sufficient to explain the component of increased conductance not attributable to stretch.

One problem in addressing this question is that GAG concentration alone (~4 mg (ml extrafibrillar space)$^{-1}$; control) is substantially less than the total non-collagen biopolymer concentration needed to generate control synovial hydraulic resistance, viz. ~14 mg biopolymer (ml extrafibrillar space)$^{-1}$ (Levick, 1994). The discrepancy may be due, at least in part, to the presence of additional biopolymers such as structural glycoproteins and proteoglycan core protein in the interstitium, as discussed by Price *et al.* (1996). The problem can, however, be couched in terms that allow a partial answer here, namely: was the fractional dilution of the interstitial matrix sufficient to explain the increase in conductance that is not due to geometrical deformation? In other words, if all other biopolymers in the extrafibrillar matrix (glycoproteins etc.) were diluted to the same average extent as those assayed here, namely to 0·544 of control (Table 2, mean), would the increase in conductance at 25 cmH_2O be accounted for?

For a GAG matrix or a swelling biological membrane the relation between concentration, C, and specific hydraulic conductivity, κ, takes the form:

$$\kappa = aC^{-b}, \quad (1)$$

where b is between 1·7 (data of Bert & Fatt, 1970; swelling biological membranes) and 2·354 (chondroitin sulphate proteoglycan data of Zamparo & Comper, 1989). Thus, a 0·544-fold decrease in concentration can be expected to

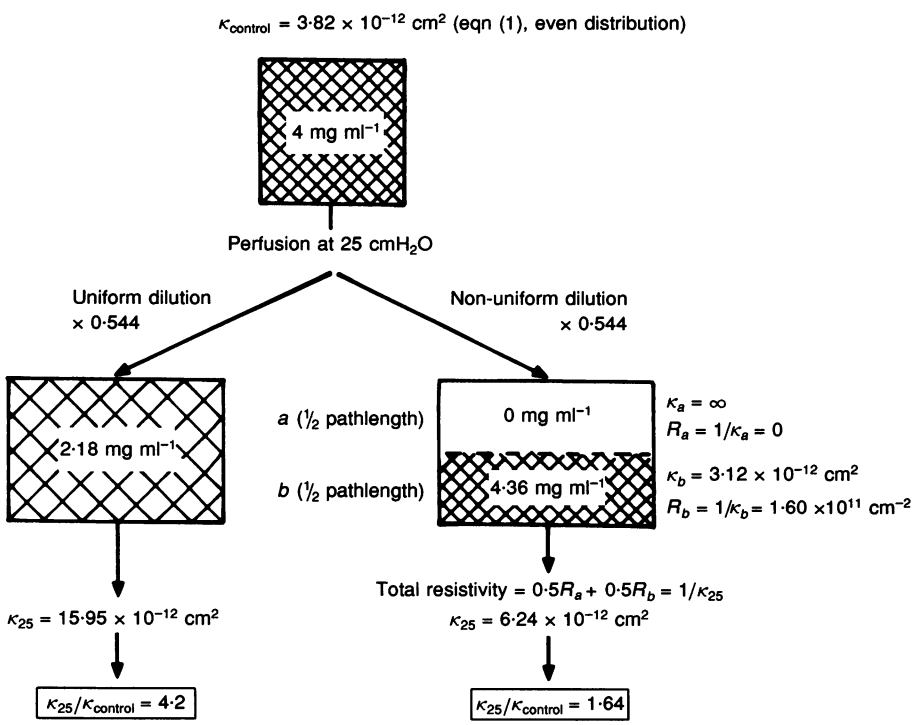

Figure 5. Hydraulic consequences of a uniform dilution of the interstitial polymer matrix *versus* non-uniform dilution in axis normal to flow

In the right-hand panel the total GAG concentration is reduced by the same factor as on the left (0·544, experimental result), but a non-uniform distribution in the direction of flow has been introduced. Hydraulic conductance (κ) increases by a smaller factor for the non-uniform distribution than for the uniform distribution. Numerical values are calculated from eqn (1) for $a = 8{\cdot}66 \times 10^{-18}$ and $b = 2{\cdot}354$ (chondroitin sulphate proteoglycan data of Zamparo & Comper, 1989). R, specific hydraulic resistivity.

cause a 2·8- to 4·2-fold rise in hydraulic conductivity. The actual rise in conductance here, from below yield pressure to 25 cmH_2O, was 5·23-fold, of which stretch alone can account for a 2·67-fold rise (Levick, 1991). If the 2·67-fold, stretch-induced rise was simply multiplied by the increase predicted from uniform matrix dilution, an increase in conductance of 7·7- to 11·2-fold would be predicted. On this first-order assessment, therefore, the observed matrix dilution factor is not only large enough to explain the non-geometric component of the conductance rise but even appears excessive, the observed rise in conductance being only 5·23-fold.

One probable reason for the above overprediction is that the first-order calculation applies to a uniformly distributed material, whereas in synovium perfused with saline at 25 cmH_2O a biopolymer concentration gradient may develop normal to the surface, with the lowest concentration closest to the perfused joint cavity (Levick, 1991). Because the relation between resistivity ($1/\kappa$) and concentration is non-linear (eqn (1)), and resistances in series summate, the increase in conductance (reciprocal of sum of resistances) induced by a rise in hydration is substantially less when a concentration gradient develops than when concentration is uniform. This is illustrated for a simple two-zone case in Fig. 5. If the proteoglycan concentration were to decrease uniformly to 0·544 of control, conductance would rise 4·2-fold (eqn (1), using proteoglycan parameters; Fig. 5, left). If, however, the same average dilution were accompanied by a downstream shift of the entire proteoglycan mass into the downstream half of the tissue (for the purpose of this illustration), the overall conductance would rise only 1·64-fold (Fig. 5, right). This simplified example shows that the imposition of a matrix concentration gradient normal to flow by hydraulic drag can considerably attenuate the rise in conductance that accompanies matrix dilution, and this may reconcile the observed mean matrix dilution and stretching with the size of the conductance increase. No histochemical evidence bearing on this issue is available at present.

Conclusion

The results show that the increased synovial conductance upon perfusion with saline at high pathological pressures (yield phenomenon) is caused partly by a reduction in interstitial GAG concentration. It remains to be determined whether synovial matrix dilution occurs progressively with increase in intra-articular pressure above yield pressure, in parallel with hydraulic conductance. Certainly, in de-endothelialized aorta wall the albumin distribution volume (indicative of matrix hydration) increased with increasing intraluminal pressure and transluminal flow (Tedgui & Lever, 1987). It may also be asked whether matrix dilution might have actually occurred here at low pressure due to the introduction of saline into the joint cavity. Further analyses could answer this, but two pointers against it already exist. First, the control tissue was in fact exposed to saline at atmospheric pressure for some minutes prior to sampling (see flushing procedure, Methods), so exposure to saline *per se* does not explain the difference observed. Second, there is limited evidence that synovial lining conductance at subatmospheric pressure in joints not perfused with saline is similar to that in joints perfused with saline below 9 cmH_2O (Knight & Levick, 1985), which would be unlikely if exposure to saline *per se* increased hydration (and hence conductance). On the available evidence, therefore, it seems likely that substantial matrix dilution is not merely the result of exposure to saline *per se*, but requires a pathologically raised intra-articular pressure.

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