Interstitial exclusion of macromolecules studied by graded centrifugation of rat tail tendon

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Interstitial exclusion of macromolecules studied by graded centrifugation of rat tail tendon. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2794–H2803, 1997.—Mechanical compression of cartilage and tendon has been shown to expel fluid both from collagen fibrils and from the extracellular space. As reported previously, albumin (Alb) concentration and colloid osmotic pressure in tendon fluid (TF) expelled by repeated centrifugations fell progressively at increasing centrifugation force (G = 600, 2,400, and 13,100), suggesting either molecular sieving in compressed tendon or increasing centrifugation force (G) expelled by repeated centrifugations fell progressively at concentration and colloid osmotic pressure in tendon fluid (TF). As reported previously, albumin (Alb), immunoglobulin G (IgG), and hyaluronan (hyaluronic acid; HA) with molecular weight (MW) ranging from 341 to 5 \times 10^6 strongly favored the exclusion hypothesis; the fraction of Alb, IgG, and HA-free fluid (excluded) was already 0.23–0.36 in the first centrifugate, increasing to 0.73–0.82 in the second and third centrifugates, with each space contributing about equally to the total centrifugate volume. The calculations also indicated that Alb-, IgG-, and Ap-free fluid was mobilized from extracellular space by increasing overlap of excluded territories. An excess of HA in tendon compared with that estimated from centrifugate concentrations suggests a large bound or immobilized HA fraction.

Collagen fibrils; hyaluronan; proteoglycans; plasma proteins; aprotinin

It is well known that the mobility and distribution of macromolecules (e.g., plasma proteins) in tissue interstitial spaces are greatly restricted by matrix components, hyaluronan (hyaluronic acid; HA), proteoglycans (PG), and collagen (4). However, it has been difficult to evaluate the contributions of these components to the overall restriction or the extent to which the composition of interstitial fluid varies in different structural associations.

Studies based on lymph or fluid collected by implanted wicks have provided valuable information about the composition and dynamics of the most mobile fraction of interstitial fluid in various organs and tissues. "Free" fluid, the fraction most directly involved in interstitial fluid turnover (3). In 1991, one of us (K. Aukland; Ref. 2) published a study exploring the use of progressive centrifugation to sample successively less mobile fractions of interstitial fluid in rat tail tendon, a nearly acellular tissue. With increasing centrifugal force and time, colloid osmotic pressure and concentrations of total plasma protein, albumin (Alb), and HA all decreased as centrifugate volume accumulated. Intercepts of zero centrifugate volume appeared to reflect concentrations in free interstitial fluid, i.e., similar to that of lymph or wick fluid. A similar fall in protein concentration (measured as a fall in colloid osmotic pressure) was reported by another of us (H. Wiig; Ref. 32) for centrifugates of rabbit cornea, another nearly acellular tissue. However, it was not clear from either study whether the progressive fall in macromolecular concentrations was caused by increasing contributions of fluid from macromolecule-excluding spaces (e.g., fluid between fibers of tightly packed collagen bundles) or by sieving of free interstitial fluid by progressively compressed matrix components. Significant dilution by intracellular fluid in these tissues is unlikely, because <5% of tissue volume is composed of cells.

In the present study we have therefore extended these observations to include a wide range of molecular sizes, from 51Cr-EDTA [molecular weight (MW) 341], aprotinin (Ap; MW 6,500), Alb (MW 69,000), and immunoglobulin G (IgG; MW 156,000) to HA (MW \approx 5 \times 10^6). Both sieving and excluded space at the surface of collagen fibrils and PG should increase with increasing size of the test molecules. If centrifugation causes denser packing of collagen fibrils, the excluded space would be expected to be more reduced for larger molecules, i.e., the concentration of the largest molecules in the expelled fluid should show greatest reduction. On the other hand, a similar dilution of all test molecules should result from admixture of fluid contained within the collagen fibrils. Because the center-to-center distance (lateral spacing) of the collagen molecules in tail tendon fibrils is only \approx 13 A (8, 9), the fibrils should probably not allow occupancy of any of the molecules tested here, with the exception of 51Cr-EDTA. Furthermore, as shown by Maroudas et al. (15), the lateral spacing and extracellular water content of cartilage can be considerably reduced by mechanical or osmotic compression. In kangaroo tail tendon, the lateral spacing has also been shown to be reduced by evaporation (29). The pressures required in these studies suggested that this intracellular fluid might be mobilized by high centrifugation rate only. This apparently conflicts with our previous finding that centrifugation three times at 2,400 G gave a pattern similar to that obtained with increasing revolutions per minute (rpm), leading to the conclusion that the fall in Alb concentration was a function of the amount of fluid removed from the tendon (2). To elucidate this question we have now tested the effect of three short-lasting consecutive high-speed runs ("express centrifugation") in paired...
comparison with the “standard” 15-min periods at increasing G.

METHODS

Tail tendon was obtained from male or female Wistar rats (200–400 g) kept on standard laboratory diet and with free access to water. Anesthesia was induced by intraperitoneal Inactin (80–100 mg/kg) or pentobarbital sodium (50 mg/kg), followed by supplementary intravenous injections when needed. Polyethylene catheters were placed in the right carotid artery and jugular vein for blood sampling and infusions.

Before the tail was removed, the rat was exsanguinated in the head-down position by severing the aorta through an abdominal incision. The tail was cut off and immediately transferred to an incubator kept at room temperature (20–24°C) and 100% humidity. The tail was divided into three sections, each ~3 cm long. When these sections were flexed, the tendons would protrude from their sleeves and could easily be pulled out. To obtain two comparable samples, tendons from the right half of each segment were collected and placed in a preweighed 2-ml centrifuge tube, provided with a basket of nylon mesh (pore size 15 × 20 μm) designed to keep the tissue sample up from the bottom of the tube (2), which was then immediately capped. Tendons from the contralateral halves of all three segments were collected in another basket. After reweighing, the tubes were centrifuged at room temperature and then immediately brought back into the incubator. The basket containing the sample was transferred to another preweighed centrifuge tube, and the tendon fluid accumulated at the bottom was collected in glass capillaries that were immediately closed at both ends. After reweighing, the second tube was centrifuged, and the procedure was repeated by centrifugation of the basket in a third tube. As described previously (2), the protocol provided data on the initial and subsequent weights of the tendon sample, each centrifuged volume, as well as any fluid loss by evaporation.

Typical tissue sample weights were 0.15–0.30 g, giving three tendon fluid samples of 5–15 μl each.

Centrifugation Protocols

The speed and duration of the three centrifugations were chosen to provide similar volumes of tendon fluid.

Standard centrifugation. Standard centrifugation periods were 15 min at rates of 3,000, 6,000, and 14,000 rpm, corresponding to 600, 2,400, and 13,100 G, respectively. The mechanical force applied to the tissue is not well defined: a 250-mg tissue sample will have a height of ~6 mm when placed in the nylon basket. That means that standard centrifugations exposed the bottom of the tissue pellet to pressures of 0.4, 1.6, and 9.2 bar above atmospheric pressure (~1 bar). However, the pressure will decline linearly toward zero in the top layer, giving average pressures of ~50% of bottom pressure. It seems likely, therefore, that more fluid could be derived from deep than from superficial layers of the tissue sample, even if mechanical and osmotic forces within the tissue may tend to even out such differences. We confirmed the previous observation that loosening and turning around the tissue sample within the ~5-min lapse between the centrifugations did not influence the concentration pattern for either Alb or HA, indicating that each centrifugation will start with full fluid equilibration within the sample (2). To evaluate to what extent a steady state was obtained by 15-min centrifugation, i.e., whether more fluid would have been obtained by more prolonged centrifugation, we compared centrifugate volumes obtained on samples from the same tail with centrifugation periods of 5-, 10-, and 15-min duration. In five tails, the total volumes obtained with 5- and 10-min centrifugations reached 67 ± 4 and 87 ± 4%, respectively, of those obtained with 15-min centrifugation, indicating near but not complete equilibrium at 15 min. Prolonging centrifugations beyond 15 min was deemed undesirable because of potential evaporative water loss.

To evaluate the effect of varying hydration on removal of macromolecules from the tendon, the standard centrifugation was repeated after a volume of 0.9% saline corresponding to that lost in the first three centrifugations was added. This will be referred to as “rehydrated.” In another series, one-half of the tail tendon was “prehydrated” by adding saline corresponding to 8–15% of tendon sample weight 1–2 h before the first centrifugation, which was usually reduced to a speed of 1,500 or 2,000 rpm, and the other one-half was exposed to standard centrifugation.

Express centrifugation. Express centrifugation consisted of 2 min at 9,000 rpm, 2 min at 12,000 rpm, and 5 min at 14,000 rpm (G = 5,400, 9,600, and 13,100, respectively).

Macromolecules and Analyses

Endogenous plasma Alb was determined in plasma and centrifugate by the fluorimetric 8-anilino-1-naphthalenesulfonic acid method of Rees et al. (28) as modified by Aukland and Fadnes (3).

HA in centrifugate and tendon samples was determined with a specific radioimmunoassay (HA-Test 50, Pharmacia, Uppsala, Sweden), after papain digestion of freeze-dried specimens (26). The MW of HA in centrifugate was estimated by gel chromatography, using a G1000 column (Pharmacia Biotech) packed with Sephacryl S-400 High Resolution and elution by phosphate-buffered saline with pH 7.5 or 11.6. Blue dextran (Sigma) and bovine serum albumin were used as markers. Total uronic acid in tail tendon was determined as described by Reed et al. (25), and collagen was determined according to Woessner (35).

IgG was labeled with 125I and infused for 5–7 days with an implanted osmotic minipump (Alzet). The implantation technique, 125I-labeling, gamma-counting procedure, and the resulting plasma 125I-IgG concentration have been described in detail by Wiig et al. (33, 34).

Ap (Sigma) with MW 6,500 was labeled with 125I by the iodogen technique and infused intravenously in rats for 2–4 h together with 51Cr-EDTA. Because this might not provide complete plasma-interstitial equilibration in the poorly perfused rat tail, in some experiments the tail was stored at 4°C in humidified atmosphere for 24 h before dissection and centrifugation.

Free 125I in the infusion fluid was checked with gel chromatography and kept below 2% by dialysis. To prevent breakdown in the kidney or urinary excretion, both renal pedicles were ligated before 125I-Ap infusion. Radioactivity was measured in a Cobra II Auto-Gamma counting system (Packard Instruments, Meriden, CT).

RESULTS

Albumin: Effect of Centrifugation Protocol

In each of six animals one-half of the tail tendon was centrifuged according to the standard centrifugation protocol (3,000, 6,000, and 14,000 rpm, each for 15 min), and the other one-half was centrifuged at 9,000 rpm for 2 min, 12,000 rpm for 2 min, and 14,000 rpm for 5 min (express centrifugation).
The Alb concentration obtained with standard centrifugation fell from 41% of plasma concentration in the first to 13% in the third centrifugation, as shown in Fig. 1, where the Alb concentration is plotted as a function of the accumulated volume of centrifuged fluid (tendon fluid weight; TFW) as a fraction of the initial tendon sample weight (TSW0).

During express centrifugation, the first tendon fluid sample (TF I) had significantly lower concentration, and little further reduction was obtained in TF II and TF III. Thus, in spite of the completely different concentration profiles, the total amounts of fluid and Alb removed from the tendon were not radically different.

All comparisons of HA, IgG, Ap, and 51Cr-EDTA with Alb were made with standard centrifugation.

HA and Alb

As shown in Fig. 2, the fall in HA concentration in successive samples obtained with standard centrifugation was slightly steeper than for Alb (P > 0.05). Because concentration in plasma is irrelevant in the case of HA, different experiments were compared by calculating the concentrations of both HA and Alb in the two last centrifugations (TF II and TF III) as ratios of that in TF I. The average absolute HA concentration in centrifugate extrapolated back to “zero centrifugation” (2) was 0.25 (SE 0.06) mg/ml. In a separate series, the tendon sample was loosened and turned upside down after centrifugation I–III. This did not alter the concentrations in TF II and TF III of either HA or Alb compared with those obtained in paired control samples from the same tail (n = 5).

Rehydration. The difference between the HA and Alb patterns was slightly more marked after rehydration (Fig. 2B), i.e., when the whole centrifugation sequence was repeated after saline corresponding to the volume lost in the first sequence was added, on average 7.5% of TSW0. The absolute concentration in the initial sample after rehydration (normalized to 1) varied considerably relative to that in the first sequence, but the average ratios of 0.88 for Alb and 1.02 for HA were not significantly different from 1. Quite remarkably, the amount of Alb and HA removed by centrifugation after rehydration was not significantly different from that in the preceding control centrifugation (Alb 106% and HA 116% of control).

Prehydration. Seven samples from the same tendons as those included in the control group (Fig. 2A) were prehydrated by adding saline corresponding to an average of 12.2 ± 4.4% of initial wet weight. The centrifugate volume obtained by the initial centrifugation was more than doubled compared with control, whereas concentration in the initial centrifugate was reduced to 87 and 88% of control for Alb and HA, respectively. As shown in Fig. 2C, the concentration of HA fell significantly more than that of Alb in the second and third centrifugations. The accumulated volume of 17.3% of TSW0 exceeded that of euhydrated controls by 100%, and the total amounts of Alb and HA removed were 204 and 185% of control, respectively.

Gel chromatography of centrifugate on Sephacyr-400 gave a single sharp HA peak in all centrifugations, clearly preceding blue dextran (MW ≈ 2 × 10^6) and was not distinguishable from Healon, the standard HA (MW ≈ 5 × 10^6) provided by Pharmacia for radioimmunoassay (Fig. 3). The pattern was the same in centrifugations I–III. Changing elution buffer pH from 7.5 to 11.6 did not influence the pattern.

IgG and Alb

In two rats, 125I-labeled IgG was infused for 168 h by implanted osmotic minipumps. One sample from each rat was centrifuged before and after rehydration, and another two samples from the same tails were centrifuged after prehydration (11.7 and 9.1% of initial wet wt). As shown in Fig. 4, the fall in IgG concentration from centrifugation I to II and III was similar to that of Alb in the same samples. However, in TF III, IgG fell more than Alb in 10 of the 12 samples, giving a statistically significant difference in paired comparison (P < 0.05).

Aprotinin and Alb

125I-Ap was determined together with Alb in 12 samples from 6 rats after 2–4 h of constant intravenous infusion, followed in 3 experiments by 24-h storage of the tail at 4°C for equilibration within the tendons. Ap concentrations showed greater scatter than that obtained with Alb. The relative fall in concentration from centrifugation I to II was on average less than one-half of that observed for Alb, whereas the reduction from TF II to TF III was similar to that of Alb (Fig. 5).
pattern was similar with or without overnight storage before centrifugation.

$^{51}$Cr-EDTA

$^{51}$Cr-EDTA, with a molecular radius ($r$) of 0.47 nm (21), was infused intravenously together with $^{125}$I-Ap in the six experiments described in Aprotinin and Alb and in six additional rats, providing 24 samples. The average concentration ratios were TF II/TF I = 1.05 ± 0.030 and TF III/TF I = 0.92 ± 0.04, the latter ratio being significantly different from 1.00.

Comparison of All Test Substances

The determination of Alb concentration in all tendon fluid samples permits comparison of the other test substances. Figure 6 is based on the concentrations of HA, IgG, Ap, and $^{51}$Cr-EDTA in centrifugations I–III, with all concentrations given as fractions of their zero centrifugation intercepts (2) and plotted against their paired Alb concentrations. The lines are drawn to best visual fit and demonstrate the closely similar pattern for Alb, IgG, and HA. As already mentioned above, Ap concentration fell moderately from the first to the second centrifugate, and then fell almost in proportion to Alb from TF II to TF III. Also, the HA concentration curve tends to parallel Alb concentration in TF II and TF III.

Tendon Composition

Duplicate measurements of uronic acid in tendons from nine tails gave 1.72 ± 0.14 mg/g dry weight. The average HA concentration was 0.83 ± 0.05 mg/g dry weight. With an HA-to-uronic acid ratio of 2.28, the HA will account for 0.36 mg uronic acid per gram dry tendon. The remaining uronic acid is presumably derived from PG, corresponding to a glycosaminoglycan (GAG) moiety of 3.54 mg/g dry tendon. The content of HA-GAG is practically identical to that reported by Parry et al. (23), whereas PG-GAG is almost 50% higher. The dominating PG in tendon is dermatan sulfate (23), which has a protein moiety of 30% (1), or 1.52 mg/g dry weight. Thus the total GAG content will be 5.06 mg/g dry weight. Based on a dry weight-to-wet weight ratio of 0.38 (2) and the assumption that HA and
PG are confined to an extracellular-extrafibrillar space of 0.35 ml/g TSW (see Table 2), the HA concentration in this space may be calculated at 0.90 mg/ml. The PG concentration will be 5.50 mg/ml, of which the GAG and protein moieties will contribute 3.85 and 1.65 mg/ml, respectively. Collagen concentration in six samples averaged 31.3 (SD 0.8) mg/g wet weight.

DISCUSSION

The rat tail tendon provides a reproducible and simple connective tissue model, consisting mainly of collagen and water. It contains no blood vessels and has a cell volume of ≤5% (23) and thus shares many properties with cartilage and cornea. The partition of fluid between collagen fibrils and the extrafibrillar matrix in these tissues has previously been studied by measuring macromolecular distribution spaces and by X-ray estimates of the spacing of collagen molecules within the fibrils (see, e.g., Refs. 15, 29). Reduction of tissue fluid volume has been produced experimentally by mechanical or osmotic pressure or by evaporation. The centrifugation technique used here and described in detail in a previous study (2) offers complementary
Fluid Removal

A striking feature observed with repeated centrifugations is the steep increase in resistance against fluid removal. Thus, to obtain similar centrifuge volumes in three subsequent 15-min centrifugations, the force (G) has to be increased by a factor of 4 and 22 in centrifugations II and III, respectively. Still, the accumulated centrifugate volume was only 7–10% of tendon wet weight, or 11–16% of total tissue water. A similar resistance against fluid removal observed with compression of cartilage has been attributed to increasing concentration and osmotic pressure of immobilized PG (15). However, the PG content of tendon is <10% of that in cartilage, suggesting that mechanical forces against compression may be more important in tendon. The resistance against water removal also limits the removal of macromolecules. Nevertheless, even if ~20% of tendon Alb is removed in one standard centrifugation sequence, the concentration in the available space may remain constant or even increase because of simultaneous water loss. This explains the finding that after rehydration of the tendon sample by addition of saline corresponding to the volume lost in the first centrifugation sequence, renewed centrifugations gave similar volumes and Alb concentration patterns (Fig. 2).

Dilution of Macromolecules in Centrifugate

The fall in Alb concentration to 20–30% of the initial level means that for each centrifugation increasing amounts of water are removed relative to Alb. This excess of Alb-free fluid could result either from 1) sieving, i.e., by holding back Alb molecules in the tendon sample, or from 2) addition of fluid from an Alb-excluded phase. In the previous study, both these explanations seemed adequate, even though some HA analyses gave a pattern similar to that of Alb and seemed to favor the exclusion hypothesis (2). The present experiments strongly support that suspicion.

Sieving hypothesis. The resistance toward filtration through a porous membrane may be greatly increased when the solution contains large molecules that are prevented from or hindered in passing through the pores, especially at high filtration pressure and flow. This may also occur in biological membranes, as demonstrated for transsynovial fluid filtration in the rabbit knee joint (16). Addition of HA increased hydraulic resistance, strongly suggesting partial molecular sieving in the synovial matrix, “leading to accumulation of a resistive filter cake of hyaluronan chains at the tissue-cavity interface” (16). Such accumulation at the filtering site would obviously lower the HA concentration in the filtrate and might well explain the reduction in HA concentration in tendon fluid observed at increasing centrifugation rate. However, as recently discussed by Levick and McDonald (13), the degree of sieving would be strongly dependent on molecular size. In their model membrane of cylindrical rods, they estimated sieving coefficients (filtrate-to-filtrand concentration ratios) at high flow rates of ~0.7 for Alb [molecular radius (r) = 3.6 nm], ~0.35 for IgG (r = 5.5 nm), and 0.05 for α2-macroglobulin (r = 9.1 nm). This does not fit with the practically identical centrifugation patterns for Alb and IgG. Even less compatible with sieving is the marked fall in Ap concentration and the relatively well maintained HA concentration in the third centrifugation (Fig. 6). According to the Levick-McDonald model, Ap (r = 1.3 nm) should not be appreciably sieved.

Quantification of Free Fluid in Centrifugate

As shown in the previous study (2), the Alb concentration in the three standard centrifugation samples plotted against accumulated tendon fluid volume showed an approximately exponential fall. Moreover, evidence was presented that extrapolation of concentration to zero centrifugation (C0) gave the concentration in the tendon Alb available space, on average ~60% of the concentration in plasma (2, 32). (A value of 50% can be obtained from the standard centrifugation curve shown in Fig. 1.) The fall in Alb concentration during subsequent centrifugations may be considered to be caused by addition of Alb-free fluid (FF). The amount of FF added to each sample per gram wet tendon (TSW0) may be illustrated as shown in Fig. 7 and quantified as

$$\text{FF} = \left[ \frac{(C_0 - C)}{C_0} \right] (TFW/TSW_0)$$

where TFW and C are volume and Alb concentration of each sample, respectively. In other words, FF is the volume of water that has to be removed to restore the solute concentration to C0. Although relevant plasma concentrations of the other test substances were not available, we adopted a similar estimate of C0 and calculation of HA-, IgG- and Ap-free fluid according to Eq. 1 for these substances. In the case of 51Cr-Eyta, calculation of free fluid was based on the reduction of the concentration in TF III relative to that in TF I.

As shown in Table 1, the absolute total amount of Alb-free water added varied from 2.9 to 5.0% of wet weight in the four series, clearly depending on the total amount of centrifugate. Thus the fraction of total Alb-free fluid was within 0.45 to 0.54, and it may be noted that a similar fraction was obtained with express centrifugation and with the standard procedure, both estimated from the C0 obtained by the latter procedure. HA- and IgG-free fluid exceeded that of Alb by 15 and 11%, respectively, whereas Ap-free fluid was 43% lower. The fractions of free fluid in the three centrifugates were practically identical for Alb, HA, and IgG, but increased for Ap relative to Alb from centrifugations I through III, confirming the impression gained from Fig. 6.

Origin of Free Fluid

Before a discussion of the origin of solute-free (excluded) water, it seems pertinent to briefly consider the structure and fluid spaces of the rat tail tendon. The
main component of the tendon fibrils is triple-helical collagen I molecules lined up in parallel in groups of five or seven, forming microfibrils, which again are organized into well-defined round fibrils with diameter varying from 10 to 200 nm (8, 9, 10, 17, 22). Most relevant for the present purpose is the finding that water makes up 40–50% of the fibril volume and that the packing of the microfibrils is so tight that this fluid space is inaccessible for polyethylene glycol molecules with a MW of 4,000 (10). This strongly suggests that Ap (MW 6,500) also is completely excluded from fibril water. On the other hand, 51Cr-EDTA seems to have free access, based on the finding of a 51Cr-EDTA space of 92% of total tendon water (2). Cellular volume may in part account for the difference. To what extent PG and HA penetrate into the fibril is not known. The extrafibrillar space contains scattered, branching histocytes. Their relative volume falls during maturation and accounts for 5% of total tendon volume at the age of 12 wk (17).

Quantitative estimates of the various spaces listed in Table 2 are mainly based on distribution volumes measured in a previous study (2). The extracellular volume of 0.57 ml/g wet weight was calculated to be distributed with 0.22 and 0.35 ml/g on intra- and extracellular space, respectively. Alb space was estimated at 38% of extracellular fluid volume, indicating an Alb-free (excluded) volume of 62% (2). Somewhat lower exclusion, 54%, was obtained by Wiig et al. (33), who also obtained a similar exclusion (55%) of IgG. If complete exclusion of Alb and IgG from intracellular fluid (0.22 ml/g wet wt) is assumed, it may be calculated that these proteins are excluded from 37%, or 0.13 ml/g wet weight, of extracellular-extrafibrillar fluid (Table 2), leaving an Alb space of 0.22 ml/g wet weight. Thus, even without adding an intracellular fluid volume of 0.03–0.05 ml/g wet weight, there is clearly plenty of Alb-free fluid to account for the total of ~0.05 ml/g wet weight obtained by three centrifugations (Table 1). The origin of the slight but statistically significant 51Cr-EDTA space will be discussed in a later paper.

Table 1. Solute-free fluid in centrifugate

<table>
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<tr>
<th>Solute</th>
<th>MW $\times 10^{-3}$</th>
<th>$r$, nm</th>
<th>$\Sigma_{TF}/TSW_0$</th>
<th>$\Sigma_{FF}/TSW_0$</th>
<th>$\Sigma_{FF}/\Sigma_{TF}$</th>
<th>$FF_{I-III}/TF_{I-III}$</th>
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All ratios are expressed $\times 100$. TF, volume of centrifuged tendon fluid; TSW$_0$, initial tendon sample weight; FF, solute-free fluid; I–III, centrifugation number; MW, molecular weight; $r$, molecular radius; HA, hyaluronan (hyaluronic acid); Alb, albumin; IgG, immunoglobulin G; Ap, aprotinin; Alb$_{Express}$, Alb from express centrifugation; Alb$_{STD}$, Alb from standard centrifugation. *Including euhydrated and rehydrated.

Table 2. Tendon composition

<table>
<thead>
<tr>
<th>Description</th>
<th>Explanation</th>
<th>References</th>
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<td>Observed values</td>
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<tr>
<td>Total water</td>
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<td>Alb available volume</td>
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<td>Alb XV</td>
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<td>Alb-free fraction</td>
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All values are expressed in milliliters per gram wet weight except for collagen weight (CW), which is expressed in grams per gram wet weight. ECV, extracellular water; AlbXV, Alb-free ECV; CV, collagen volume; FV, fibrillar volume; IFV, intrafibrillar water; (EF, EC), extracellular, extracellular water.
EDTA-free fluid in TF III is not clear. Possible sources are intracellular fluid, incomplete equilibration in the extracellular fluid, or extracellular exclusion resulting mainly from a net negative charge of 1.5 eq/mol on the Cr-EDTA complex (14).

As noted above, Ap should also be excluded from the extracellular space. Nevertheless, little Ap-free fluid was obtained in the two first centrifugates (Table 1, Fig. 6), indicating that these portions were mainly derived from the extracellular space. Apparently, higher centrifugation force is needed to expel fluid from the collagen fibrils. This conclusion is supported by the marked fall in Alb concentration already obtained in the first portion with short-lasting high-speed centrifugation (express centrifugation, Fig. 1). It also agrees well with X-ray diffraction studies showing that the initial fluid lost from kangaroo tail tendon during dehydration and from cartilage during mechanical compression is derived mainly from the extracellular space (15, 29).

If it is assumed that 90% of Ap-free fluid was derived from the intracellular space (Table 3), the contribution from extracellular Alb-containing and Alb-free spaces may be calculated for the series with simultaneous measurements of Ap and Alb (Table 1, Fig. 5), based on the tendon spaces listed in Table 2. As shown in Fig. 8, fluid was most readily expelled from the extracellular Alb-containing space, whereas the intracellular space contributed less than its share of total tendon extracellular volume. Thus only 8% of the extracellular fluid was expelled, whereas Alb-free and Alb-containing extracellular fluid contributed 13 and 19% of their respective initial volumes. Nevertheless, the intracellular fluid contributed 52% of the Alb-free fluid but only 23% of the total centrifugate volume.

Removal of excluded water from the extracellular space seems at first glance to be quite problematic. According to the classical concepts of Ogston and co-workers (see, e.g., Ref. 19) the excluded volume should consist of fluid spaces surrounding, e.g., PG and collagen fibril surfaces, keeping the center of dissolved macromolecules at a minimum distance corresponding to their radii. Although removal of fluid will increase the concentration of such fixed excluding structures, their mass will remain unchanged and would therefore be expected to exclude Alb, IgG, and HA from the same volume as before. Therefore, the only way by which the amount of free fluid could be reduced would be that increasing density of the excluding structures leads to overlap of their excluded territories (4).

In an attempt to evaluate the chance for such overlap, we have calculated available and excluded volume fractions in the extracellular-extracellular space. The calculations were based on the concentrations of collagen, HA, and PG presented above and the fluid spaces shown in Table 2. The distribution of collagen fibril radii was based on histograms presented by Moore and De Beaux (17) for 6- and 12-wk-old hooded Lister rats and those reported by Parry and Craig (22) for 8-wk-old Sprague-Dawley rats. (In the latter case we used an extracellular space of 0.20 ml/g wet wt, as estimated by these authors, instead of the 0.35 ml/g wet wt shown in Table 2.) Exclusion of Alb, IgG, and Ap by collagen fibrils was calculated according to Curry (Ref. 6, equations 6.9–6.11). Exclusion of GAG (chain radius = 0.55 nm; Ref. 20) by collagen was obtained as available volume according to Levick (Ref. 12, equation 6) to calculate the partition coefficient and excluded volume fraction. To roughly evaluate the effect of electrostatic repulsion of negatively charged molecules, the exclusion of Alb by GAG (HA + PG) was calculated by adding one Debye length (0.8 nm) to the radius of both species (6, 18). No attention was paid to the positive charge on the Ap molecule.

As shown in Table 3, the sum of excluded fractions depends strongly on whether the calculation is based on the distribution of collagen fibril radii reported by Parry and Craig (22) or by Moore and De Beaux (17), the latter having a much greater fraction of thin fibrils.
The large exclusion of Alb, IgG, and Ap by PG and HA depends strongly on the assumption that the GAG are evenly distributed in the extracellular space. Although we have found no direct evidence for that assumption, it may be noted that the production sites, the tenoblasts, are undoubtedly located outside the fibrils.

The small calculated exclusion of Alb compared with that of IgG based on molecular size alone conflicts with their similar distribution volumes as reported by Wiig et al. (33, 34). Better agreement is obtained by including the effect of negative charges on Alb distribution, which also fits well with the similar centrifugation patterns observed for IgG and Alb (Fig. 4, Table 1). As expected, little extracellular exclusion was obtained for Ap, explaining the small amount of Ap-free fluid observed at low-rate centrifugation (Table 1).

Regardless of the uncertainties of the values presented in Table 3, the cumulated exclusion of IgG and Alb from the extracellular space of 50–70% clearly exceeds the 37% calculated from measurements of distribution spaces (Table 2). This leads to the important conclusion that there is considerable overlap of excluded extracellular volumes already in the normal state, indicating that centrifugation and reduction of the extracellular space will give even more overlap and thereby provide more IgG- and Alb-free fluid for the centrifugate.

The fall in HA concentration in successive centrifugates was slightly steeper than that of IgG (Fig. 6), suggesting even more overlap of HA than of IgG-excluded territories. In fact, a rough estimate showed that HA behaved like a globular molecule with radius 7–9 nm. This is radically different from the accepted picture of HA as “a stiffened helical configuration, which gives the molecule an overall expanded coil structure in solution,” making up a highly hydrated sphere with a radius of 200 nm (11). Based on this model and the assumptions that the GAG chains have a radius of 0.55 nm (20) and a persistence length (length “stiff segments”) of 4 nm (5), calculations according to Ogston (Ref. 19, equation 3) gave an HA exclusion by PG and collagen fibrils of no more than 10–16% of the extracellular space (Table 3). However, if the negative charge is taken into account by adding 0.8 nm to the radii of both PG and HA chains, one obtains an HA exclusion by PG alone of 24%. Even higher exclusions by PG, 38 and 59%, are obtained if the persistence length of HA is increased to 8 and 16 nm, respectively (30). Also, the calculated exclusion of HA by collagen is increased to 14, 24, and 47% by increasing persistence length to 4, 8, and 16 nm, respectively.

The calculations above clearly indicate sufficient overlap of excluded territories to explain the HA-free fluid derived from the extracellular space. However, the assumptions may have been stretched so far that other mechanisms such as steric restriction should be considered. We believe that formation of a “filter cake” of HA at the tendon surface has been effectively excluded by the finding that loosening and turning the tendon pellet between the centrifugations had no effect on the HA concentration in the following fluid samples. On the other hand, we cannot exclude that fluid removal could lead to increasing internal entanglement and immobilization of HA.

Another intriguing observation was the high whole tendon HA content, which, if confined to the extracellular space, would give a concentration four to five times higher than that in tendon fluid, even when extrapolated to zero centrifugation. This suggests some kind of binding or immobilization of 80–90% of tendon HA, possibly through HA network formation or binding to interstitial cells (30, 31). Also, studies of lymph drainage from several tissues have indicated a large HA fraction that seems immobilized but not so firmly bound as to prevent mobilization during hydration and increased lymph flow (7, 24, 27).

In summary, the present observations strongly support the hypothesis that dilution of macromolecular solutes in fluid extracted from rat tail tendon by progressive centrifugation is caused by increasing mobilization of tissue water from which these molecules are excluded. The alternative explanation of molecular sieving by compacted interstitial matrix is rejected. The critical evidence is that concentrations of aprotinin (MW 6,500), albumin (MW 69,000), immunoglobulin G (MW 156,000), and hyaluronan (MW 5 × 10^6), all excluded, fall nearly in parallel as centrifugation proceeds and centrifugal force is increased (Fig. 6). As the tendon is compressed, increasing proportions of macromolecule-free fluid are added to the centrifugate. The concentration of 51Cr-EDTA (MW 341), which is not excluded, remains nearly constant. If the decreases in concentration were caused by progressive sieving of interstitial fluid, they would be greater for the larger macromolecules (6, 12). There are two potential sources of macromolecule-excluded fluid in tendon, 1) fluid within the interstices of collagen fibrils and 2) fluid within a macrofibril's radius of the surfaces of fibrils and of glycosaminoglycan molecules in the extracellular space. The molecular size cutoff for penetration into collagen fibrils is relatively low (10). Aprotinin and larger molecules are excluded; of the substances studied, only 51Cr-EDTA is able to enter. In contrast, exclusion zones around the collagen fibrils and proteoglycan molecules might be expected to increase over the full range of macromolecular sizes tested (19). The closely similar behavior of the anionic hyaluronan and albumin molecules to that of immunoglobulin G (Fig. 6) may in part result from repulsion to negatively charged proteoglycans in the extracellular fluid and may in part reflect contribution of completely macromolecule-free interstitial fluid. Intrafibril fluid is mobilized mainly at high centrifugation rate (TF II–III) and contributes ~25% of the total centrifuged fluid and 50% of its macromolecular excluded volume.

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