Effect of intra-articular hyaluronan on pressure–flow relation across synovium in anaesthetized rabbits

J. N. McDonald and J. R. Levick*

Department of Physiology, St George's Hospital Medical School, London SW17 0RE, UK

1. Hyaluronan is the major polysaccharide of synovial fluid, responsible for its high viscosity. The effect of hyaluronan on fluid transport across the synovial lining of the joint was investigated. Rate of fluid absorption from the joint cavity ($\dot{Q}_s$) was measured at intra-articular pressures (P_j) of up to 24 cmH_2O in knees of anaesthetized rabbits, in the presence or absence of hyaluronan in intra-articular infusates.

2. Viscometry studies *in vitro* showed that the commercial hyaluronan used had a molecular weight of 549 000–774 000, a radius of gyration of 48–99 nm and a critical concentration for molecular overlap of 1·3 g l^{-1}.

3. With intra-articular Krebs solution (control) or subnormal, subcritical concentrations of hyaluronan (0·5 g l^{-1}), flow increased with pressure. Hyaluronan reduced the fluid escape rate by reducing slope $d\dot{Q}_s/dP_j$ by 32–64% relative to Krebs solution.

4. At normal to high hyaluronan concentrations (3–6 g l^{-1}) and low pressures, hyaluronan again reduced slope $d\dot{Q}_s/dP_j$, by 39–64%. The reduction in slope was slight, however, when compared with the reduction in bulk fluidity (1/relative viscosity). Fluidity at high shear rates was reduced to 6% of control values by 6 g l^{-1} hyaluronan. The effect on slope did not correlate significantly with the effect on fluidity.

5. At pressures above ~12 cmH_2O, 3–6 g l^{-1} hyaluronan altered the shape of the pressure–flow relation: a flow plateau developed. In some joints raising pressure even reduced trans-synovial flow slightly. The pressure required to drive unit trans-synovial flow (an index of outflow resistance) increased 2·5-fold between 5 and 25 cmH_2O in the presence of hyaluronan. By contrast, in the absence of hyaluronan the outflow resistance fell as pressure was raised.

6. It is suggested that the increasing resistance to flow in the presence of hyaluronan may be caused by partial molecular sieving of hyaluronan by the small porosities of the synovial interstitial matrix, leading to accumulation of a resistive filter cake of hyaluronan chains at the tissue–cavity interface. Since hyaluronan impedes fluid escape when pressure is raised, it may serve to preserve synovial fluid volume *in vivo*, e.g. during sustained joint flexion.

Diathrodial joints depend on synovial fluid for their normal operation, the fluid supplying nutrients to the articular cartilage and lubricating the cartilage and synovial lining of the cavity. The glycosaminoglycan hyaluronan is an important lubricating component of synovial fluid (Radin, Paul, Swann & Schottstaedt, 1971; Cooke, Dowson & Wright, 1976; Roberts, Unsworth & Mian, 1982) and is secreted by the synovial lining. The joint lining, or 'synovium', is a thin sheet of vascularized mesenchyme whose function is to regulate synovial fluid volume and composition. Production of synovial fluid begins with ultrafiltration of plasma across capillaries within the synovium, and the fluid then percolates into the joint cavity through the extracellular matrix. The matrix occupies broad spaces between the cells lining the surface, and some of the cells (B cells) secrete hyaluronan into the fluid, giving it a characteristic 'synovial' quality, meaning 'with egg' (i.e. very viscous). Fluid leaves the joint cavity by flowing out through the synovial interstitial spaces when the pressure gradient is favourable, to reach a lymphatic plexus located in the subsynovium. The subsynovium is a layer of loose areolar connective tissue, adipose or fibrous tissue, depending on location.

* To whom correspondence should be addressed.

Trans-synovial flow has been studied to date by infusing physiological electrolyte solutions into the joint cavity, with or without added plasma proteins; however, hyaluronan has always been omitted in order to facilitate the unravelling of the basic hydrodynamic processes (Edlund, 1949; Levick, 1987*a*, 1994). The present work addresses the more complex but important issue of how hyaluronan affects flow across the synovial lining. Hyaluronan is a vast non-sulphated polysaccharide of molecular weight $2{\cdot}5 \times 10^6$–$2{\cdot}7 \times 10^6$ in rabbit synovial fluid, and even more in man and horse, namely 7×10^6 (Sunblad, 1953; Denlinger, 1982; Balazs & Denlinger, 1985; Dahl, Dahl, Engström-Laurent & Granath, 1985). The concentration of hyaluronan in rabbit synovial fluid is 3–5 g l^{-1} (Sunblad, 1953; Knox, Levick & McDonald, 1988). The rabbit hyaluronan molecule comprises a chain of around 8000 acetylglucosamine–glucuronate disaccharide units. The chain adopts a random coil configuration in solution, of diameter 130–210 nm, and thereby encompasses a large volume of solvent. Resistance to solvent flow through the interstices of the coiled molecule is high. At concentrations >1 g l^{-1} adjacent molecular domains overlap due to their large diameter, and the chains then form an effectively continuous network permeating the entire body of solvent. These characteristics produce a highly viscous, non-Newtonian fluid showing shear-dependent viscosity (Ogston & Stanier, 1950; Bollett, 1956; Balazs & Denlinger, 1985).

Because of hyaluronan's remarkable properties, it has long been suspected that hyaluronan might influence trans-synovial flow (Levick, 1983; Henderson & Edwards, 1987), but this has only recently been investigated experimentally. In an initial study at an intra-articular pressure of 6 cmH_2O (top of the physiological range), it was found that physiological concentrations of hyaluronan reduced the rate of fluid absorption from the rabbit knee by up to 45%, but the reduction was much less than the reduction in bulk fluidity (1/viscosity; McDonald & Levick, 1994). It was thought that this disparity might be due to partial reflection of intra-articular hyaluronan by synovium, and preliminary electron micrographs of synovium after hyaluronan infusion supported this view. In this study the action of hyaluronan is explored more fully by defining the pressure *versus* trans-synovial flow relation in the presence and absence of intra-articular hyaluronan.

METHODS

Materials

Human umbilical grade III hyaluronan (Sigma) was used for most experiments *in vitro* and *in vivo*. The hyaluronan was dissolved in Krebs solution of osmolarity 283 mosmol l^{-1} at pH 7·4 (for composition see Knight, Levick & McDonald, 1988). A broad-spectrum antibiotic (gentamycin, 40 mg l^{-1}; Roussel Laboratory, Harrow, UK) was added to preclude bacterial degradation. Since the chain length of hyaluronan is variable, the molecular weight of the commercial sample was assessed *in vitro*, together with viscosity, osmotic pressure, solvated molecular radius and critical concentration for domain overlap.

Physical measurements on hyaluronan *in vitro*

Colloid osmotic pressure measurements. An electronic membrane osmometer (Knox *et al.* 1988; Knight *et al.* 1988) was fitted with a PM10 membrane (exclusion >10 kDa; Amicon, Lexington, USA) and calibrated with a water column; observations are reported, therefore, in centimetres of water (1 cmH_2O = 98 Pa). Repeated measurements of a commercial standard (human serum albumin, 50 g l^{-1}; stated oncotic pressure 24·5 ± 0·6 cmH_2O at 22 °C; Instrumentation Laboratory Inc., Lexington, USA) gave a value of 24·5 ± 0·5 cmH_2O at 21 °C (mean ± S.D., $n = 6$). The coefficient of variation for repeated measurements using a solution of mean oncotic pressure 10 cmH_2O was 3%, rising to 6% at 2 cmH_2O. The hyaluronan solutions generated stable oncotic pressures and these were measured at 20–22 °C.

Capillary tube viscometry. Horizontal capillary viscometers of internal radius (r) 0·056, 0·174 and 0·506 mm were immersed in a water-bath at 35 °C (normal joint temperature) and operated as described previously (McDonald & Levick, 1993). The driving pressure ΔP (a water column of 0–100 cmH_2O) was measured to ±0·1 cmH_2O and results for sucrose standards of relative viscosity 1·9–37 fell within ±1·5% of tabled viscosities. Wall shear stress (S_w) and wall shear rate ($\dot{\gamma}$) were calculated as follows:

$$S_w = \Delta P r / 2l, \quad (1)$$

$$\dot{\gamma}_w = 4\dot{Q}/\pi r^3, \quad (2)$$

where l is tube length and $\dot{Q}$ is flow (Philippoff, Han, Barnett & Dulfano, 1970). The viscosities are quoted relative to the carrier medium, Krebs solution, which itself had a viscosity of 1·059 relative to distilled water at 35 °C.

Low-shear rotational viscometry. The shear rate in a capillary viscometer varies radially and very low wall shear rates are difficult to achieve. In order to measure viscosity at very low uniform shear rates (2·4–128 s^{-1}) a Low-Shear 30 concentric cylinder viscometer was employed (cup 1, bob 1; Contraves A.G., Zürich, Switzerland). The viscometer was thermostated to 35 °C and calibrated according to the manufacturer's tables.

Evaluation of intrinsic viscosity and molecular size. Intrinsic viscosity, $[\eta]$, represents the space occupied by a gram of solute at the limit of extreme dilution. In molecular terms $[\eta]$ is a measure of the ratio of effective hydrodynamic volume of a polymer molecule to its molecular weight. This ratio increases with polymer chain length (Laurent, Ryan & Pietruszkiewicz, 1960; Flory, 1971). The weight-average molecular weight of a polymer such as hyaluronan (M_w) is related to $[\eta]$ in millilitres per gram by the Mark–Houwink equation:

$$[\eta] = k M_w^{\,a}, \quad (3)$$

where k is 0·0228 and a is 0·816 for hyaluronan in 0·2 M saline (Cleland & Wang, 1970). Closely similar values are predicted from the parameters of k and a (0·012 and 0·86, respectively) determined in 0·15 M saline by Wik (cited by Bert & Pearce, 1984). The intrinsic viscosity of the grade III umbilical hyaluronan used *in vivo* was evaluated in this study from measurements of relative viscosity at a series of different concentrations and shear rates, using the dual extrapolation procedure of Haug & Smidsrød (1962) (see Results).

The effective radius of the solute in solution can be evaluated from intrinsic viscosity. According to Flory's self-avoiding random-walk model of polymer configuration, the radius of gyration, r_g, for a long neutral flexible chain in dilute solution is described by:

$$r_g^3 = [\eta] M / 8{\cdot}84 N_A, \quad (4)$$

where M is molecular weight and N_A is Avogadro's number (Flory, 1971). For an ionic polymer in an electrolyte solution r_g should be influenced by electrostatic effects and electrolyte shielding, and Johnson, Kamm, Ethier & Pedley (1987) found that the following relation described hyaluronan data:

$$r_g = 0{\cdot}025\, M^{3/5}\, S^{-0{\cdot}08}, \quad (5)$$

where S is salinity (molarity). Hydrodynamically, the isolated molecule behaves like a solid particle with a radius of 50–65% of r_g according to Johnson *et al.* (1987).

Critical overlap concentration. At low concentrations (C) the individual hyaluronan molecules are separated from each other by solvent (the dilute regime). Because of their very large volume domains, however, hyaluronan molecules come to span the entire solvent space at a certain critical concentration, C^*, and above C^* the solution contains a continuous network of polymer chains (the semi-dilute regime, see inset to Fig. 2*D*). The point of transition, C^*, depends on the volume domain of the hydrated molecule and therefore chain length. C^* can be evaluated graphically or by calculation. (i) Graphical method. Defining specific viscosity, η_{sp}, as relative viscosity minus 1, and $(\eta_{sp})_{\dot{\gamma}=0}$ as specific viscosity at zero shear rate, a plot is made of $\log(\eta_{sp})_{\dot{\gamma}=0}$ as a function of $\log C$. The onset of intermolecular coupling is marked by an abrupt steepening of this relation (Morris, Rees & Welch, 1980). (ii) C^* can also be calculated, on the principle that molecular entanglement occurs when the concentration of polymer chains in the whole solution reaches the same value as the local concentration of polymer within the volume domain of the molecule, V_d (de Gennes, 1979). This leads to:

$$C^* = n/V_d = n/(4/3)\pi r_g^3, \quad (6)$$

where n is the number of monomers per molecule and C^* here has units of number of monomers per unit volume of solution. Substitution of eqn (4) into eqn (6) and re-expression of C^* in grams per millilitre gives the simple relation: $C^*[\eta] = 2{\cdot}1$. This contrasts with the intuitive expression, $C^*[\eta] = 1$, used by Granger, Laine & Laine (1985).

Experiments *in vivo*

Overview. Krebs solution (control) was infused into the cavity of the knee joint of one hindlimb, and hyaluronan solution was infused into the opposite knee. The flow of infusate out of the joint cavity across the synovial lining was measured at a series of increasing intra-articular pressures, and the trans-synovial pressure–flow relation plotted for each knee. Three concentrations of hyaluronan in Krebs solution were investigated, namely 0·5 g l^{-1} (low, as sometimes found in arthritic effusions), 3 g l^{-1} (a typical normal value) and 6 g l^{-1} (raised); five to seven animals were studied at each concentration.

Animal preparation. As described previously (Levick, 1979) New Zealand White rabbits (2·0–3·2 kg body weight) of either sex were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, I.V.) plus urethane (500 mg kg^{-1}, I.V.), tracheostomized and maintained by smaller half-hourly doses, in conformity with animal welfare regulations. Core temperature was controlled by a Harvard animal blanket and rectal thermistor (Harvard Instruments, South Natick, MA, USA). With the animal supine the hindlimbs were secured with the knees at 100–130 deg extension, an angle naturally adopted by the relaxed limb. Two cannulae (21-gauge hypodermic needles with terminal lateral perforations) were inserted into the suprapatellar joint space (Fig. 1). One needle was connected to a Gould–Statham P23 pressure transducer (Oxnard, CA, USA) level with the joint to measure intra-articular fluid pressure (P_j, $\pm 0{\cdot}2$ cmH_2O). The other was connected to an infusion reservoir, the vertical height of which controlled P_j. Flow of test solution from the infusion reservoir into the joint cavity, $\dot{Q}_{in}$, was recorded by an intervening photoelectric drop counter (drop size 10·2 μl at flows < 300 $\mu l\ min^{-1}$). In the case of 3–6 g l^{-1} hyaluronan the infusion line was filled with hyaluronan solution only from the infusion cannula to the drop counter chamber; the reservoir and drop needle still contained Krebs solution, so that drop size was unaltered. Because the volume infused into the cavity over a 3 h experiment was only ~4 cm^3 and the intervening 150 cm of tubing had a volume of 24 cm^3, the fluid entering the cavity was not diluted during this procedure (confirmed by dye studies).

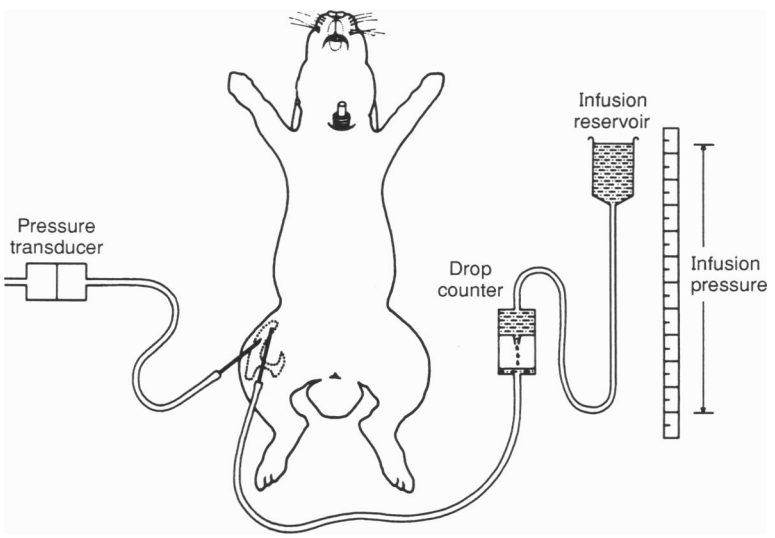

Figure 1
Preparation for studying trans-synovial flow *versus* pressure relation in an anaesthetized rabbit.

Pressure and flow were recorded on a SE6008 ultraviolet oscillograph (SE Laboratories, Feltham, UK). Correct insertion of cannulae was confirmed by dissection postmortem. Previous studies have shown that it is unnecessary to warm the infusate prior to infusion because intra-articular heat transfer is rapid and efficient (McDonald & Levick, 1993).

Calculation of trans-synovial flow, $\dot{Q}_s$. When a step rise in infusion pressure is imposed, the flow of infusate into the joint cavity consists of two phases (Fig. 3 of this study and Levick, 1979). Initially there is a fast inflow caused by elastic expansion of the cavity. This inflow declines rapidly as P_j rises to its new steady value. Once P_j has stabilized (a minute or so for Krebs or dilute hyaluronan solutions; the slower filling by more concentrated hyaluronan solutions is addressed later), there is a slower inflow that is due chiefly to trans-synovial absorption of fluid. A small part of the second phase, however, is caused by viscous creep of the cavity walls, particularly in the first few minutes. The inflow attributable to wall creep has been measured by experiments with non-absorbed oil. The residual creep still present after 15–20 min ($\dot{Q}_{creep}$, in microlitres per minute) was found to be related to intra-articular pressure by:

$$\dot{Q}_{creep} = 0{\cdot}23\,P_j + 0{\cdot}4, \quad (7)$$

where P_j is joint fluid pressure in centimetres of water (Levick, 1979). In this study, inflows recorded 15–20 min after a pressure step were corrected by subtraction of $\dot{Q}_{creep}$ to give the steady-state trans-synovial flow ($\dot{Q}_s$): $\dot{Q}_s = \dot{Q}_{in} - \dot{Q}_{creep}$. The relative magnitude of this correction depended on the absolute flow; at 60 μl min^{-1} (e.g. Krebs solution at a P_j of 20 cmH_2O) creep represented an 8% correction, whereas at 3·5 μl min^{-1} (hyaluronan solution at a P_j of 3 cmH_2O) the creep rate of 0·86 μl min^{-1} constituted a 25% correction.

Protocol for determining pressure–flow relations. In each animal one knee was infused with Krebs solution (control) and the other with test solution; the order was varied. An initial infusion of 250–500 μl raised P_j from its endogenous subatmospheric pressure to between 0 (i.e. atmospheric pressure) and 2·5 cmH_2O, the lowest pressure that generated a recordable absorption rate. Since endogenous fluid volume is only ~24 μl (Knox *et al.* 1988), endogenous fluid was diluted 10- to 20-fold and had little influence on the subsequent experiment. In some cases the cavity was then drained (to wash out the highly diluted endogenous material) and refilled, but since this produced no consistent change in the pressure–flow relation flushing was not adopted routinely. In a previous study of sixteen joints infused with hyaluronan-free solutions it was found that neither residual native hyaluronan nor hyaluronan secretion rate were large enough to alter intra-articular viscosity significantly over the 2–3 h required for an experiment (McDonald & Levick, 1993). The net rate of secretion and/or leaching of endogenous hyaluronan into rabbit limb joints is slow, of the order of 6–18 μg h^{-1} (Denlinger, 1982; Knox *et al.* 1988).

Intra-articular pressure was then increased in steps, usually 1·5–2·0 cmH_2O, by raising the infusion reservoir at 15–20 min intervals. Trans-synovial flow was calculated at the end of each period. In the case of the very viscous 3–6 g l^{-1} hyaluronan solutions, gravity-driven filling of the synovial cavity on raising the reservoir was slow. To achieve the new equilibrium pressure reasonably quickly (i.e. as quickly as for Krebs solution), a small volume of infusate was injected manually from a syringe through a 3-way tap on the infusion line; the joint was then reconnected to the infusion reservoir (Fig. 3*C*). Experiments continued until P_j had been raised to ~24 cmH_2O, which is in the range found in pathological effusions.

After the last flow was recorded (~3 h) a sample of intra-articular fluid was aspirated for viscosity measurements. This check was important because the viscosity of the hyaluronan solution tended to decrease significantly during the experiment, even in the infusion reservoir. For the 6 g l^{-1} hyaluronan solution, viscosity fell on average by 29% of the initial value over ~3 h ($P = 0{\cdot}02$; Student's paired t test), and the corresponding falls for 3 and 0·5 g l^{-1} hyaluronan were 16% ($P = 0{\cdot}03$) and 6% ($P = 0{\cdot}01$), respectively. The decrease was attributed to a slow depolymerization, not prevented by gentamycin or 1 g l^{-1} sodium azide, a potent metabolic poison.

Analysis of pressure–flow relation and other statistical methods. The problem of how best to characterize trans-synovial pressure–flow relations for saline solutions mathematically (to allow statistical comparisons) was considered by Edlund (1949) and Levick (1979). Such relations often showed a sharp bend at a certain pressure (yield pressure, P_y) with little or inconsistent curvature above and below this pressure, so that the empirical expedient of representing the relation by two straight lines, one above and the other below the yield point, has been adopted in the past. To conform with this practice, the relations for Krebs solution above and below P_y in this study were fitted by linear regression analysis, P_y being determined by inspection of the plot. If the slope change developed over a range of pressures rather than at a single pressure, the mid-range was adopted as yield point. The relation for the 3–6 g l^{-1} hyaluronan solution had a different shape and its analysis is considered further in the Results. Regression slopes were compared by Student's paired t test, with $P < 0{\cdot}05$ accepted as a significant difference. Results are expressed as means ± S.E.M. throughout.

To describe the curvilinear relations between concentration and oncotic pressure or viscosity, non-linear regression analysis by the simplex optimization method was used, as implemented in Fully Interactive Regression Statistics (Serious Statistical Software, Willaston, South Wirral, UK).

RESULTS

Observations *in vitro*

Hyaluronan oncotic pressure

The colloid osmotic (oncotic) pressures of umbilical grade III hyaluronan solutions are shown in Fig. 2*A*. At physiological concentration the osmotic pressure was very small, viz. 0·9 cmH_2O at 3 g l^{-1}. The osmotic pressure–concentration relation was non-linear (Laurent & Ogston, 1963; Shaw & Schy, 1977). The first virial coefficient for the curve was evaluated to provide an estimate of the number-average molecular weight, M_n, of the sample. Using regression of π/C on C (method of Granger *et al.* 1985) the first virial coefficient was found to be 0·206 ± 0·023 (mean ± S.E.M.). If a third-order polynomial was fitted, the coefficient was 0·141, but inspection of residuals indicated a poorer fit. The first

virial coefficient equals RT/M_n, where R is the gas constant and T the absolute temperature. From this, M_n was calculated to be 120 000–176 000.

Viscosity studies *in vitro*

Viscosities of grade III hyaluronan solutions of concentration 0·5–6·0 g l^{-1} are shown as a function of shear rate (2·4–5000 s^{-1}) in Fig. 2*B*. As expected, these non-Newtonian solutions showed a rise in viscosity at low shear rates; this was less pronounced at low concentrations. Grade I umbilical hyaluronan, which was not used *in vivo* because of its high cost, was much more viscous than grade III hyaluronan; at 3 g l^{-1} and a shear stress of 2·9 N m^{-2} (29 dyn cm^{-2}), the relative viscosity of grade I hyaluronan was 88 whereas the relative viscosity of grade III hyaluronan was only 10, indicating that the grade III preparation was relatively depolymerized. The viscosity data enabled estimation of $[\eta]$, from which an estimate of molecular size weighted towards the longer chains was obtained. To evaluate $[\eta]$, Haug–Smidsrød plots were constructed from the results in Fig. 2*B*, i.e. plots of the reduced viscosity, η_{sp}/C, as a function of $\sqrt{(\dot{\gamma}\eta_{sp}/C)}$, where η_{sp} is specific viscosity (see Methods). Linear regression analysis and extrapolation gave reduced viscosity at zero shear rate. The latter is plotted on a logarithmic scale as a function of C in Fig. 2*C*. The intercept, intrinsic viscosity at zero shear rate, $[\eta]_0$, was 1456 ml g^{-1} (95% confidence limits, 1242–1707 ml g^{-1}). When η_{sp}/C was plotted as a function of C without prior extrapolation to zero shear rate (as was common practice

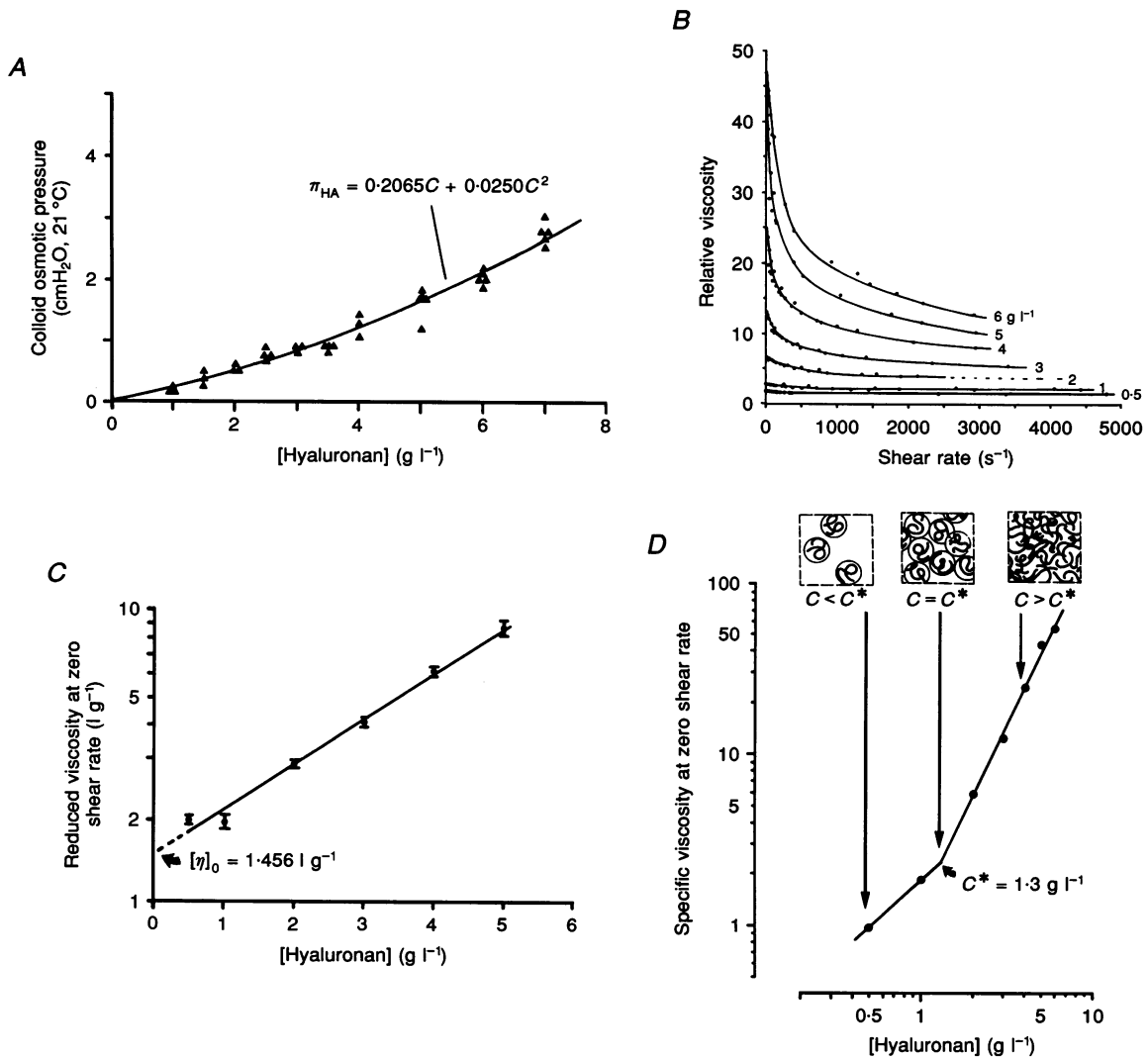

Figure 2. Physical properties of solutions of Sigma grade III umbilical hyaluronan

A, colloid osmotic pressure (π_{HA}) as a function of concentration. *B*, relative viscosity *versus* shear rate over the range of concentrations used. *C*, evaluation of intrinsic viscosity at zero shear rate, $[\eta]_0$, from intercept of plot of reduced viscosity at zero shear rate, $(\eta_{sp}/C)_{\dot{\gamma}=0}$, *versus* concentration. *D*, evaluation of critical overlap concentration (C^*) by graphical method (see text).

in earlier work on hyaluronan), the apparent intrinsic viscosity $[\eta]_{app}$ decreased as shear rate increased and was only 800 ml g^{-1} at 1000 s^{-1}. Dependence of $[\eta]_{app}$ on shear rate is characteristic of hyaluronan of true $[\eta]$ greater than 1400 ml g^{-1} according to Cleland & Wang (1970), and this is in keeping with our estimate of 1456 ml g^{-1} for this sample.

Molecular weight of hyaluronan sample

For a $[\eta]$ of 1456 ml g^{-1}, eqn (3) predicts a sample mean M_w of 774 000, which is roughly a quarter of the normal M_w of rabbit hyaluronan. A slightly lower molecular weight is indicated by the following argument. In evaluating the Mark–Howink parameters for eqn (3), Cleland & Wang (1970) did not in general use Haug–Smidsrød plots to extrapolate η_{sp}/C to zero shear rate (although a correction was applied to large M_w material, and shear rates down to 3 s^{-1} were used, so that any discrepancies between their approach and the present one may be small). Haug & Smidsrød (1962) showed that, with non-Newtonian fluids (alginate solutions), $[\eta]_{app}$ determined without extrapolating η_{sp}/C to zero shear rate was ~87% of $[\eta]_0$ when the latter was 1500 ml g^{-1}. The use of true $[\eta]_0$ in eqn (3) may therefore have led to an overestimation of molecular size. To assess this, results for η_{sp}/C at a fixed shear stress of 2·9 N m^{-2} (comparable to the shear in the larger capillary viscometer used by Cleland & Wang) were plotted against C and extrapolated to $C = 0$ without correction to zero shear rate. This gave a $[\eta]_{app}$ of 1100 ml g^{-1} and, from eqn (3), a M_w of 549 000.

Radius of molecular domain

Taking the viscometric mean M_w to be within the range 549 000–774 000, it follows that there were 1448–2042 disaccharide units (weight, 379) per molecule. Since the unit disaccharide length is 0·95 nm, the linear chain length was 1·4–1·9 μm. According to Flory's self-avoiding random-walk model for neutral polymer configuration

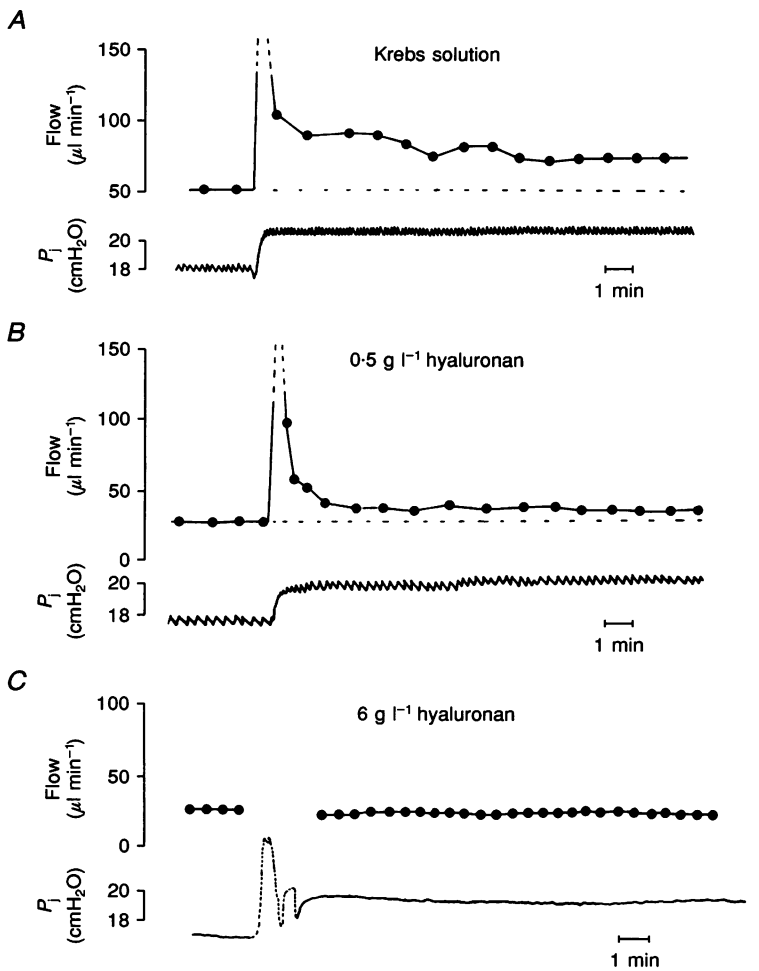

Figure 3. Effect of raising infusion pressure (reservoir height) on pressure in joint cavity (lower traces) and flow into joint cavity (upper traces)

Pressure steps were approximately from 18 to 20 cmH_2O. *A*, Krebs solution; *B*, 0·5 g l^{-1} hyaluronan solution; *C*, 6 g l^{-1} hyaluronan solution, with filling speeded by a manual injection. Traces in *A* and *C* are from opposite joints of the same animal. Traces show that (i) hyaluronan reduces trans-synovial flow in steady-state conditions, and (ii) at 6 g l^{-1} hyaluronan, raising pressure did not raise trans-synovial flow.

(eqn (4)), such a polymer has a r_g of 48–60 nm in dilute solution. For an ionic polymer in an electrolyte solution of 0·15 M salinity the model of Johnson *et al.* (1987), which incorporates electrostatic effects and electrolyte shielding, predicted larger radii, of 81–99 nm (eqn (5)).

Corresponding molecular parameters calculated from the osmometric value for M_n, namely 120 000–176 000, were: 316–464 disaccharides per molecule; linear chain length, 0·3–0·4 μm; and r_g in 0·15 M saline, 32–41 nm. Osmometry thus indicated a very voluminous molecular domain, approximately 10 times the linear dimension of albumin. The ratio M_w/M_n is an index of polydispersity, i.e. the amount of variation in molecular size within the sample, and this appeared to be substantial.

Critical overlap concentration

The critical concentration, C^*, above which the solution contains an effectively continuous network of polymer chains, was assessed using both the graphical method and calculation. (i) When $\log(\eta_{sp})_{\dot{\gamma}=0}$ is plotted as a function of $\log C$ there is a marked kink at the onset of intermolecular coupling (Morris *et al.* 1980). This gave a value of C^* of approximately 1·3 g l^{-1}; see Fig. 2*D*. (ii) Since this latter value depended strongly on two points below 1 g l^{-1}, C^* was checked by calculation based on de Gennes' expression (eqn (6)). Substituting values of n of 2042, r_g of 60×10^{-7} cm and converting to grams per litre by factor $379/N_A$ (379 is monomer mass), C^* was calculated to be 1·4 g l^{-1} (or 1·96 g l^{-1} for $[\eta]_{app}$-derived values). This is in fair agreement with Fig. 2*D*. If the larger estimate of r_g from the model of Johnson *et al.* (1987) was used, C^* was calculated to be 0·3–0·4 g l^{-1}, which is less compatible with Fig. 2*D*. The Flory model (eqn (4)) thus described the results better than the model of Johnson *et al.* (eqn (5)).

Results *in vivo*

Flow *versus* time curves

The effect of raising infusion pressure on flow into the joint cavity is illustrated in Fig. 3. The initial pressure transient for 3–6 g l^{-1} hyaluronan (Fig. 3*C*) differed from that of Fig. 3*A* and *B* merely because in the latter the cavity was filled under a gravity feed, whereas filling with 3–6 g l^{-1} hyaluronan was hastened by manual injection (see Methods). After P_j reached a stable value two points were noted. First, hyaluronan reduced the outflow across the synovial lining, i.e. the absolute flows were lower for hyaluronan solution than for Krebs solution at similar pressures. Second, whereas the steady-state rate of absorption of Krebs solution or 0·5 g l^{-1} hyaluronan increased on raising pressure, that for 3–6 g l^{-1} hyaluronan was no higher after raising joint pressure than before, at the pressure illustrated.

These differing characteristics were revealed more clearly upon analysing the pressure–flow relations. The relation for 0·5 g l^{-1} hyaluronan is considered first because it differs least from the control curve (Krebs solution).

Effect of dilute (0·5 g l^{-1}) hyaluronan on the pressure–flow relation

Figure 4 shows trans-synovial flow as a function of P_j when one knee was infused with Krebs solution and the other with dilute hyaluronan solution (same animal). Hyaluronan reduced the rate of escape of fluid through the synovial lining but the shape of the relation was not affected in this animal; both the control and hyaluronan relations had steeper slopes at $P_j > 10$–12 cmH_2O (P_y) than at lower pressures. This pattern was typical of five out of seven paired experiments and these five are summarized in Table 1. The ratio of the slope $d\dot{Q}_s/dP_j$ below P_y to the slope above P_y in the presence of hyaluronan averaged 0·35 ± 0·12 $\mu l\ min^{-1}\ cmH_2O^{-1}$ in these five joints ($P = 0·03$, sign test). The other two out of seven animals behaved differently and are described in the next section. Factors contributing to the slope change, namely synovial stretching and interstitial matrix dilution, have been reported previously (Edlund, 1949; Levick, 1991; Mason, Price & Levick, 1994).

Hyaluronan reduced trans-synovial flow by altering the slope of the pressure–flow relation rather than the intercept. Below P_y, 0·5 g l^{-1} hyaluronan reduced slope $d\dot{Q}_s/dP_j$ to 36 ± 12% of that for Krebs solution. Above P_y, hyaluronan reduced the slope to 68 ± 14% of that for

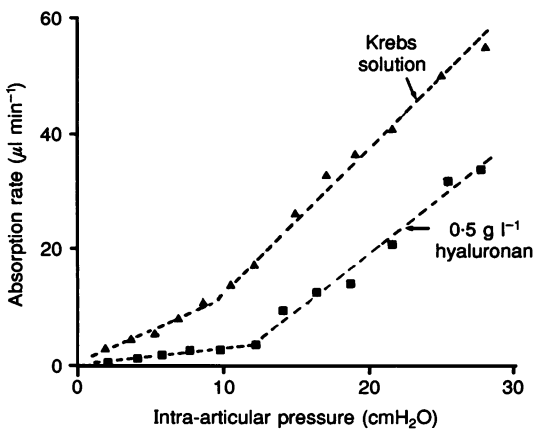

Figure 4. Trans-synovial flow (absorption) as a function of intra-articular pressure for Krebs solution and dilute hyaluronan in opposite knees of the same animal

The increase in slope with pressure was significant for each liquid ($P < 0·02$ for Krebs solution, $P < 0·04$ for hyaluronan solution, paired t tests).

Table 1. Relation between intra-articular pressure (P_j) and trans-synovial flow ($\dot{Q}_s$) in the presence and absence of 0·5 g l^{-1} hyaluronan

Infusate	$d\dot{Q}_s/dP_j$ (μl min^{-1} cmH_2O^{-1})		Intercept at $P_j = 0$ (μl min^{-1})	P_y (cmH_2O)
	$< P_y$	$> P_y$		
Hyaluronan	0·30 ± 0·11*	1·25 ± 0·38†	−0·29 ± 0·28	7·52 ± 1·26
Krebs solution	1·01 ± 0·19	1·76 ± 0·27	−0·01 ± 0·28	8·18 ± 1·04

Values are given as means ± S.E.M.; $n = 5$ pairs of knees. * Probability that hyaluronan does not reduce slope (compared with Krebs solution); $P = 0·01$. † Probability that hyaluronan does not reduce slope; $P = 0·04$. Paired t test, one tailed.

Krebs solution. The intercept of the relation at zero pressure was not altered significantly, nor was P_y. The reduction of slope $d\dot{Q}_s/dP_j$ to 36–68% of the control value was roughly comparable with the reduction in intra-articular fluidity caused by hyaluronan, although this was probably fortuitous (see later). The relative viscosity of the intra-articular aspirate was 2·2 ± 0·1 at 2·9 N m^{-2}, giving a relative fluidity of 46%, a value relatively independent of shear rate at this concentration (Fig. 2*B*). The relative viscosity of the aspirated intra-articular hyaluronan solution at the end of the experiment (mean, 2·2) was in every case greater than that infused (1·7 ± 0·2, at 2·9 N m^{-2}), with the rise in viscosity averaging 29% ($P = 0·01$, paired t test). The colloid osmotic pressure of the hyaluronan solution was only 0·1 cmH_2O.

Effect of physiological hyaluronan concentrations on the pressure–flow relation

At concentrations of hyaluronan that bracket the physiological range (3 g l^{-1}, $n = 5$; 6 g l^{-1}, $n = 5$) and in two out of seven animals at 0·5 g l^{-1}, hyaluronan not only reduced trans-synovial outflow but also profoundly altered the *shape* of the pressure–flow relation, as illustrated in Fig. 5. The shape of the relation at low and high intra-articular pressures appeared to be different, and results at low P_j are described first.

Low pressure. 'Low pressure' was defined as a pressure below P_y in the opposite Krebs solution-infused joint; a clear yield point was not usually observable in the hyaluronan relation itself at these concentrations. At low pressures, solutions of 3–6 g l^{-1} hyaluronan reduced slope $d\dot{Q}_s/dP_j$ to 48·8 ± 15·8% of that for Krebs solution ($P = 0·01$, $n = 10$, t test). Comparing the two hyaluronan concentrations, the slope was reduced to 61 ± 27% of the control by 3 g l^{-1} hyaluronan and to 36 ± 18% of the control by 6 g l^{-1} hyaluronan but the difference was not statistically significant ($P = 0·46$, unpaired t test). Absolute values are given in Table 2. Although the slope reduction was substantial and important physiologically, it was remarkably small compared with the change in bulk fluidity. The relative fluidity of 3 g l^{-1} hyaluronan was only 9·7 ± 1% of the control (aspirate relative viscosity, 10·8 ± 1·1 at 2·9 N m^{-2}) and its colloid osmotic pressure was 0·9 cmH_2O. For 6 g l^{-1} hyaluronan the relative fluidity was 6·2 ± 0·3% of the control, viscosity

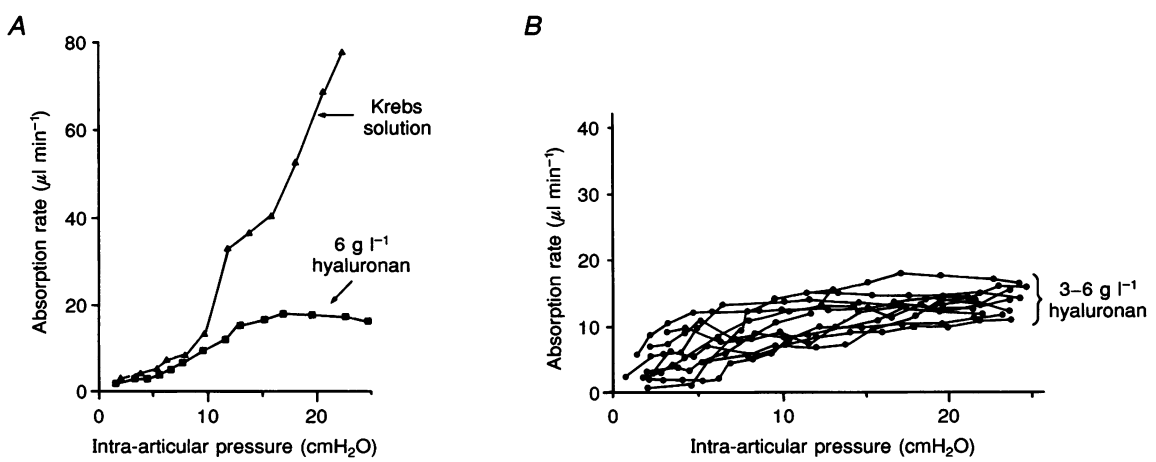

Figure 5. Trans-synovial flow as a function of intra-articular pressure

A, results for Krebs solution and 6 g l^{-1} hyaluronan solution in opposite knees of same animal. *B*, results from ten joints infused with 3–6 g l^{-1} hyaluronan solution.

Table 2. Relation between intra-articular pressure (P_j) and trans-synovial flow ($\dot{Q}_s$) in the presence and absence of 3 or 6 g l^{-1} hyaluronan

Infusate	$d\dot{Q}_s/dP_j$ (μl min^{-1} cmH_2O^{-1})	
	$< P_y$*	$> P_y$*
3 g l^{-1} hyaluronan	0·63 ± 0·29	0·05 ± 0·12
Krebs solution (opposite knee)	1·30 ± 0·24	2·58 ± 0·41
6 g l^{-1} hyaluronan	0·63 ± 0·33	−0·01 ± 0·09
Krebs solution (opposite knee)	2·16 ± 0·51	3·71 ± 0·67
3 and 6 g l^{-1} hyaluronan pooled †	0·63 ± 0·21	0·02 ± 0.07
Krebs solution (opposite knee)	1·73 ± 0·30	3·14 ± 0·41

Values are given as means ± S.E.M.; $n = 5$ pairs of knees ($n = 10$ for pooled data). *P_y refers here to yield pressure for Krebs solution (9·0 ± 1·3 cmH_2O for 5 joints in 3 g l^{-1} series; 6·8 ± 1·2 cmH_2O in 6 g l^{-1} series). No distinct point of slope change was detectable in the hyaluronan curves. $d\dot{Q}_s/dP_j$ was assessed by regression analysis. † Differences between 3 and 6 g l^{-1} were not statistically significant. Differences between pooled hyaluronan and Krebs solution results were statistically significant, both below and above P_y ($P < 0·02$, paired t test). Changes in slope $d\dot{Q}_s/dP_j$ with pressure were significant both for Krebs solution ($P < 0·01$, paired t test) and for hyaluronan ($P = 0·04$).

17·4 ± 2·1 at 2·9 N m^{-2} and colloid osmotic pressure 1·9 cmH_2O. It might be thought that comparison of slopes *in vivo* and fluidity *in vitro* is a spurious procedure because the shear rates *in vivo* probably differed from those in the viscometer (e.g. 600 s^{-1} at 2·9 N m^{-2}). Figure 2*B* shows, however, that extremely high shear rates would be needed to raise the relative fluidity of these solutions to 0·4–0·6, the value required to match the fractional slope change.

The fractional reduction in slope by hyaluronan at concentrations between 0·5 and 6 g l^{-1} is plotted as a function of relative fluidity in Fig. 6. The lack of relation is obvious, and a regression line through the data had a slope of −0·18 ± 0·41, which was not significantly different from zero ($P = 0·67$). The regression slope was significantly different from 1 ($P < 0·02$, t test), which is the slope relating conductance to fluidity for Darcy's law. Thus the action of hyaluronan bore no simple relation to its effect on bulk fluidity.

The intercept of the pressure–flow relation was unaffected by 3–6 g l^{-1} hyaluronan; there were no significant differences between the intercepts for Krebs solution, 3 or 6 g l^{-1} hyaluronan solutions, and no significant correlation between the intercept and the slight colloid osmotic pressure of hyaluronan, taking the whole range from 0·5 to 6 g l^{-1}.

High pressures. When pressure was raised above ~12 cmH_2O, a new phenomenon was observed in the presence of 3–6 g l^{-1} hyaluronan. In most cases the absorption rate increased by smaller and smaller amounts with each increment in pressure, then developed a plateau or near-plateau at a flow of 11–17 μl min^{-1} (Fig. 5*B*). Indeed, in four joints, raising the pressure above 12–16 cmH_2O actually caused the absorption rate to fall slightly, one such case being illustrated in Fig. 5*A*. As a result, instead of the slope $d\dot{Q}_s/dP_j$ increasing above 7–9 cmH_2O, as happened in the Krebs solution-infused contralateral joints ($P < 0·01$; Table 2), the slope *decreased* above these pressures in the hyaluronan-infused joints ($P = 0·04$, paired t test). The effects of 3 and 6 g l^{-1} hyaluronan on $d\dot{Q}_s/dP_j$ were not significantly different from each other ($P = 0·72$, unpaired t test) and the mean value of $d\dot{Q}_s/dP_j$ for 3–6 g l^{-1} hyaluronan at pressures above contralateral P_y was only 0·6% of that for Krebs solution ($P = 0·0002$, paired t test).

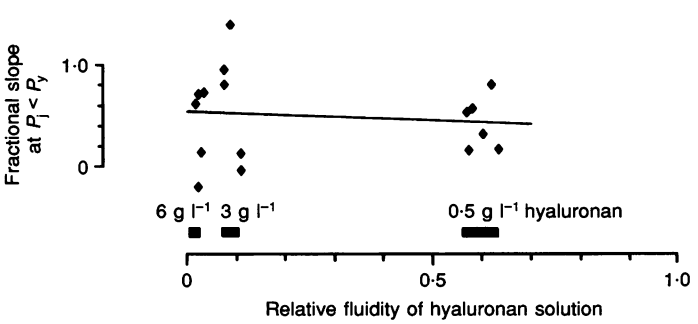

Figure 6. Effect of hyaluronan on regression slope $d\dot{Q}_s/dP_j$ at low intra-articular pressures, plotted *versus* its effect on relative fluidity (1/relative viscosity)

Fractional slope is $d\dot{Q}_s/dP_j$ in presence of hyaluronan expressed as fraction of $d\dot{Q}_s/dP_j$ in its absence (Krebs solution alone). There was no significant correlation between fractional slope and fluidity (see text).

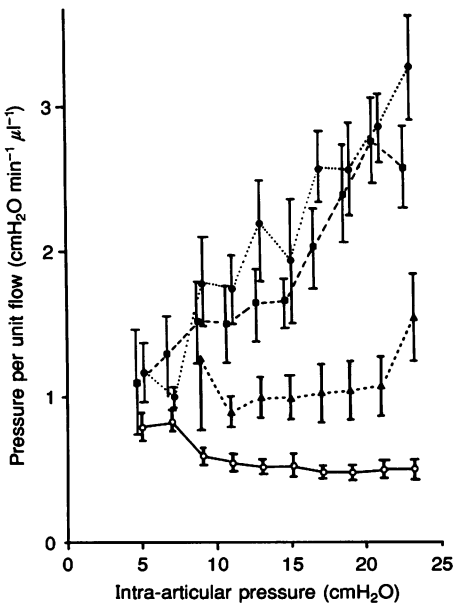

Figure 7. Pressure required to drive *unit* flow ($P_j/\dot{Q}_s$) across synovial lining (resistance parameter) as a function of intra-articular pressure

Results are shown for Krebs solution (○), 0·5 (▲), 3 (●) and 6 g l^{-1} (■) hyaluronan solution. Means of all experiments ± S.E.M. (error bars).

Inspection of Fig. 5*B* indicated that resistance to trans-synovial flow increased as pressure was raised in the presence of hyaluronan. To quantify this, the intra-articular pressure needed to drive unit flow across the synovial lining was analysed at 2 cmH_2O intervals. (This is equivalent to calculating outflow resistance if subsynovial pressure is zero; see Discussion.) Mean values are presented in Fig. 7. For Krebs solution the resistance parameter decreased on raising pressure, in keeping with previous work (Knight & Levick, 1985). With 3–6 g l^{-1} hyaluronan, by contrast, the resistance parameter increased progressively as pressure was raised, from 1·2 cmH_2O min^{-1} μl^{-1} at 5 cmH_2O to 3·0 cmH_2O min^{-1} μl^{-1} at 23 cmH_2O. The presence of hyaluronan also increased the variability of the results, as shown by the standard error bars.

DISCUSSION

Molecular size of commercial hyaluronan sample

Although osmometry is known to be a poor method for estimating M_n when macromolecular size is $>10^6$, owing to the dominating influence of second and third virial coefficients (Ogston, 1966), the oncotic pressures measured here indicated that much of the grade III umbilical hyaluronan was of relatively small molecular size. Others have had similar findings with commercial hyaluronan preparations (Shaw & Schy, 1977; Goldberg & Toole, 1987). The relatively small molecular weight of the sample was confirmed by viscometry; the intrinsic viscosity of the sample was much smaller than that of undegraded rabbit synovial hyaluronan (3740–4060 ml g^{-1}; Sunblad, 1953; Balazs & Denlinger, 1985), The chain length of the sample was clearly relatively low, and further work on discrete hyaluronan fractions will be needed to assess the physiological importance of hyaluronan chain length for the effects observed *in vivo*.

Physiological significance

Hyaluronan greatly reduced the rate of drainage of fluid from the joint cavity, even at subnormal concentration and molecular size, and changed the shape of the pressure–flow relation from concave to convex towards the flow axis at physiological concentrations. Hyaluronan may therefore be important in synovial fluid turnover. By reducing or 'buffering' the rate of escape of fluid during long periods of raised P_j (i.e. many minutes), such as during sustained joint flexion, hyaluronan should help to minimize depletion of synovial fluid and conserve lubricant.

Although the initial manual infusion of 3–6 g l^{-1} hyaluronan caused a transient high pressure near the infusion needle (suprapatellar pouch; Fig. 3), several considerations indicate that this brief pressure elevation (cf. hyaluronan) is unlikely *per se* to be the primary cause of the reduced flows and conductance. (i) The transient elevation appeared to be confined to a local region near the infusion site, because it decayed quickly as infusate flowed away into more distant parts of the joint cavity. (ii) High P_j raises synovial conductance rather than reduces it (Edlund, 1949). (iii) In a different protocol both the control (Krebs) solution and an alternating series of hyaluronan solutions were infused manually using a triple-flush procedure. The control flows and fractional

reduction in trans-synovial flow by hyaluronan were similar to those seen here (McDonald & Levick, 1994). (iv) There was no initial pressure overshoot with 0·5 g l^{-1} hyaluronan, which was infused entirely by gravity, yet a plateau developed in two out of seven cases.

Mechanism by which hyaluronan reduces trans-synovial flow and $d\dot{Q}_s/dP_j$

The action of hyaluronan did not appear to depend on the colloid osmotic pressure or viscosity of the bulk phase. Taking *osmotic pressure* first, the reduction in absorption rate was much too great to be attributed to the slight colloid osmotic pressure of the solution, e.g. 0·1 cmH_2O for 0·5 g l^{-1} hyaluronan. Even for 6 g l^{-1}, the colloid osmotic pressure was only 1·9 cmH_2O. While the latter might in theory reduce flow at low intra-articular pressures if the lining acted as a semipermeable membrane with respect to hyaluronan, an effective osmotic pressure should alter the intercept of a pressure–flow relation rather than slope, because osmotic pressure is a thermodynamic force rather than a resistance term. There was no reduction, however, in the flow intercept at zero pressure as hyaluronan osmotic pressure was increased.

Considering *viscosity* as a possible mechanism, the slope of the pressure–flow relation across a macroscopic porous medium at low Reynolds number is linearly proportional to the reciprocal of viscosity (fluidity) according to Darcy's law. For synovium, however, the slope at low intra-articular pressures, $d\dot{Q}_s/dP_j$, bore no discernible relation to the bulk-phase viscosity of hyaluronan solution (Fig. 6); the most viscous solution had no more effect on the slope at low pressures than did the most dilute solution, and the regression line relating $d\dot{Q}_s/dP_j$ to fluidity had a gradient not significantly different from zero. A possible explanation might be that hyaluronan did not permeate the synovial interstitial pathway freely, leaving the interstitial fluid with a higher fluidity than the intra-articular bulk phase. This mechanism was proposed by McDonald & Levick (1994) to explain the disparity between the effect of intra-articular hyaluronan on trans-synovial flow and on fluidity at 6 cmH_2O in earlier experiments. Both sets of experiments indicate that the viscosity of hyaluronan is unlikely to be the chief factor reducing trans-synovial flow.

The extreme buffering of trans-synovial flow on the plateau of the pressure–flow relation provided a valuable clue to the mechanism of action of hyaluronan. Since raising pressure failed to increase flow in the steady state and even reduced it slightly in some cases, it is inferred that synovial hydraulic resistance increased with pressure in the presence of 3–6 g l^{-1} hyaluronan. The pressure required to drive unit flow across the lining, $P_j/\dot{Q}_s$, increased nearly 3-fold between the lowest and highest pressures explored, whereas the opposite happened in the absence of hyaluronan (Fig. 7). An increase in the term $P_j/\dot{Q}_s$ could in principle arise either because synovial resistance increased or because subsynovial pressure increased. The latter seems an unlikely explanation, because Krebs solution crossed the lining in far greater quantities than hyaluronan solution yet did not cause a plateau. Moreover the compliance (pressure–volume) curve for areolar connective tissue (comprising much of the subsynovium) develops a pressure plateau at just above atmospheric pressure (Aukland & Reed, 1993). If it is assumed that downstream pressure remains close to atmospheric pressure (McDonald & Levick, 1993), the plot in Fig. 7 is in effect a plot of synovial lining resistance *versus* P_j. The most likely explanation for the present findings thus seems to be that in the presence of 3–6 g l^{-1} hyaluronan the synovial hydraulic resistivity increases with each succeeding step in P_j.

Day (1952) studied the hydraulic permeability of mouse flank fascia and his findings parallel in many respects those on synovium in this study. Hyaluronidase was used to increase the permeability of the fascial membranes, after which filtration of solutions of starch or large molecular weight dextran across the membrane was found to restore a low permeability, progressively slowing the flow. Synovial fluid acted on the fascia in a similar manner to starch in Day's experiments. The effect was unrelated to bulk viscosity, just as in the present work, was reduced by a rise in temperature, was related to solute molecular size, and depended on flow (soaking had no effect). It was therefore attributed to the interstitial matrix acting as a molecular sieve, leading to retention of perfused polymers, which in turn caused a rise in interstitial resistance. We suggest that a similar mechanism may operate across the synovial lining, as discussed in the next section.

Mechanism by which hyaluronan might increase synovial hydraulic resistivity

The large radius of the hydrated hyaluronan molecule relative to the average size of the pores within the interstitial matrix (details below) suggests a mechanism by which hyaluronan could raise outflow resistivity, namely via accumulation of a 'filter cake' of hyaluronan molecules at or just within the synovial surface. As the hyaluronan solution is swept by convection into the synovial lining, water can be expected to penetrate the interstitial pores more easily than the vast hyaluronan molecules, leading to selective retention of hyaluronan near the surface. If a layer of sieved hyaluronan molecules builds up at the surface due to exclusion and reflection, and/or within the interstitium due to entanglement with the fixed biopolymers there, water is then compelled to pass through an additional barrier, the network of quasi-fixed hyaluronan chains. Since resistance to flow through

the hyaluronan molecular domain is high, formation of a hyaluronan molecular filter cake should raise synovial resistivity.

If a simple boundary polarization effect occurred, without intramatrix entanglement, a steady state would be reached when the increased solute concentration at the boundary caused diffusion of solute back into the bulk phase at a rate equal to convective transport of solute into the boundary (Pedley & Fischbarg, 1978; Tedgui & Lever, 1985; Yan, Weinbaum & Pfeffer, 1986). Raising joint pressure would transiently increase convective transport into the boundary, raising the concentration and thickness of the polarized layer and establishing a new steady state in which increased boundary resistance counteracts the increased pressure. Ultrafiltration of Dextran 70 or albumin across a semipermeable membrane produces a pressure–flow relation with a flow plateau for the above reason (Kozinski & Lightfoot, 1972; Wijmans, Nakao, Berg, Troelstra & Smolders, 1985).

The sieving process is more complex for a membrane that only partially rejects the solute. When plasma is ultrafiltered across Amicon membranes of differing selectivity, membranes UM10 and PM30, which totally exclude plasma proteins, produce pressure–flow relations with a flow plateau. The more permeable XM100 membrane, however, produces a pressure–flow relation with a short plateau followed by a fall in flow on raising pressure further (Blatt, Dravid, Michaels & Nelson, 1970). Similarly, trans-synovial flow in the steady state sometimes decreased slightly on raising pressure (Fig. 5*A*). Entanglement of convected hyaluronan within the superficial matrix might produce this.

Evidence for molecular sieving of hyaluronan

There are a number of other grounds for believing that hyaluronan might be selectively retained at the synovial surface, as follows.

(i) Solute : pore size ratio. The average r_g of hyaluronan molecules in the commercial preparation, 48–99 nm, is comparable with or larger than the estimated average dimension of the spaces within the synovial interstitial matrix. The mean hydraulic radius within the network of collagen VI microfibrils that abounds in the innermost synovial matrix is estimated to be 65–132 nm (Levick & McDonald, 1990). If the extrafibrillar proteoglycan and glycoprotein concentration in synovial interstitium is 13·9 mg ml^{-1}, as inferred recently (Levick, 1994), the mean hydraulic radius within the matrix would be as small as 41 nm.

Size comparison alone does not fully answer the sieving issue, because flexible polymers can deform and permeate cylindrical pores of radius less than half the solute's own hydrodynamic radius (Munch, Zestar & Anderson, 1979). Indeed, intra-articular injected hyaluronan can escape from the rabbit knee cavity into lymph over a 2 h period (Antonas, Fraser & Muirden, 1973), even at a M_w of $2{\cdot}8 \times 10^6$ (J. R. E. Fraser, W. G. Kimpton & T. C. Laurent, personal communication). Such permeation is thought to involve reptate (snake-like) translocation of partially uncoiled molecules (Preston & Snowden, 1973), the radius of the polymer chain itself being only ~0·6 nm. A slow permeation of hyaluronan does not, however, preclude the formation of a concentration polarization layer, since the latter merely requires a differential rate of transfer of water and solute. Partial hyaluronan permeation does, however, complicate the issue by raising questions about the effective viscosity of the interstitial fluid.

Perhaps as important as the relative size of the individual hyaluronan molecule and interstitial pore is the fact that solutions of 3–6 g l^{-1} hyaluronan exceed C^*, the critical overlap concentration. This means that the intra-articular solution comprised an effectively continuous network of overlapping polymer chains. This presumably increases the difficulty encountered by any individual hyaluronan molecule in escaping through an interstitial matrix pore. This might explain why in most joints infused with 0·5 g l^{-1} hyaluronan, which appeared to be just below C^*, the pressure–flow relation did not plateau.

Other observations supporting partial molecular sieving of hyaluronan across synovium are as follows.

(ii) Preliminary electron micrographs of synovium from two joints infused with 6 g l^{-1} hyaluronan at 6 cmH_2O showed accumulation of a Ruthenium Red-staining material (assumed to be hyaluronan) at the surface and within the most superficial few micrometres of interstitium (McDonald & Levick, 1994). This seems analogous to the observation of Day (1952) that reduction of the hydraulic permeability of connective tissue by starch was accompanied by accumulation of starch in the surface plane. Similarly, Denlinger (1982) reported large increases in the hyaluronan content of rabbit synovium following intra-articular injection of hyaluronan.

(iii) Although aspirates were not analysed biochemically in these experiments, the fact that the viscosity of the aspirate at the end of experiments with 0·5 g l^{-1} hyaluronan was in every case greater than that infused, on average by 29%, implied that the intra-articular concentration of hyaluronan had increased over the 3 h experiment. It was not possible, unfortunately, to reproduce this observation at the higher concentrations owing to the tendency of viscosity to decay with time (see Methods).

(iv) Studies *in vitro* in which hyaluronan solutions are filtered through membranes show that even when pore size is as large as 450 nm, a concentration polarization

layer forms at the surface in the presence of endogenous or Sigma grade III hyaluronan (Ogston & Shermann, 1961; Nettelbladt & Sundblad, 1967; Fraser, Murdoch, Curtain & Watt, 1977; Parker & Winlove, 1984; Johnson *et al.* 1987). It is probable, therefore, that concentration polarization will also develop with synovium, where the effective pore radius may be < 100 nm (mean hydraulic radius, see earlier).

(v) There is indirect evidence that the rate of bulk turnover of synovial fluid water and protein is an order of magnitude faster than the turnover of intra-articular hyaluronan. The turnover time for synovial fluid volume and protein is estimated to be around 1 h in rabbit and normal human knees (Levick, 1987*a*), while that for hyaluronan in rabbit shoulder is estimated to be of the order 20–28 h (Knox *et al.* 1988). Hyaluronan in the rabbit knee is estimated to have a half-life of around 27–32 h at normal volume (Denlinger, 1982), while in volume-expanded knees the half-life is between 13 h (for M_w of 6×10^6) and 10 h (for M_w of $0{\cdot}9 \times 10^6$; Brown, Laurent & Fraser, 1991). If the large difference between hyaluronan turnover time and turnover time for other constituents is correct, it follows that there must be selective retention of hyaluronan within the joint cavity.

Quantitative aspects of hyaluronan resistivity

Assuming that the rise in $P_j/\dot{Q}_s$ in Fig. 7 is due to increased hydraulic resistance of the synovial lining, the question must be asked whether a 3 times rise in resistance *in situ* is compatible with the relation between hyaluronan concentration and hydraulic resistance *in vitro*. Can accumulated hyaluronan be expected to have an effect of the observed magnitude? The hydraulic conductivity (reciprocal of resistivity) of synovial interstitium *in vivo* is estimated to be in the range 2×10^{-15} to $10{\cdot}2 \times 10^{-15}$ $m^4 s^{-1} N^{-1}$ (2×10^{-12} to $10{\cdot}2 \times 10^{-12}$ $cm^4 s^{-1} dyn^{-1}$; Levick, 1991, 1994). A hyaluronan network of concentration 3–6 g l^{-1} has a much bigger hydraulic conductivity than this, namely 38×10^{-15} to 106×10^{-15} $m^4 s^{-1} N^{-1}$ (Levick, 1987*b*), so a substantial local concentration of hyaluronan would have to develop to raise synovial resistance appreciably. However, gels formed by fitration of hyaluronan solutions across membranes *in vitro* do indeed have a high hydraulic resistance (Nettelbladt & Sundblad, 1967). For example, the hydraulic conductivity of a hyaluronan concentration polarization layer extending 2 mm upstream from a membrane studied by Parker & Winlove (1984) had a hydraulic conductivity of 7×10^{-15} $m^4 s^{-1} N^{-1}$, using a bulk-phase grade III hyaluronan concentration of 15 g l^{-1} and filtration velocity $\sim 3 \times 10^{-6}$ m s^{-1} (3–5 times the estimated trans-synovial flow velocities in this study). The conductivity of the polarized layer was comparable with synovial interstitial permeability and was several orders of magnitude smaller than that of the bulk phase. It seems not unreasonable, therefore, to postulate that concentration of hyaluronan in the vicinity of the synovial surface, coupled perhaps with the viscous effect of those hyaluronan molecules that actually succeed in permeating through the interstitium, could account for the experimental observations in this study.

Conclusion

The hyaluronan of synovial fluid exerts a major buffering effect on the escape of fluid from a joint cavity under pressure, helping to retain fluid within the cavity. The mechanism of this effect is unlikely to be purely viscous or osmotic. The mechanism could be a rise in outflow resistance caused by concentration of hyaluronan at the synovial surface and within the interstitium. Many questions remain unanswered, such as the influence of hyaluronan chain length, the fraction of the hyaluronan that permeates the lining to reach lymph, and whether the presence of hyaluronan significantly modifies the hydraulic effects of intra-articular plasma proteins. The findings also draw attention to our relatively poor understanding of the basic biophysics of the convective transport of a random coil super-molecule through a fibrous matrix.

Antonas, K. N., Fraser, J. R. E. & Muirden, K. D. (1973). Distribution of biologically labelled radioactive hyaluronic acid injected into joints. *Annals of the Rheumatic Diseases* **32**, 103–111.

Aukland, K. & Reed, R. K. (1993). Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiological Reviews* **73**, 1–78.

Balazs, E. A. & Denlinger, J. L. (1985). Sodium hyaluronate and joint function. *Journal of Equine Veterinary Science* **5**, 217–228.

Bert, J. L. & Pearce, R. H. (1984). The interstitium and microvascular exchange. In *Handbook of Physiology*, section 2, *The Cardiovascular System*, vol. IV, *The Microcirculation*, ed. Renkin, E. M. & Michel, C. C., pp. 521–548. American Physiological Society, Bethesda, MD, USA.

Blatt, W. F., Dravid, A., Michaels, A. S. & Nelsen, L. (1970). Solute polarization and cake formation in membrane ultrafiltration: causes, consequences and control techniques. In *Membrane Science and Technology*, ed. Flinn, J. E., pp. 47–95. Plenum Press, New York.

Bollet, A. J. (1956). The intrinsic viscosity of synovial fluid hyaluronic acid. *Journal of Laboratory and Clinical Medicine* **48**, 721–728.

Brown, T. J., Laurent, U. B. G. & Fraser, J. R. E. (1991). Turnover of hyaluronan in synovial joints: elimination of labelled hyaluronan from the knee joint of the rabbit. *Experimental Physiology* **76**, 125–134.

Cleland, R. L. & Wang, J. L. (1970). Ionic polysaccharides. III. Dilute solution properties of hyaluronic acid fractions. *Biopolymers* **9**, 799–810.

Cooke, A. F., Dowson, D. & Wright, V. (1976). Lubrication of synovial membrane. *Annals of the Rheumatic Diseases* **35**, 56–59.

DAHL, L. B., DAHL, I. M., ENGSTRÖM-LAURENT, A. & GRANATH, K. (1985). Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. *Annals of the Rheumatic Diseases* **44**, 817–822.

DAY, T. D. (1952). The permeability of interstitial connective tissue and the nature of the interfibrillary substance. *Journal of Physiology* **117**, 1–8.

DE GENNES, P.-G. (1979). *Scaling Concepts in Polymer Physics.* Cornell University Press, Ithaca, NY, USA.

DENLINGER, J. L. (1982). Metabolism of sodium hyaluronate in articular and ocular tissues. Doctoral dissertation, Université de Sciences et Techniques de Lille.

EDLUND, T. (1949). Studies on the absorption of colloids and fluid from rabbit knee joints. *Acta Physiologica Scandinavica* **18**, suppl. 62, 1–108.

FLORY, P. J. (1971). *Principles of Polymer Chemistry.* Cornell University Press, Ithaca, NY, USA.

FRASER, J. R. E., MURDOCH, W. S., CURTAIN, C. C. & WATT, B. S. (1977). Proteins retained with hyaluronic acid during ultrafiltration of synovial fluid. *Connective Tissue Research* **5**, 61–65.

GOLDBERG, R. L. & TOOLE, B. P. (1987). Hyaluronate inhibition of cell proliferation. *Arthritis and Rheumatism* **30**, 769–778.

GRANGER, H. J., LAINE, S. H. & LAINE, G. A. (1985). Osmotic pressure exerted by entangled polysaccharide chains. *Microcirculation Endothelium and Lymphatics* **2**, 85–105.

HAUG, A. & SMIDSRØD, O. (1962). Determination of intrinsic viscosity of alginates. *Acta Chemica Scandinavica* **16**, 1569–1578.

HENDERSON, B. & EDWARDS, J. C. W. (1987). *The Synovial Lining in Health and Disease.* Chapman and Hall, London.

JOHNSON, M., KAMM, R., ETHIER, C. R. & PEDLEY, T. (1987). Scaling laws and the effects of concentration polarization on the permeability of hyaluronic acid. *Physicochemical Hydrodynamics* **9**, 427–444.

KNIGHT, A. D. & LEVICK, J. R. (1985). Effects of fluid pressure on the hydraulic conductance of interstitium and fenestrated endothelium in the rabbit knee. *Journal of Physiology* **360**, 311–332.

KNIGHT, A. D., LEVICK, J. R. & MCDONALD, J. N. (1988). Relation between trans-synovial flow and plasma osmotic pressure, with an estimation of the albumin reflection coefficient in the rabbit knee. *Quarterly Journal of Experimental Physiology* **73**, 47–65.

KNOX, P., LEVICK, J. R. & MCDONALD, J. N. (1988). Synovial fluid – its mass, macromolecular content and pressure in major limb joints of the rabbit. *Quarterly Journal of Experimental Physiology* **73**, 33–45.

KOZINSKI, A. A. & LIGHTFOOT, E. N. (1972). Protein ultrafiltration: a general example of boundary layer filtration. *American Institute of Chemical Engineering Journal* **18**, 1030–1040.

LAURENT, T. C. & OGSTON, A. G. (1963). The interaction between polysaccharides and other macromolecules 4. The osmotic pressure of mixtures of serum albumin and hyaluronic acid. *Biochemical Journal* **89**, 249–253.

LAURENT, T. C., RYAN, M. & PIETRUSZKIEWICZ, A. (1960). Fractionation of hyaluronic acid. The polydispersity of hyaluronic acid from the bovine vitreous body. *Biochimica et Biophysica Acta* **42**, 476–485.

LEVICK, J. R. (1979). The influence of hydrostatic pressure on trans-synovial fluid movement and on capsular expansion in the rabbit knee. *Journal of Physiology* **289**, 69–82.

LEVICK, J. R. (1983). Synovial fluid dynamics: the regulation of volume and pressure. In *Studies in Joint Disease*, vol. 2, ed. MAROUDAS, A. & HOLBOROW, E. J., pp. 153–240. Pitman Medical, London.

LEVICK, J. R. (1987*a*). Synovial fluid and trans-synovial flow in stationary and moving joints. In *Joint Loading: Biology and Health of Articular Structures*, ed. HELMINEN, H., KIVIRANTA, I., TAMMI, M., SAAMAREN, A. M., PAUKONNEN, K. & JURVELIN, J., pp. 149–186. Wright and Sons, Bristol.

LEVICK, J. R. (1987*b*). Flow through interstitium and other fibrous matrices. *Quarterly Journal of Experimental Physiology* **72**, 409–438.

LEVICK, J. R. (1991). A two-dimensional morphometry-based model of interstitial and transcapillary flow in rabbit synovium. *Experimental Physiology* **76**, 905–921.

LEVICK, J. R. (1994). An analysis of the interaction between extravascular plasma protein, interstitial flow and capillary filtration; application to synovium. *Microvascular Research* **47**, 90–125.

LEVICK, J. R. & MCDONALD, J. N. (1990). The microfibrillar meshwork of the synovial lining and associated broad-banded collagen – a clue to identity. *Annals of the Rheumatic Diseases* **49**, 31–36.

MCDONALD, J. N. & LEVICK, J. R. (1993). Effect of extravascular plasma protein on pressure–flow relations across synovium in anaesthetized rabbits. *Journal of Physiology* **465**, 539–559.

MCDONALD, J. N. & LEVICK, J. R. (1994). Hyaluronan reduces fluid escape rate from rabbit knee joints disparately from its effect on fluidity. *Experimental Physiology* **79**, 103–106.

MASON, R. M., PRICE, F. M. & LEVICK, J. R. (1994). A quantitative investigation of the glycosaminoglycans of the synovium. *Transactions of Orthopaedic Research Society* **19**, 403.

MORRIS, E. R., REES, R. A. & WELSH, E. J. (1980). Conformation and dynamic interactions in hyaluronate solutions. *Journal of Molecular Biology* **138**, 383–400.

MUNCH, W. D., ZESTAR, L. P. & ANDERSON, J. L. (1979). Rejection of polyelectrolytes from microporous membranes. *Journal of Membrane Science* **5**, 77–102.

NETTELBLADT, E. & SUNDBLAD, L. (1967). On the significance of hyaluronic acid changes in the pathogenesis of joint effusions. *Opuscula Medica* **12**, 224–232.

OGSTON, A. G. (1966). On water binding. *Federation Proceedings* **25**, 986–989.

OGSTON, A. G. & SHERMAN, T. F. (1961). Effects of hyaluronic acid upon diffusion of solutes and flow of solvent. *Journal of Physiology* **156**, 67–74.

OGSTON, A. G. & STANIER, J. E. (1950). On the state of hyaluronic acid in synovial fluid. *Biochemical Journal* **46**, 364–376.

PARKER, K. H. & WINLOVE, C. P. (1984). The macromolecular basis of the hydraulic conductivity of the arterial wall. *Biorheology* **21**, 181–196.

PEDLEY, T. J. & FISCHBARG, J. (1978). The development of osmotic flow through an unstirred layer. *Journal of Theoretical Biology* **70**, 427–447.

PHILIPPOFF, W., HAN, C. D., BARNETT, B. & DULFANO, M. J. (1970). A method for determining the viscoelastic properties of biological fluids. *Biorheoleogy* **7**, 55–67.

PRESTON, B. N. & SNOWDEN, J. MCK. (1973). Diffusion properties of model extracellular systems. In *Biology of the Fibroblast*, ed. KULONEN, E. & PIKKARAINEN, J., pp. 215–225. Academic Press, London.

RADIN, E. L., PAUL, I. L., SWANN, D. A. & SCHOTTSTAEDT, E. S. (1971). Lubrication of synovial membrane. *Annals of the Rheumatic Diseases* **30**, 322–325.

ROBERTS, B. J., UNSWORTH, A. & MIAN, N. (1982). Modes of lubrication in human hip joints. *Annals of the Rheumatic Diseases* **41**, 217–224.

SHAW, M. & SCHY, A. (1977). Exclusion in hyaluronate gels. *Biophysical Journal* **17**, 47–55.

SUNBLAD, L. (1953). Studies on hyaluronic acid in synovial fluids. *Acta Societatis Medicorum Upsaliensis* **58**, 113–218.

TEDGUI, A. & LEVER, M. J. (1985). The interaction of convection and diffusion in the transport of ^{131}I-albumin within the media of the rabbit thoracic aorta. *Circulation Research* **57**, 856–863.

WIJMANS, J. G., NAKAO, S., VAN DEN BERG, J. W. A., TROELSTRA, F. R. & SMOLDERS, C. A. (1985). Hydrodynamic resistance of concentration polarization layers in ultrafiltration. *Journal of Membrane Science* **22**, 117–135.

YAN, Z.-Y., WEINBAUM, S. & PFEFFER, R. (1986). On the fine structure of osmosis including three-dimensional pore entrance and exit behaviour. *Journal of Fluid Mechanics* **162**, 415–438.

Acknowledgements

Support by the Arthritis and Rheumatism Council and by the Wellcome Trust (grants 031333/Z/90 and 039033/Z/93) is gratefully acknowledged.

Received 12 May 1994; accepted 7 November 1994.