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Mechanical Properties of the Collagen Network in Human Articular Cartilage as Measured by Osmotic Stress Technique

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We have used an isotropic osmotic stress technique to assess the swelling pressures of human articular cartilage over a wide range of hydrations in order to determine from these measurements, for the first time, the tensile stress in the collagen network, P_c, as a function of hydration. Osmotic stress was applied by means of calibrated solutions of polyethylene glycol. Calculations of osmotic stress were based on the balance, at equilibrium, between the applied stress, the collagen stress, and the proteoglycan osmotic pressure, π_{PG}, acting within the extrafibrillar matrix compartment. P_c vs hydration was determined for several normal human samples, both native and trypsin-treated, and for cartilage from one osteoarthritic (OA) joint. We found that for normal cartilage the collagen network does not become "limp" until the volume of cartilage has decreased by 20–25% of its initial value and that its contribution to the balance of forces in cartilage therefore must be taken into account over a much wider range of hydrations than was previously thought. For normal cartilage, the P_c vs hydration curves exhibit a steep increase with increasing hydration; trypsin treatment does not change their slope, showing that PG concentration does not influence the inherent stiffness of the collagen network. By contrast, the curves for OA specimens are considerably shallower and displaced to higher hydrations. Our findings thus highlight the role of the stiffness of the collagen network in limiting hydration in normal cartilage and ensuring a high PG concentration in the matrix, which is essential for effective load-bearing and is lost in OA.

Key Words: **human cartilage; hydration; collagen network; proteoglycan; osmotic pressure; osteoarthritis; degeneration; connective tissue; tension; swelling.**

More than a quarter of a century has passed since Ogston (1) originally proposed the idea that in connective tissues the collagen network resists the osmotic swelling pressure exerted by proteoglycans (PG):[2] "So we may arrive at a model of connective tissue, as consisting of a relatively coarse fibrous network capable of resisting tension (internal or external), but not compression, filled with a much finer, molecular network of polysaccharide fibres trapped within it." Ogston clearly stated that the high concentration of polysaccharides in cartilage must produce a high swelling pressure which keeps cartilage "inflated" (1).

Hence, Maroudas deduced that the increased hydration of osteoarthritic (OA) cartilage, compared with normal cartilage, must be due to the weakening of the collagen network, which allows the tissue to swell, in spite of the lower content of glycosaminoglycans—the main hydrophilic components of cartilage (2–4).

Surprisingly, although the basic concepts (and some qualitative data) have been in existence for a long pe-

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[2] Abbreviations used: PG, proteoglycans; OA, osteoarthritic; PEG, polyethylene glycol; EF, extrafibrillar; IF, intrafibrillar.

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riod, this is the first time that they have been used as the basis of a systematic, quantitative approach to determine the tensile stresses, P_c, in the collagen network of articular cartilage, as a function of hydration. Our method here is to first obtain experimental measurements of cartilage swelling pressure vs hydration by applying isotropic osmotic pressure to the tissue. Then we calculate P_c both from these data and from our earlier experimental data on the osmotic pressure of extracted proteoglycans. An important characteristic of our treatment is that we can quantify, for the same cartilage specimen, both the osmotic pressure of its PG, π_{PG}, and the tensile stresses in its collagen network, P_c, over a wide range of tissue hydrations, by making use of the balance of forces in cartilage (5, 6).

MATERIALS AND METHODS

Rationale of the Method and Calculations

The physiological role of the collagen network is to resist swelling or stretching of the cartilage. Our primary experimental tool to characterize this behavior employs an isotropic osmotic stress technique in which tissue specimens are exposed to osmotically active polyethylene glycol (PEG) solutions. Equilibrium hydration is achieved when the hydrostatic pressure caused by the tensile stress exerted by the collagen network, P_c together with the externally applied osmotic stress π_{PEG}, both of which tend to squeeze fluid out of the tissue, are balanced by the osmotic pressure of the cartilage PG, viz., π_{PG}.

Thus,

$$P_c + \pi_{PEG} = \pi_{PG} \text{ or } P_c = \pi_{PG} - \pi_{PEG}. \quad [1]$$

The above equation is at the basis of our method for determining P_c. Since it was originally formulated (7), this equation has been used only at the two extremes of hydration: at the one extreme, when cartilage is fully hydrated, i.e., in the initial unloaded equilibrium (when $\pi_{PEG} = 0$) in order to estimate P_c from π_{PG}, (e.g., 8), and, at the other extreme, in order to show that at low tissue hydrations π_{PG} and π_{PEG} become equal to each other, and that, therefore, under these conditions P_c tends to zero (e.g., 5, 9). In the intermediate regime only qualitative conclusions were drawn (10). It is for the first time now that Eq. [1] is being used specifically to assess P_c quantitatively over the entire range of cartilage hydrations within which the collagen network is under tension.

In order to make use of Eq. [1] to calculate P_c for a given cartilage specimen, at a given hydration level, in addition to determining π_{PEG} from current experiment, one also needs to be able to calculate π_{PG} in the tissue corresponding to the same hydration. The procedure for doing this has been described previously (e.g., 5), but the main features and those assumptions which are relevant to the present work will be briefly summarized below.

Our analysis is based on a two-compartment model of the cartilage matrix (e.g., 11–13) and several simplifying assumptions. (i) The matrix consists of an extrafibrillar (EF) and an intrafibrillar (IF) compartment, each with uniform composition, material properties, and structure, the PG being present in the EF space alone. (ii) The PG osmotic pressure, π_{PG}, *within* the EF space is the same as the osmotic pressure of *isolated* PGs at the same concentration (or FCD) (5, 8, 11). (iii) The relationship between π_{PG} and FCD, based on pooled data for PG extracted from normal aged adult human femoral heads (14, 15) applies to all of our specimens. (iv) Intrafibrillar water content is not constant, but varies with the osmotic stress, π_{EF}, applied to the outside of the fibrils (11, 12); it does not, however, explicitly depend on P_c. (v) The collagen network within the specimen is isotropic and homogeneous. In this study, we have deliberately chosen cartilage specimens from the middle zone, in which the proteoglycan content is substantially uniform (16), and in which the collagen network is known not to be strongly oriented.

In order to determine π_{PG} for insertion into Eq. [1], we have four equations relating π_{PG}, m_{EFH_2O}, m_{IFH_2O} and FCD_{eff} to one another (where $m_{EF\,H_2O}$ and $m_{IF\,H_2O}$ are the respective masses of the EF and IF water; and FCD_{eff} is the fixed charge density in milliequivalents per gram of EF water). Essentially as in our previous publications (5), these equations are:

$$FCD_{eff} = FCD_{TOTAL\,H_2O}\left[\frac{m_{TOTAL\,H_2O}}{m_{EF\,H_2O}}\right] \quad [2]$$

$$m_{EF\,H_2O} = M_{TOTAL} - m_{IF\,H_2O} - M_{dry} \quad [3]$$

$$\pi_{PG} = f_1(FCD) \quad [4]$$

$$m_{IF\,H_2O} = f_2(\pi_{EF}), \quad [5]$$

where M_{TOTAL} and M_{dry} represent the total wet and dry tissue mass, respectively, and the other symbols have been defined above.

Equations [2] and [3] are based on conservation of charge and mass, respectively, in a cartilage specimen. Equation [4] is an empirical relationship between the osmotic pressure of extracted PG and concentration in solution (expressed as FCD); it is used to determine the variation of π_{PG} with FDC_{eff} during isotropic compression. Equation [4] represents an empirical relationship between IF water and the pressure prevailing in the EF compartment. (In native cartilage π_{EF} is equal to π_{PG}.) Curve-fitting equations to represent relationships [4] and [5] were derived from experimental data (see Procedures, below).

Once all independent parameters (π_{PEG}, $FCD_{TOTAL\,H_2O}$, M_{TOTAL}, M_{dry}, and m_c) have been measured for each specimen, then the values of the four dependent variables ($m_{EF\,H_2O}$, $m_{IF\,H_2O}$, FCD_{eff}, and π_{PG}) are completely specified at each equilibrium state by Eqs. [2] to [5]. These equations are solved here by guessing initial equilibrium values and iterating until final equilibrium values of $m_{IF\,H_2O}$, $m_{EF\,H_2O}$, π_{PG}, and FCD_{eff} are obtained; then, using π_{PEG} and π_{PG} in Eq. [1], we calculate P_c. The use of Eqs. [1] to [5] is illustrated in Appendix I.

We define a collagen network hydration parameter, $(V_{TOTAL} - V_c)/V_c$, that describes the degree of collagen network inflation. In a simple polymer gel, network hydration is usually defined as the volume of the fluid encompassed by the network divided by the volume of the polymer. Since cartilage is a composite material, this concept must be extended in a meaningful way. Given that one of our aims is to compare different cartilage samples whose composition may vary (e.g., normal vs OA, old vs young), tissue volume must be expressed in relation to a parameter which will remain constant. We have chosen collagen as such a reference substance since its rate of turnover in cartilage is known to be extremely low (17–19) and its quantity in a given joint remains relatively constant throughout life. Therefore, we define the collagen network hydration parameter as total tissue volume, excluding collagen, divided by dry collagen volume: or,

$$(V_{TOTAL} - V_c)/V_c = \frac{m_{TOTAL\,H_2O}v_{H_2O} + m_{GAG}v_{GAG} + m_p v_p}{m_c v_c}, \quad [6]$$

where v_{H_2O}, v_{GAG}, v_p, and v_c are the partial specific volumes of water, glycosaminoglycans, noncollageneous proteins, and collagen, respectively.

According to the above definition, the calculation of the network

TABLE I

Some Characteristics and Chemical Composition of a Typical Specimen from 90-Year-Old Hip

Initial wet weight of specimen M_o (g)	0.01880, measured
Dry weight of specimen, M_{dry} (g)	0.00628, measured
Weight of water, $m_{TOTAL\ H_2O}$ (g)	0.01252, calculated
Collagen weight, m_C (g)	0.00326, measured
Specific volume of collagen, v_c (cm^3/g)	0.695, obtained from (11) and (22)[a]
Collagen volume, V_c (cm^3)	0.00227, calculated
GAG weight, m_{GAG} (g)	0.00115, estimated from previous data[b]
Specific volume of GAG, v_{GAG} (cm^3/g)	0.54, obtained from (23)
GAG volume, V_{GAG} (cm^3)	0.00062, calculated
Weight of noncollagenous protein, m_p g	0.00187, calculated[c]
Specific volume of noncollagenous protein (cm^3/g)	0.74, see[d]
Volume of noncollagenous protein cm^3	0.00138, calculated
Total dry tissue volume V_{dry} ($V_{dry} = V_{TOTAL\ protein} + V_{GAG}$) ($cm^3$)	0.00427, calculated
Total tissue volume, V_{TOTAL} ($V_{TOTAL} = V_{dry} + V_{TOTAL\ H_2O}$) ($cm^3$)	0.01679, calculated

[a] The specific volume of collagen type II is slightly lower than that of collagen type I because of the extent of its glycosylation (0.695 as compared with 0.709 cm^3/g).

[b] Mean GAG content was taken as 18.4% by dry weight, value typical of middle zone non-fibrillated elderly human femoral head cartilage and consistent with present FCD measurements (author's unpublished data, also (20, 21)).

[c] Noncollagenous proteins comprise both PG protein and other matrix proteins and their content, m_p, was calculated as follows: $m_p = M_{dry} - m_c - m_{GAG}$.

[d] The mean specific volume of noncollagenous proteins in cartilage was taken as 0.74 cm^3/g. Note that the density of proteins does not vary much, most published values lying within 5% of the above mean value (24).

hydration parameter requires that both the GAG and the collagen contents be known as well as the partial specific volumes of all the components. In our case, we had at our disposal the compositional data for femoral head cartilage from our previous work (16, 20, 21) and the literature values for the partial specific volumes. These data, as well as various parameters calculated for a typical specimen, are given in Table I and Appendix I.

For cases where detailed compositional data are lacking, it may be more convenient (though less rigorous) to define a network hydration parameter in terms of mass rather than volume, viz., $(M_{TOTAL} - m_c)/m_c$ instead of $(V_{TOTAL} - V_c)/V_c$. To calculate it, one would only require the knowledge of total tissue mass and collagen concentration.

Detailed Experimental Procedures

Tissue sources. Femoral heads (ages 40, 55, and 90 years) were obtained at operations for femoral neck fractures or at postmortem. Femoral condyles were obtained at an operation for total joint replacement. Joints were immediately placed in plastic bags, sealed, and then kept frozen at −20°C. Before handling, joints were soaked in physiological saline at 4°C for 1 to 2 h. This freezing and thawing protocol has been shown to preserve structural and mechanical properties of the tissue (25).

Mild enzymatic treatment of the cartilage specimens was performed by incubation in trypsin solution (1 mg trypsin per milliliter of 0.15 M NaCl) for approximately 5 h at room temperature, with subsequent washings in 0.15 M NaCl solution, containing enzyme inhibitors (soybean trypsin inhibitor, 1 mg/ml solution + 1 mM EDTA, 2 mM PMSF, 10 mM *N*-ethylmaleimide). This mild treatment resulted in partial removal of the PG, and a concomitant decrease of FCD of approximately 40% (present data). PG removal was uniform throughout the thickness of the specimens, as checked on adjacent samples, which were sliced into 200-μm slices after enzyme treatment and tested individually for final FCD. Note that less mild trypsin treatments, carried out for longer periods of time and at higher temperatures in order to remove all of the PG, were shown not to affect collagen fibril structure, composition, or uniaxial tension response of the tissue (11, 26–28).

Specimen preparation. Full-depth cartilage plugs, approximately 7 mm in diameter, were cored from the superior surface of the normal femoral head. The cartilage showed no sign of fibrillation as assessed by the methods of Byers *et al.* (29, 30) and india ink staining (31). Similar plugs were obtained from OA femoral condyles, the surfaces of which were carefully characterized by the same procedures. In the case of OA specimens, the surface appearance ranged from nearly intact to severely fibrillated.

Both normal and OA plugs were sliced on a freezing microtome (Leitz), 400-μm sections being removed from the cartilage surface as well as from the deep zone. The test specimens thus consisted of the central, ~1-mm-thick slice, representing mainly "middle zone" cartilage. We chose to use sections from the middle zone because in normal cartilage the PG contents and fixed-charge densities are known to be relatively uniform in this region (2, 16, 32) and in OA specimens this zone is usually less disrupted than the surface. We weighed the sections as cut, and then soaked them in 0.15 M NaCl (physiological saline) overnight, and again measured their equilibrium weight. The saline solution contained enzyme inhibitors (1 mM EDTA, 2 mM PMSF, 10 mM ethylmaleimide).

Chemical reagents. PEG (20,000 Da) was obtained from Fluka (Switzerland) and purified by ultrafiltration through a 3000-Da membrane. Trypsin and proteinase inhibitors were obtained from Sigma Chemicals (St. Louis, MO), radioactively labeled ^{22}Na was from Amersham International (UK), and "Spectrapor" dialysis tubing (M_r cutoff 1000 Da) was from Spectrum Medical (CA).

Calibration of PEG solutions. Both tissue swelling pressure and the osmotic pressure of the proteoglycan solutions were measured by equilibrium dialysis against PEG solutions of known osmotic pressure. PEG calibration was carried out in specially adapted stirred ultrafiltration cell (Amicon). The cell is fitted with a semipermeable

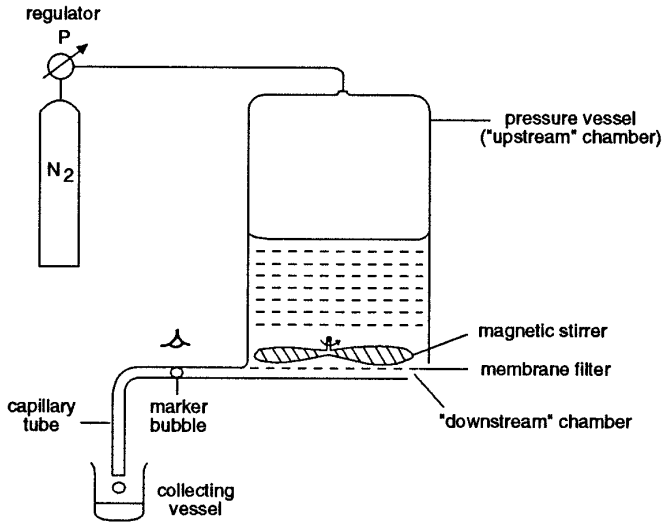

FIG. 1. Apparatus for measuring the osmotic pressure of PEG solutions.

membrane and is capable of withstanding pressures up to 5 atm. The apparatus we used is shown schematically in Fig. 1. The "upstream" chamber is fitted with a magnetic stirrer, located just above the membrane. The membrane is initially wetted with the NaCl solution and the small capacity (approx 0.5 ml) "downstream" chamber is filled with the above solution, care being taken to exclude air bubbles from the chamber and the membrane. The exit consists of capillary tubing in which an air bubble is trapped as a marker of equilibrium. Initially, the upstream chamber contains a very thin layer of pure NaCl solution. To this we add approximately 50–80 ml of the PEG solution of a given concentration whose osmotic pressure we wish to measure. Air pressure is applied and the stirrer is started. By trial and error we find the applied pressure which will just balance the difference in osmotic pressure between the PEG solution in the upstream chamber and the pure NaCl solution in the downstream compartment. When the two pressure differentials become equal, the bubble in the capillary stops moving (as in a "null" method). Sufficient time is allowed for equilibrium to be achieved and the exact air pressure is then measured, using an accurate pressure gauge, previously calibrated. Aliquots of the PEG solution are removed from the cell and freeze-dried in order to check the exact PEG concentration. At equilibrium there is no diffusion of NaCl across the membrane, as determined by conductivity measurements carried out on fluid periodically removed from the downstream compartment. The equilibrium NaCl concentration in the downstream compartment was found to be always higher than that in the PEG compartment, due to excluded volume effects, as anticipated from equations given by Wells for equilibrium in three component systems (33). Our experimental data for the osmotic pressure of 20,000-Da PEG in 0.15 M NaCl at 4°C (5, 14, 34) lie very close to those of Parsegian *et al.* for PEG in water at 7°C (35) (Fig. 2). The π_{PEG} vs PEG concentration data in 0.015 and 0.15 M NaCl were found to coincide (authors' unpublished data).

Note that in our calculations of P_c we used π_{PEG} values derived from Parsegian's curve since the latter extends to higher pressures than our own experimental data.

Equilibrium dialysis of cartilage specimens against PEG solutions. PEG solutions were prepared in 0.15 and 0.015 M NaCl, ranging from 3 g PEG in 100 g solvent ($\pi_{PEG} \approx 0.1$ atm) to 27.5 g PEG in 100 g solvent ($\pi_{PEG} \approx 8.35$ atm). All solutions contained 0.01% azide as antibacterial agent.

Since PEG has been found to penetrate cartilage, the tissue samples were not placed directly in the PEG solution, but were separated from it by dialysis tubing (Spectrapor, 1000-Da cutoff), which minimizes PEG penetration (15). Control experiments showed that differences between dry tissue weight determined before and after dialysis against PEG were negligible. This ensured that under our experimental conditions there was indeed no significant PEG penetration.

Cartilage slices were equilibrated in each PEG solution for approximately 48 h at 4°C as this has been found to be sufficient to achieve equilibrium hydration, while at the same time minimizing PEG penetration. At the end of each test, specimens were removed from the dialysis tubing and weighed immediately. They were then reequilibrated in 0.15 M NaCl and reweighed to ensure that they had returned to their baseline weight. At this stage, the slices were ready for equilibration in a solution of different PEG concentration. This process was repeated several times. All weighings were carried out as previously described (36): tissue samples, placed in preweighed stoppered vials, were weighed to five decimal places using an analytical balance. Reproducibility was between 0.2 and 0.3%. Note that we periodically weighed the specimens after reequilibration in 0.15 M NaCl (which contained enzyme inhibitors) to ensure that there were no significant changes in tissue weight during our series of tests, and periodically measured FCD to ensure that no PG was lost. We discarded from the study those samples that exhibited significant changes in weight and/or FCD. Such samples constituted less than 20% of the total number of normal test specimens. However, while normal specimens could be tested in several PEG solutions and repeatedly reequilibrated in normal saline in between these tests, without changes in FCD or water content, OA specimens were more liable to lose PG when left in contact with saline solution. Therefore, for the latter specimens, we tried to minimize such contact, often transferring the cartilage sample directly from one PEG solution to another (lower to higher concentration), without reequilibration in 0.15 M NaCl.

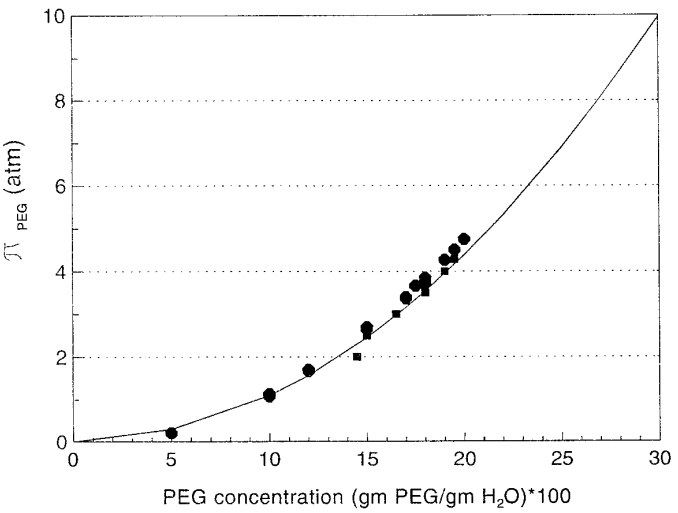

FIG. 2. Osmotic pressure of PEG solutions (π_{PEG}) as a function of PEG concentration. ■, Ramon and Maroudas (1989, unpublished data); ●, Erlich (1993) (14). (—) The curve was obtained from the equation $\log y = 7.338[x/(x + 100)]^{0.21} - 4.3957$, where $y = \pi_{PEG}$ (atm) and x is the concentration of PEG in grams per 100 g H_2O. This equation is directly derived from the empirical expression given by Parsegian *et al.* (35), the constants being adjusted for the difference in the units used. Parsegian's equation is based on data obtained for PEG in water at 7°C.

To obtain dry weights, the specimens were dried to constant weight in a freeze drier. This usually took 24 to 36 h, depending on the initial tissue weight. Sometimes the samples were freeze-dried several times during a series of experiments (e.g., to check for PEG penetration) and subsequently rehydrated. This treatment was found not to alter any of the tissue properties that we were testing.

The experimental procedure is straightforward but success depends on extremely careful, accurate, and reproducible weight determinations, both wet and dry. For wet weight it is essential, before one weighs the sample, to blot both surfaces to remove all extraneous liquid: blotting gently so as not to express the interstitial fluid, yet swiftly so as not to allow loss of water through surface evaporation.

Most of the experiments were carried out in 0.15 M NaCl. However, to extend the range of P_c to higher hydrations, some specimens were also equilibrated in 0.015 M NaCl. Without PEG, the equilibrium in 0.015 M NaCl is described by:

$$P_{C(SW)}^{(0.015M)} = \pi_{PG(SW)}^{(0.015M)}. \quad [7]$$

$P_{C(SW)}^{(0.015M)}$ and $\pi_{PG(SW)}^{(0.015M)}$ represent respectively the collagen tensile stress and PG osmotic pressure in the swollen state in 0.015 M NaCl. Clearly, to determine P_c we need to know the corresponding π_{PG}.

Unfortunately, at present we have no data on the osmotic pressure of extracted PG in hypotonic solutions. Therefore, an indirect procedure was used, which is described in Appendix II.

Determination of cartilage FCD. Measurements of total fixed-charge density (meq/g total tissue) were obtained by means of the tracer cation method, using ^{22}Na in hypotonic saline (37).

Osmotic pressure and FCD of PG solutions. In the present work, we have not made these measurements, but have used experimental results from studies previously carried out in our laboratory (14, 15). In these studies, the procedure for osmotic pressure measurements had consisted of confining the PG, prepared as described in (15), in low pore dialysis tubing and measuring their equilibrium concentration after exposure to PEG solutions of known osmotic pressure. The concentration of PG was then translated into FCD by determining the latter for each separate PG preparation. The FCD was determined by "spiking" one of the PEG–0.15 M NaCl solutions used for the osmotic pressure measurements with radioactively labeled ^{22}Na and ^{36}Cl. The details of the procedure are given in Refs. (15), (34), and (38).

The data we have used are those of Urban *et al.* (15), which have been recalculated,[1] as well as more recent experimental results (14; also authors' unpublished data). The combined data were fitted with a quadratic function

$$\pi_{PG} = C_1 \times FCD + C_2 \times FCD,^3 \quad [8]$$

where $C_1 = 3.51$ and $C_2 = 19.30$ by using nonlinear regression to obtain the virial coefficients C_1 and C_2. (C_0 and higher order terms were found to be negligible for the present range of concentrations).

[3] Urban *et al.* (15) originally calculated the osmotic pressures of PEG solutions using virial coefficients reported for 25°C by Edmond and Ogston (39) and assumed that these virial coefficients did not vary with temperature. Subsequently, direct measurements of PEG osmotic pressure at lower temperatures were performed by Parsegian *et al.* (35), and independently in our laboratory (5, 14, 34, 40). The assumption that the virial coefficients were temperature-independent were shown to be incorrect. We recalculated the data of Urban *et al.* (15) using new experimental calibrations of PEG solutions (see Fig. 2).

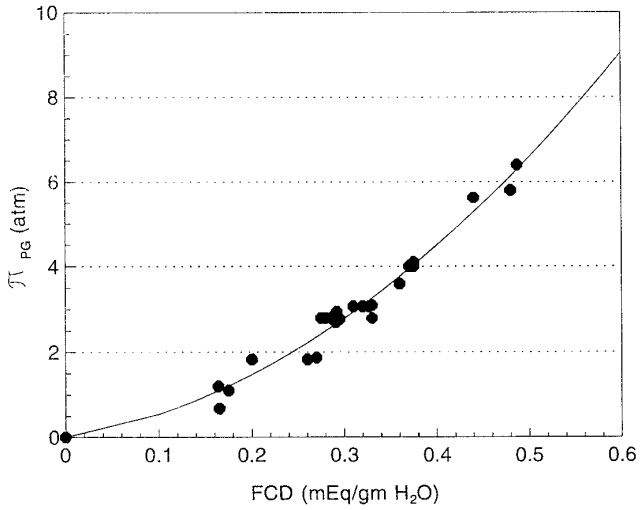

FIG. 3. Osmotic pressure, π_{PG}, of solutions of proteoglycans extracted from human adult femoral head cartilage as a function of fixed charge density (FCD). Fitted curve: $\pi_{PG} = 19.30*(FCD)^2 + 3.51*(FCD)$. ($R^2 = 0.98$) (Gen Fit, xl STAT).

The data set and the fitted quadratic function are shown in Fig. 3 (note that this empirical relationship includes both electrostatic and entropic contributions). Equation [6] supersedes the relationships previously given for PG osmotic pressure (e.g., in (15) and (5)) because it is based on an up-to-date calibration of PEG solutions and includes more experimental data for hip PG.

It should be noted that for polyelectrolyte solutions the electrostatic component of osmotic pressure increases with an increase in the intercharge distance on the polyelectrolyte molecule (15, 41). In the case of PG solutions, this effect appears in the dependence of π_{PG}, not only on FCD, but also on the molar ratio of KS—which has one negatively charged group per disaccharide—to CS—which has two such groups (15, 34, 42). In the present work we have used the curve of π_{PG} vs FCD for the PG extracted from aging human hip and knee cartilage, for which the CS to KS ratio is known to be close to unity (15, 16, 43). This curve may not be accurate for OA cartilage where the molar KS:CS ratio is usually less than 1 and π_{PG} may therefore be somewhat less than we have estimated.

Collagen Analysis

Cartilage samples were hydrolyzed in 6 M HCl at 110°C for 20–24 h, dried overnight in a Speed Vac (Savant, Farmingdale), and dissolved in 0.1 M sodium borate buffer (pH 8.0). A 200-ml aliquot containing 0.76 mg tissue (dry weight) was derivatized with 200 ml 6 mM γ-fluorenylmethyl chloroformate (Fluka, Switzerland) in acetone for 5 min at room temperature. Termination of the reaction was performed by extraction with pentane. Amino acid analysis was performed according to Miller *et al.* (44). The amount of collagen (in mg) was determined from the amount of hydroxyproline, assuming 300 residues per triple helical molecule with a molecular weight of 300,000 Da.

Intrafibrillar Water Determination

Intrafibrillar water content was obtained from collagen spacing data for human articular cartilage as measured by low-angle equatorial X-ray scattering at varying levels of osmotic stress.

It was shown by Katz *et al.* (12) and Maroudas *et al.* (11) that

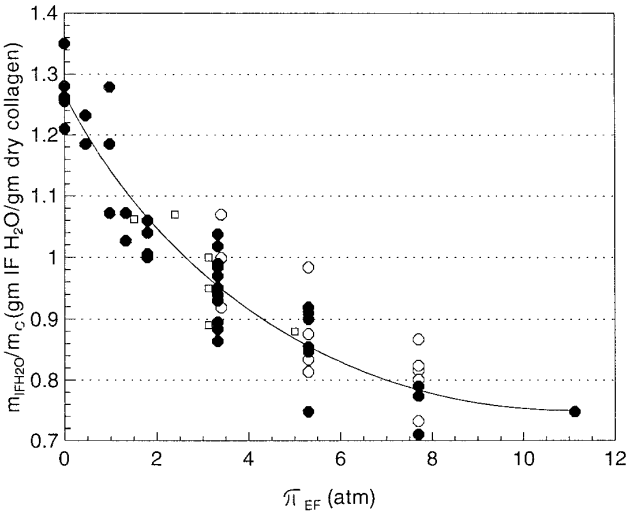

FIG. 4. Intrafibrillar water as a function of applied osmotic stress, π_{PEG}, representing the extrafibrillar osmotic pressure, π_{EF}. ●, PG-depleted samples; ○, native samples. Fitted curve: $m_{IFH_2O}/m_c = 0.726 - 0.538 \exp(-0.258*\pi_{PEG})$ ($R^2 = 0.86$). (Gen Fit, xl STAT). □, native samples, extrafibrillar osmotic pressure π_{EF} given by π_{PG}, see text (points not included for curve-fitting).

collagen fibril hydration (defined as the mass of IF water, m_{IFH_2O} divided by the mass of dry collagen, m_c) is not a constant, but is a function of the osmotic stress acting on the collagen fibrils and that the relationship between IF water and EF osmotic pressure is the same whether the osmotic pressure is due to the activity of the PG in the EF space or whether it is caused by the externally applied, osmotically active PEG when no PG are present in the tissue. It should be borne in mind that, in accordance with Eq. [1], for osmotically compressed native specimens under conditions when $P_c \rightarrow 0$, $\pi_{EF} = \pi_{PG} = \pi_{PEG}$. The curve in Fig. 4 showing IF water as a function of π_{EF} is based on several sets of previously published data for normal human cartilage (11, 45) as well as on more recent unpublished results, all of which we have combined here. The curve was obtained using π_{PEG} as the EF stress, π_{EF}. Each point represents the mean of several replicate samples from the same joint. For high PEG concentrations ($\pi_{PG} > 7$ atm), in addition to the data for PG-free specimens, we were able to include values of m_{IF}/m_c vs π_{PEG} also for native specimens, since in this case $P_c \rightarrow 0$ and π_{EF} approaches π_{PEG}. For lower PEG concentrations, mainly data for PG-depleted specimens were used. However, some results for native samples of relatively low FCD were also included, such that P_c could again be neglected and π_{EF} could be assumed equal to π_{PEG}.

For native cartilage, we also show in Fig. 4 some experimental values of IF water (45, and recent unpublished data) corresponding to values of $\pi_{EF} = \pi_{PG}$, as calculated from measured FCD and hydration, by the procedure given in Appendix I. As can be seen, these π_{PG} values lie in the same range as the π_{PEG} data.

We fitted the IF water content to an exponentially decaying function of the osmotic pressure in the EF compartment, π_{EF}, using nonlinear regression

$$\frac{m_{IFH_2O}}{m_c} = C_3 + C_4 \times \exp(-C_5 \times \pi_{EF}), \qquad [9]$$

where $C_3 = 0.726$, $C_4 = 0.538$, and $C_5 = 0.258$ (atm^{-1}). Both the data and the fitted function are shown in Fig. 4. The use of an exponentially decaying function in this regime of intermolecular spacing is consistent with recent findings of Leikin *et al.* (46).

Although the curve in Fig. 4 was derived from data obtained on normal cartilage alone, we have assumed in the present study that it can also be used for estimating the intrafibrillar water in OA cartilage. That this assumption is justified has been shown in a recent study in which we demonstrated that at equivalent FCD's IF water in specimens of OA cartilage is the same as in samples of normal tissue (Maroudas, Wachtel, and Schneiderman, unpublished data).

It should be emphasized that the empirical functions for both π_{PG} and $m_{IF\,H_2O}/m_c$ (Eqs. [8] and [9], respectively) given here are based on the most complete collection of results available to date, derived from a number of different investigations and including unpublished data. They supersede previous relationships given in [5], which were based on more limited information.

RESULTS

When normal cartilage specimens (full depth) were excised from the joint and equilibrated in 0.15 M NaCl, they swelled by less than 1% (by tissue weight). On the other hand, initial swelling of the OA cartilage specimens in 0.15 M NaCl was as high as 7%. Normal specimens transferred from 0.15 to 0.015 M NaCl swelled by 1 to 2%, while OA specimens swelled by up to 10%. This agrees with previous findings (3, 4).

Figure 5 shows the measured equilibrium pressures: P_c, π_{PG}, and π_{PEG} vs normalized tissue wet weight,

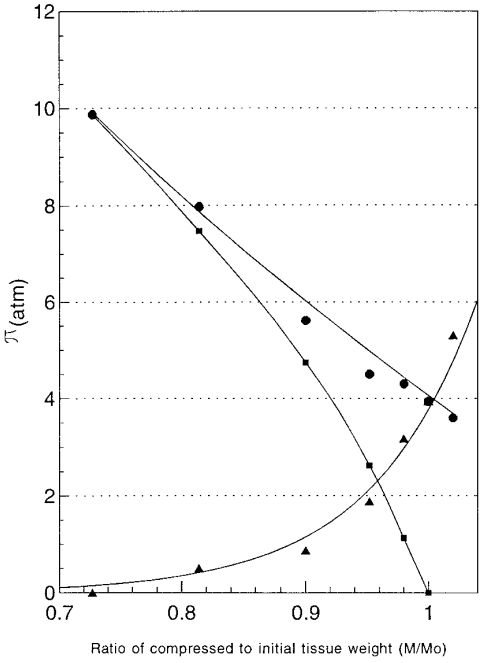

FIG. 5. "Balance of forces" in normal adult cartilage at equilibrium: osmotic pressure of cartilage proteoglycans, π_{PG}, collagen tensile stress, P_c, and applied osmotic stress, π_{PEG}, in relation to decreasing tissue weight under compression. ●, π_{PG}; ■, π_{PEG}; ▲, P_c. Age of donor: 55 years.

M/M_0, for a typical normal cartilage specimen from the middle zone. The point of intersection of the P_c and π_{PG} curves represents the state of unloaded equilibrium ($\pi_{PEG} = 0$) in normal saline, when $M/M_0 = 1$, where $\pi_{PEG} = 0$. While the tissue is unstrained, the collagen network is in tension: $P_c = \pi_{PG} = 3.9$ atm. As we increase π_{PEG} from 0 atm, P_c decreases and π_{PG} increases. P_c approaches 0 atm after a decrease in M/M_0 of about 20% (i.e., a decrease in hydration of about 30%). As we increase π_{PEG} further, π_{PG} approaches π_{PEG}, and consequently P_c remains close to zero. The coincidence of π_{PG} and π_{PEG} has been previously observed at low tissue hydrations (8, 10, 11) and is consistent with the Ogston picture of cartilage according to which its collagen matrix can exert tension to restrain PGs, but not support compression. Note that the P_c vs M/M_0 curve is highly nonlinear and monotonically increasing in the regime in which the network is in tension—in the "inflated" state. Conversely, the equilibrium π_{PG} vs M/M_0 curve is monotonically decreasing. Unlike the π_{PG} vs FCD curve for extracted PG which has a quadratic term, the π_{PG} vs M/M_0 curve for the tissue is practically linear. This is because, while the total water content decreases, the relative proportion of EF water increases. These competing effects lead to a less steep increase in FCD_{eff} than would be expected if there were no water repartitioning between these compartments.

The shape of the π_{PEG} vs M/M_0 curve represents the equilibrium response of the *whole tissue* (PG + collagen network) to the applied isotropic osmotic stress, the initial slope (at $M/M_0 = 1$) being ~0.4 atm per percentage change in weight. This is approximately equivalent to a stiffness modulus of 4 MN/m^2, which is within the range of values quoted for human femoral head on the basis of mechanical testing (47).

While for a single cartilage plug, it is illuminating to examine properties of the collagen network and PGs as a function of normalized tissue weight, it is difficult to use normalized tissue weight to make meaningful comparisons of tissue properties among different cartilage specimens. Therefore, in subsequent figures, we chose to plot P_C, π_{PG} and other quantities of interest against tissue hydration, $(V_{TOTAL} - V_c)V_c$, as defined in Eq. [6].

Figure 6 shows a comparison of plots of P_c and π_{PG} vs $(V_{TOTAL} - V_c)/V_c$ for a normal 55-year-old and an OA specimen (68 years of age, surface grossly fibrillated, middle zone visually intact). For the normal specimen, equilibrium in the absence of osmotic loading occurs at a hydration ($(V_{TOTAL} - V_c)/V_c$) of 6.29, whereas for the OA specimen, equilibrium in the absence of loading occurs at a hydration of 10.98, which represents more than a twofold increase in the ratio of total tissue water to dry weight (see Table II). In considering the relative equilibrium stiffness of unloaded normal and OA cartilage, it is useful to compute the differences in the slopes

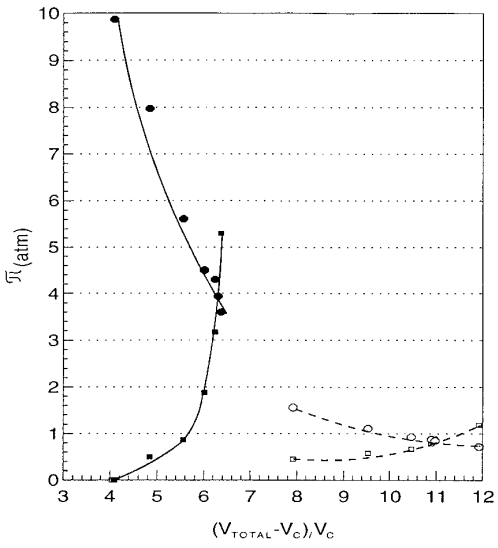

FIG. 6. Osmotic pressure of cartilage proteoglycans and collagen tensile stress for normal and OA cartilage as a function of $(V_{TOTAL} - V_c)/V_c$. ●, π_{PG} normal, 55 years; ■, P_c normal, 55 years; ○, π_{PG} OA, 68 years; □, P_c OA, 68 years.

between P_c vs $(V_{TOTAL} - V_c)/V_c$ and π_{PG} vs $(V_{TOTAL} - V_c)/V_c$ at initial equilibrium (equivalent to stiffness moduli). For the normal specimen, this difference is approximately 0.5 atm per percentage change in hydration, whereas for the OA specimen this difference is 0.1 atm per percentage change in hydration.

Another observation relating to tissue stiffness is the relative response of tissue hydration to a change in the ionic strength of the surrounding medium from 0.15 to 0.015 M: In the normal tissue specimen, the resulting change of 1 atm in π_{PG} produces a change in volume of approximately 1.8%, whereas in the OA tissue specimen, a smaller change in π_{PG} (0.5 atm) produces a much larger change in tissue volume (~10%). While we have obtained data from only one OA joint in the present study, these results are qualitatively consistent with our previous findings on hydration and swelling of OA cartilage based on a large number of joints (e.g., 3, 4, 8, 48).

Figure 7 shows curves of P_c vs $(V_{TOTAL} - V_c)/V_c$ measured in normal cartilage specimens of different ages. Results for two to seven specimens from each joint are included in the figure. The curves representing tissues from widely differing age groups are qualitatively similar, both in shape and extent. Thus, for instance, it should be noted in all cases that P_c tends to zero only when the normalized volume has decreased by as much as 20–25% of its initial value. While no clear trends in the shape of the curves with age were seen, there is a small but significant increase in the unloaded equilibrium value of P_c with age (see Table II), consistent with previous findings (8, 10).

TABLE II

Characteristics of Normal Native, Normal Trypsin-Treated, and Osteoarthritic Specimens in 0.15 M NaCl in the Absence of Compression

Age (years)	H_2O/dry wt (g/g)	$\frac{V_{TOTAL} - V_c}{V_c}$	FCD per total tissue wt (meq/g)	FCD per dry wt (meq/g)	FCD_{eff} (meq/g)	$P_c^c = \pi_{PG}$ (atm)
90, $n = 8$	1.90 ± 0.10	6.14 ± 0.12	0.201 ± 0.003	0.584 ± 0.016	0.405 ± 0.009	4.48 ± 0.23
55, $n = 7$	2.01 ± 0.08	6.29 ± 1.41	0.178 ± 0.019	0.540 ± 0.057	0.364 ± 0.014	3.67 ± 0.41
40, $n = 2$	2.45 ± 0.04	6.30 ± 0.06	0.154 ± 0.013	0.528 ± 0.049	0.278 ± 0.025	2.47 ± 0.48
Mean, $n = 17$	2.01 ± 0.23	6.22 ± 0.66	0.187 ± 0.015	0.559 ± 0.063	0.37 ± 0.072	3.91 ± 0.54
90, $n = 4$ After trypsin treatment	2.13 ± 0.14	5.92 ± 0.22	0.128 ± 0.009	0.402 ± 0.017	0.265 ± 0.043	2.21 ± 0.24
68 OA-1[a]	4.56	10.98[b]	0.094	0.52	0.138	0.88
OA-2	3.52	7.23	0.080	0.36	0.139	0.88
OA-3	3.44	10.25	0.122	0.54	0.192	1.40
OA-4	3.64	8.48[b]	0.123	0.57	0.202	1.51
Mean	3.79 ± 0.35	9.24 ± 2.01	0.105 ± 0.018	0.50 ± 0.07	0.167 ± 0.034	1.16 ± 0.34

[a] The OA specimens from which our slices were prepared had the following characteristics: OA-1, coarsely fibrillated; OA-2, superficially fibrillated; OA-3, superficially fibrillated; OA-4, superficially fibrillated. They all came from the same joint (68 years).

[b] For calculating volumes, we assumed v_c to be the same for OA as for normal cartilage: even if there are changes, for instance in the degree of glycosylation of collagen in OA, the resulting difference in v_c is expected to be minimal (since, for instance, the difference in v_c between collagen type I and collagen type II, which is far more glycosylated, is less than 2%).

[c] The difference in P_c between the normal specimens and OA specimens is highly significant ($P < 0.0005$ t test). The differences in P_c among the normal specimens of different ages are also highly significant ($P < 0.0025$ t test).

Figure 8 shows P_c vs $(V_{TOTAL} - V_c)/V_c$ curves for (i) four normal native specimens (age 90 years), (ii) four specimens from the same 90-year-old normal joint but treated with trypsin, and (iii) four specimens from one OA joint but with different surface characteristics. The mild trypsin treatment was aimed at reducing the GAG content to the level found in some of the OA specimens, while not affecting the collagen network. This treatment lowered the FCD by approximately 30–40% and decreased tissue volume by approximately 5%. The treated and untreated normal specimens show a similar dependence of P_c on hydration; however, owing to the reduction in the PG content of the treated specimens, the initial (unloaded equilibrium) hydration is lower than that in the untreated specimens. Where the hydration levels of the treated and untreated normal specimens do coincide, so do the values of P_c. The similarity in the P_c vs $(V_{TOTAL} - V_c)/V_c$ curves of the treated and untreated specimens, differing widely in FCD, shows that neither the FCD nor the GAG content per se influences the stiffness of the collagen network. In addition, since FCD is known to affect the intermolecular collagen spacing, it appears that the tensile stiffness of the collagen network is not sensitive to variations in this parameter.

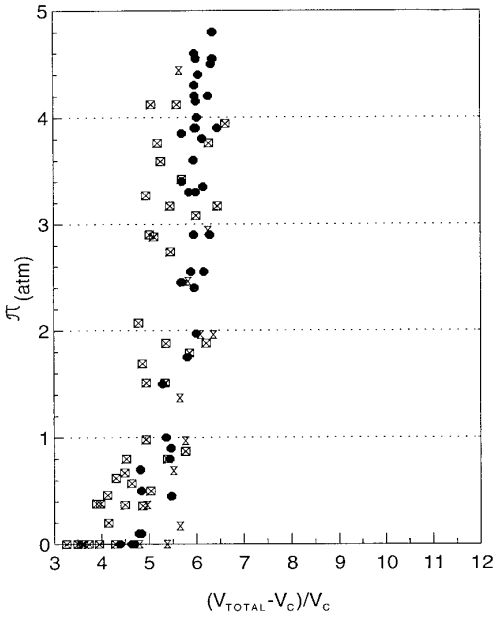

FIG. 7. Collagen tensile stress as a function of $(V_{TOTAL} - V_c)/V_c$ for normal cartilage from different age groups. ●, 90 years old; ⊠, 55 years old; ×, 40 years old.

In comparison to the normal specimens—whether native or trypsin-treated—P_c vs $(V_{TOTAL} - V_c)/V_c$ curves for OA specimens in Fig. 8 show a marked reduction in slope and shift to higher hydrations. It is particularly interesting that the OA specimen corresponding to the curve shifted furthest to the right and having a very

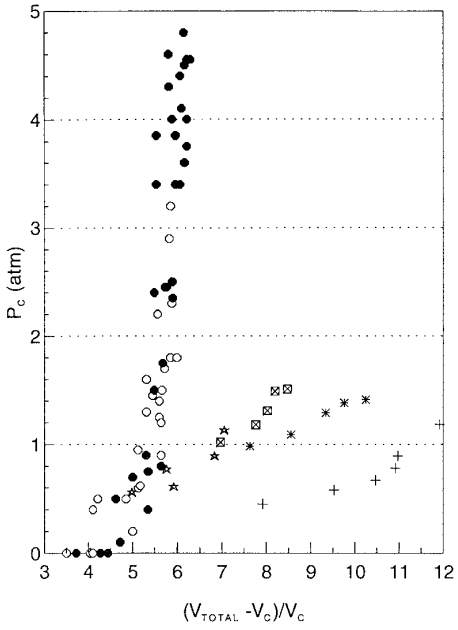

FIG. 8. Collagen tensile stress as a function of $(V_{TOTAL} - V_c)/V_c$ for normal native, normal trypsin-treated, and OA specimens. ●, normal native; ○, normal trypsin-treated; +, OA-1; ★, OA-2; *, OA-3; ⊠, OA-4.

shallow slope (OA-1) has a relatively high FCD per dry weight, similar to that found in normal native samples (see Table II). On the other hand, the OA specimen from a neighboring region on the joint, corresponding to the curve with a *steeper* slope (OA-2), has a lower FCD per dry weight, similar to that of the samples that have been trypsin-treated. Thus, there appears to be no relation between the shape of the P_c vs $(V_{TOTAL} - V_c)/V_c$ cuve and the GAG content based on dry tissue weight.

Our OA data do not extend to a sufficiently low level of hydration to show the collagen network behavior in this regime, so it is difficult to ascertain at what hydration P_c reaches zero, but there is an indication that π_{PG} and thus P_c are overestimated in the OA specimens (this is discussed below).

DISCUSSION

Some Comments on Methodology

This is the first time that use has been made of the "balance of forces" equation (Eq. [1]) to quantify P_c in cartilage over the entire range of hydrations over which the collagen network exerts a tensile stress. For this purpose, in addition to measuring the cartilage swelling pressure over a wide range of hydrations, we have considerably updated the relationships necessary for calculating P_c from the above data, viz., the relationship between π_{PG} and FCD for extracted PG in solution and the relationship between IF water and EF stress for human cartilage (Eqs. [8] and [9]).

One cautionary reminder concerns calculating P_c by taking the difference of π_{PG} and π_{PEG} when these differences are small. One source of error could arise from the fact that pooled average data were used to calculate π_{PG}, in which, for instance, differences in KS/CS ratios were not taken into account (see Materials and Methods). However, we do not think that in our study of normal cartilage this factor could contribute to major inaccuracies for the following reasons. It is well known (e.g., 20, 43) that the main source of variation in the CS to KS ratio is the age of the cartilage donor: for our normal specimens the age ranges in the present study, and in the study in which the osmotic pressure of the isolated PGs was measured (ages 50–80 years), overlap to a large extent.[4] In addition, for normal specimens, we have a reassuring indication that our calculated values of π_{PG} are accurate, since, in the low hydration regime—where collagen tension should be close to zero—we find that the difference $(\pi_{PG} - \pi_{PEG})$ approaches zero as expected. This makes us feel more confident about the accuracy of our calculated values of P_c at higher hydrations, where collagen is in tension. In the case of OA specimens, we have at present no information about the specific characteristics of their π_{PG} vs FCD curve, so we had to use data for PG from normal tissue. However, we do know that the KS/CS ratio decreases in OA (16, 49), which should lead to a lower value of π_{PG} for a given level of FCD, compared with PG from normal cartilage, on which we base our calculations (14, 15). This is consistent with an overestimate of π_{PG} and hence P_c in OA specimens.

One of our plans for refining this methodology in the future is to use PG extracted from each joint rather than PG extracted from many specimens. The availability of such data will further improve the accuracy of the empirical relationship between PG composition and osmotic pressure and, in a more general manner, can help to clarify the effects of PG structure and composition on chemical activity.

Another point concerns our definition of the "collagen network hydration." As mentioned under Materials and Methods, we chose to define it in relation to the volume of dry collagen because in adult cartilage the quantity of collagen remains constant, even in OA. If one uses a parameter which is easier to determine experimentally, viz., total hydration per total dry tissue volume, one does in fact obtain qualitatively similar trends, but quantitation can be inaccurate and misleading. For instance, in OA cartilage, because some

[4] Variability in the middle zone in the CS-to-KS ratio in this age range does not exceed 20%.

PG are lost and dry tissue volume per dry collagen volume decreases, the hydration based on total dry volume would show a higher water content compared with normal than is in fact the case.

Finally, it must be borne in mind that the conclusions in this study are based on the use of isotropic osmotic compression. Isotropic compression clearly does not reflect the physiological situation and *a priori* does not seem to be similar to *in vitro* mechanical testing. However, in actual fact, analogous results have been obtained using both mechanical and osmotic compression. Thus, when cartilage was loaded in uniaxial unconfined compression, at equilibrium the applied mechanical pressure was found to balance quantitatively π_{PG} in the regime of large deformations (50, 51), indicating that here too the overall P_c term tends to zero, as is the case when osmotic stress is used (e.g., 5, 10). Although in the above mechanical tests, the intermediate hydration regime was not quantitatively investigated, similar qualitative trends were seen to those observed in the presence of osmotic compression. In any case, the general rationale underlying the present experiments can also be extended to the use of static mechanical compression. In fact, it should be possible to derive new information on the effects of collagen anisotropy on the tensile stresses in the collagen network by applying different modes of mechanical compression to cartilage (e.g., confined versus unconfined) and determining P_c.

For more highly oriented tissues, such as the annulus fibrosus, the isotropic osmotic stress technique may yield information of limited physiological relevance. The use of mechanical compression, however, may present difficulties due to loss of the PG during the tests (38).

Physiological Implications

Our experimental results show that for normal unloaded cartilage at physiological ionic strength the slope of P_c vs $(V_{tot} - V_c)/V_c$ is significantly steeper than that of π_{PG} vs $(V_{TOTAL} - V_c)/V_c$ at their point of intersection. Thus, the collagen network effectively fixes or "clamps" the equilibrium hydration to lie within a very narrow range despite large changes in PG osmotic pressure. This result helps us understand three important phenomena. First, it explains why excised normal adult human cartilage plugs immersed in 0.15 M NaCl show little initial swelling, although the osmotic pressure gradients are altered as a result of removal from the intact joint (4). Second, we can see why normal adult human cartilage swells by only 1–2% when it is transferred from physiological saline into hypotonic solution, in spite of the fact that this treatment leads to a considerable increase in the electrostatic component of π_{PG} (e.g., 48). Third, and most important, the stiffness of the collagen network ensures that *in vivo* normal adult human cartilage maintains dimensional stability when small changes in proteoglycan content occur (such as in normal turnover): and, conversely, it follows that if collagen stiffness is decreased (as in OA), then the same variations in PG osmotic pressure cause the cartilage to swell or shrink by a larger amount.

Moreover, it is important to note that the shape of the P_c vs $(V_{TOTAL} - V_c)/V_c$ curve largely determines the PG concentration at equilibrium by controlling the volume of the hydrated tissue–and *not* vice versa: In normal specimens, high PG concentration, high FCD, and high π_{PG} can be developed owing to high collagen network stiffness. This means that during static load-bearing, because of the high GAG osmotic pressure, changes in tissue thickness and chondrocyte volume will remain relatively small.

On the other hand, in OA specimens, in which the collagen network is impaired, for the same FCD per dry weight, $(V_{TOTAL} - V_c)/V_c$ at equilibrium is approximately twice as high that as in normal specimens, and overall FCD, and hence FCD_{eff} are halved (see Table I). This shows that OA cartilage cannot develop a high effective PG concentration. Even if PG quantity (i.e., the ratio of PG-to-dry collagen) were to increase, the tissue would simply imbibe more water, and swell to a larger volume, without a significant increase in PG concentration. Hence, load-bearing capacity of the tissue would not increase.

The trypsin treatment, which reduced the FCD per dry weight to the lowest level found among our OA specimens (see Table II), did not result in any discernible change in P_c vs $(V_{tot} - V_c)/V_c$ at equilibrium. Hence, there was no increase in hydration, and therefore, FCD_{eff} was considerably higher than in the OA specimens at unloaded equilibrium (see Table II).

In our present study, we have confirmed a previous finding that the unloaded equilibrium values of $P_c = \pi_{PG}$ increase with age (8, 10). More data are needed to draw conclusions as to the slopes of the P_c vs $(V_{TOTAL} - V_c)/V_c$ for different age groups, though our present data do not reveal any significant age-related differences.

One important general point should be noted: while it was previously thought, on the basis of preliminary tests (A.M. and co-workers, e.g., 6, 10) that in normal cartilage the collagen tensile stresses are relieved when the initial cartilage volume is reduced by a small amount (approximately 5%), the present investigation has shown that the collagen network does not in fact become "limp" until the volume has decreased by much more, viz., 20–25%. Therefore, the range of hydration values over which the collagen network contribution to the "balance of forces" needs to be taken into account is very significant indeed.

So far, we have discussed some physiological consequences of the stiffness of the collagen network in human articular cartilage. However, this work also raises important biochemical and structural questions which, for the time being, must be left unanswered. What specific molecular and ultrastructural factors give rise to human articular cartilage's singularly high resistance to swelling? and how do these factors change during development, aging, and degeneration? We know that the "backbone" of the network consists of fibrillar assemblies of collagen II, with some collagen XI (52, 53). These molecules undergo posttranslational changes with age, such as intermolecular cross-linking (54). The network contains, in addition, a number of other molecular constituents, such as collagen IX, decorin, and fibromodulin, which may act as supramolecular cross-bridges (54). Ultimately, we would like to know what role these various constituents (and their interactions) play in determining the mechanical properties of the collagen network that we are now in a position to measure.

Cartilage Is Not a Soil

The distinction between the behavior of the aggregate tissue and its individual constituents, e.g., as displayed in Fig. 5, shed light on existing biomechanical models of cartilage. Following McCutchen's seminal suggestion that when loaded, cartilage deforms by expressing or squeezing out water like a sponge or a soil (55), subsequent biomechanical models (56) have treated cartilage literally as a soil, consisting of a "pore fluid" phase and a single "elastic network" phase. As our present study demonstrates, to understand the equilibrium swelling behavior of cartilage (and other extracellular matrices) requires separating its "solid" matrix phase into at least two constituents which possess distinct physiochemical properties, and perform distinct physiological functions. Their aggregation into a single "solid-like" phase (56, 57) can obscure these differences. One significant consequence of our simultaneous measurements of PG and collagen network properties is that it can help answer whether hydration changes in OA cartilage are caused by a loss of PGs or of collagen network integrity. Our data suggest the latter.

While our findings pertain directly to equilibrium loading, they have significant consequences for nonequilibrium loading regimes, particularly since the collagen network acts to restrain the PGs over a relatively wide range of hydrations. For all cases in which $P_c \neq 0$, the applied compressive load is assisted by the collagen network tension. This effect is not accounted for in existing biomechanical models, but must affect the driving pressure distribution in the tissue.

APPENDIX I

Changes in Hydration, PG Osmotic Pressure, and Collagen Tension during Compression of a Specimen from 90-Year-Old Hip (as Characterized in Table 1)

g PEG/ 100 g H_2O	π_{PEG} (atm)	M_0 (g)	M/M_0 (g)	$m_{TOTAL\ H_2O}$ (g)	F (g IF water per g collagen)	$m_{IF\ H_2O}$ (g)	$m_{EF\ H_2O}$ (g)	$FCD_{TOTAL\ H_2O}$	FCD_{eff}[c] (meq per g EF water)	π_{PG} (atm)	P_c (atm)	$(V_{TOTAL}-V_c)/V_c$
0	0	0.01880	1	0.01252	0.8968	0.00292	0.00960	0.304	0.398	4.45	4.45	6.40
8	0.71	0.01879	1	0.01251	0.8968	0.00292	0.00959	0.305	0.398	4.45	3.74	6.40
10	1.10	0.01868	0.994	0.01240	0.8935	0.00291	0.00949	0.308	0.402	4.52	3.42	6.34
12	1.57	0.01842	0.980	0.01214	0.8854	0.00289	0.00925	0.314	0.412	4.71	3.14	6.23
15	2.45	0.01805	0.960	0.01177	0.8730	0.00285	0.00892	0.324	0.428	5.03	2.58	6.07
20	4.38	0.01745	0.928	0.01117	0.8534	0.00278	0.00839	0.341	0.455	5.58	1.20	5.80
20	4.38	0.01712	0.911	0.01084	0.8427	0.00275	0.00809	0.352	0.471	5.92	1.54	5.66
25	6.89	0.01600	0.851	0.00972	0.8045	0.00262	0.00710	0.393	0.538	7.46	0.57	5.16
27	8.05	0.01556	0.828	0.00928	0.7904	0.00258	0.00670	0.411	0.569	8.23	0	4.97

[a] M_0, wet tissue weight after equilibration in 0.15 M NaCl solution; M, wet tissue weight after equilibration in PEG solution; F, is defined as the ratio: $m_{IF\ H_2O}/m_c$.

[b] Measured FCD per total (uncompressed) tissue weight: 0.203 meq/g.

[c] Trial and error procedure for calculating FCD_{eff} and hence π_{PG} and P_c: For each value of applied stress, π_{PEG}, the first step in calculating P_c is to determine F. This is carried out by trial and error as follows: we guess a value for F, proceed to calculate $m_{IF\ H_2O}$ and hence $m_{EF\ H_2O}(m_{EF\ H_2O} = m_{total\ H_2O} - m_{IF\ H_2O})$; we then calculate FCD_{eff}, based on $m_{EF\ H_2O}$, obtain π_{PG} from Eq. [8] (Fig. 3), and check from Eq. [9] (Fig. 4) whether the value of F we guessed corresponds to the calculated value of π_{PG}. If not, a second value is chosen and the cycle of calculations is repeated until the two values of F coincide. We then proceed to the definitive calculation of FCD_{eff} and hence π_{PG} and P_c.

APPENDIX II

Procedure for Determining P_c in Cartilage in Hypotonic Solutions

In essence the method consists of finding, by trial and error, the PEG concentration in 0.015 M NaCl (and hence π_{PEG}) which was just sufficient to shrink a given cartilage specimen to the same water content as it had in 0.15 M NaCl, without PEG. The equilibrium in 0.015 M NaCl in the presence of PEG is described by the equation:

$$P_{c(o)}^{(0.015M)} + \pi_{PEG(o)}^{0.015M} = \pi_{PG(o)}^{0.015M}. \qquad [A.1]$$

where the subscript (o) corresponds to initial equilibrium volume in 0.15 M NaCl, in the absence of PEG.

If we assume P_c is a function of tissue volume alone, then $P_{c(o)}^{(0.015M)} = P_{c(o)}^{(0.15M)}$, the latter quantity having already been determined. We also found that the pressure-concentration relationship for PEG solutions in 0.015 M NaCl is the same as in 0.15 M NaCl (authors' unpublished data); therefore, we know $\pi_{PEG(o)}^{(0.015M)}$ from data in Fig. 2. Thus, we calculate $\pi_{PG(o)}^{(0.015M)}$ from Eq. [A.1].

For normal specimens, the increase in water content due to swelling in 0.015 M NaCl is of the order of only 1 to 2%, so that the change in FCD_{EFF} and hence π_{PG} from the unswollen state is very small and can be neglected: thus, we can assume that $\pi_{PG(SW)}^{0.015M} \approx \pi_{PG(o)}^{0.015M}$ and substitute the latter value into Eq. [7] to obtain $P_{c(SW)}^{(0.015M)}$ corresponding to the increase in the tissue volume.

In the case of OA specimens, where the increase in hydration due to swelling in 0.015 M NaCl can reach 10%, one cannot asume $\pi_{PG(SW)}^{0.015M} \approx \pi_{PG(o)}^{0.015M}$. To estimate $\pi_{PG(o)}^{0.015M}$ we assume a linear relationship of $\pi_{PG}^{(0.015M)}$ vs hydration over the range of swelling in question and calculate $\pi_{PG(SW)}^{0.015M}$ from $\pi_{PG(o)}^{0.015M}$ in this manner.

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