Distribution spaces for hyaluronan and albumin in rat tail tendons

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Auckland, Knut, Olav Tenstad, and Helge Wiig. Distribution spaces for hyaluronan and albumin in rat tail tendons. Am J Physiol Heart Circ Physiol 281: H1589–H1597, 2001.—A low concentration of hyaluronan (HA) in lymph compared with tissue suggests a large bound fraction. To investigate the distribution and mobility of HA and serum albumin (Alb), we eluted the rat tail tendon with a series of 15 successive centrifugations, each preceded by the addition of 0.15 M NaCl (15% of initial wet wt). The eluate concentration fell exponentially versus the accumulated eluate, allowing estimation of the maximal elutable amount (E_{HA} and E_{Alb}). Alb elution was practically complete from a space of ~28% of wet wt at all centrifugation rates. Twenty percent of HA was elutable at 500 rpm, apparently from the same space as Alb, increasing to 40% at >4,000 rpm. This pattern was not significantly influenced by using 2 M NaCl or by the addition of plasma or metabolic inhibitors. Without prehydration and centrifugation at high revolutions per minute, both Alb and HA concentrations fell rapidly toward zero, presumably in part reflecting mobilization of HA- and Alb-free fluid from the collagen intrafibrillar space (3). We conclude that with prehydration the fibrils swell, increasing the intramolecular spaces to become “penetrable” to HA and allowing removal of HA-containing fluid when the fibrils are compressed by the next centrifugation at high revolutions per minute, increasing E_{HA} from 23 to 45%. Chemical binding presumably explains the unelutable 55% of tendon HA. Intrafibrillar HA may act to stabilize the fibrillar volume.

extracellular space; collagen; glycosaminoglycans; centrifugation

THE HIGH-MOLECULAR-WEIGHT POLYSACCHARIDE hyaluronan (HA, previously called hyaluronic acid) is apparently present in all connective tissues, where it is produced by connective tissue cells (8). However, despite extensive research, there are still great uncertainties as to the function(s) of HA as well as its distribution within tissues. There is no evidence for appreciable intracellular content, and, because of the high molecular weight, HA seems tacitly assumed to be excluded from the collagen fibrils, which in a fibrous tissue like tendon contains ~40% of total extracellular water (3). An extracellular and intrafibrillar location would thus seem likely.

Still, the HA concentration in lymph from the lung, skin, and intestine is low compared with tissue content, leading to the conclusion that if “free HA” was confined to the same space as serum albumin (Alb), as much as 80% of total HA would have to be bound (26). Several types and sites of binding have been demonstrated: binding to cells through specific receptors, binding to large proteoglycans or collagen, or immobilization by HA network formation (8, 10, 14, 30).

We (1, 3) have previously studied the distribution spaces in the rat tail tendon for substances with molecular weights ranging from 340 to 5 million using subsequent centrifugations at 600, 2,400, and 13,000 g. During this sequence, the concentration of serum Alb in the centrifugates plotted against the accumulated centrifugate volume fell exponentially by 70–80%. A similar pattern was obtained for IgG and HA. We concluded that the fall in centrifugate concentration reflected increasing addition of Alb- and HA-excluded fluid and that the concentration in the undisturbed interstitium could be obtained from the subsequent concentrations in centrifugate plotted against the accumulated centrifugate fluid volume (ΣTFV) by extrapolating back to “zero centrifugation” concentrations (C₀). Combined with measurement of tendon HA content, we arrived at an apparent distribution space exceeding the total tissue volume, in agreement with the lymph studies quoted above (26), clearly suggesting a bound or otherwise unavailable fraction of HA. However, this procedure was not suitable for investigating the degree and nature of the HA “binding” because the removal of both Alb and HA was strictly limited by a maximum total centrifugate volume of 6–8% of tendon wet wt only.

In the present study, we have therefore added saline to the tendon sample before each of 15 successive centrifugations at a given rate to examine how much of tendon HA and Alb could be eluted. Whereas Alb was completely eluted with all centrifugation rates, ~60% of HA was not elutable. Twenty percent of HA was elutable at all centrifugation rates, presumably derived from the extracellular-extrafibrillar space, like Alb. Removal of another 20% of HA required centrifugation rates of 4,000 rpm or more, leading to the conclusion that this portion represented extrusion from the intrafibrillar collagen space.

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METHODS

Tail Tendon Preparation and Centrifugation Technique

Tail tendons were obtained from male or female Wistar and Sprague-Dawley rats (200–400 g) kept on a standard diet with free access to water. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Before the tail was removed, the rat was exsanguinated in the head down position by severing the aorta through an abdominal incision to drain blood from the tail. The tail was cut off and immediately transferred to an incubator kept at room temperature (20–24°C) and 100% relative humidity. The tail was divided using a scalpel into three sections of ~3 cm in length. When these segments were flexed, the tendons would protrude from their sleeves and could be easily pulled out. After samples were briefly minced with a scalpel, one-half of the sample (0.3–0.6 g) was frozen and stored at −80°C for later analysis of tissue HA content. The other half was rapidly transferred to a preweighed centrifuge tube, provided with a “basket” of nylon mesh (pore size, 15 × 20 μm) designed to keep the tendon sample up from the bottom of the tube (1). The tube was immediately capped, reweighed, and spun in an Eppendorf 5417 centrifuge placed in a cold room at 4°C. After 10 or 15 min of centrifugation, the tube was brought back to the incubator, where the basket with the tendon sample was transferred to a second preweighed centrifuge tube. The tendon fluid at the bottom of the tube was collected in a glass capillary, which was immediately closed with a “basket” of nylon mesh (pore size, 15 × 20 μm) designed to keep the tendon sample up from the bottom of the tube (1). The tube was immediately capped, reweighed, and spun in an Eppendorf 5417 centrifuge placed in a cold room at 4°C. After 10 or 15 min of centrifugation, the tube was brought back to the incubator, where the basket with the tendon sample was transferred to a second preweighed centrifuge tube. The tendon fluid at the bottom of the tube was collected in a glass capillary, which was immediately closed at both ends. After reweighing, the second tube was centrifuged, and the procedure was repeated for a third centrifugation. After the centrifugation sequence was completed, the centrifuged tendon sample was frozen and stored at −80°C for later determination of HA content.

Centrifugation Protocols

Most experiments were initiated with a “standard centrifugation” protocol (1, 3) consisting of three subsequent centrifugations at 3, 6, and 14,000 rpm (600, 2,400, and 13,000 g) to estimate the C₀ of HA and Alb. This was accomplished by extrapolating back to zero centrifugation, assuming an exponential concentration decline versus accumulated centrifugate volume (cf Fig. 1B, left). It turned out, however, that the intercept could be calculated with reasonable accuracy from the first 3,000 rpm run by the formulas HA₀ = 1.26 × HA₃,000 and Alb₀ = 1.16 × Alb₃,000, based on mean values in nine experiments with a complete centrifugation sequence of 3, 6, and 14,000 rpm. After this initial step, the nylon basket was removed from the centrifuge tube and unfolded, and fluid, most often 0.15 M NaCl, was applied directly to the tendon sample with a pipette in the amount of 150 μl/g tendon wet wt, hereafter referred to as 15%.

The fluid was rapidly absorbed by the compressed tendon, and the basket was placed in another tared vial and capped within <1 min, all performed within the humidified incubator. This was then followed by a centrifugation for 10 or 15 min at the chosen revolutions per minute, another addition of saline, renewed centrifugation, etc., usually repeated 14–16 times. The tendon sample weight increased by 3–15% (average ~10%) during the first three to six centrifugations and thereafter usually remained stable.

The procedure described above might well be conceived of as a series of elutions, and, hereafter, we may refer to the added saline as elution fluid and to the centrifugate as eluate. It turned out, however, that the elution pattern was determined not only by the volume of elution fluid but also by the centrifugation force, and the variation in this parameter allowed characterization of the distribution spaces of Alb and HA in the tendon sample. Analysis of the elution curves is described in RESULTS by the detailed presentation of a single experiment.
Measurement of Alb and HA Concentrations

Endogenous plasma Alb was determined in the centrifugate using the spectrofluorimetric 8-anilino-1-naphthalenesulfonic acid method of Rees et al. (28) as modified by Aukland and Fadnes (2). HA in the centrifugate and tendon samples and in gelfiltration fractions was measured with a specific radioimmunoassay (HA Test 50, Pharmacia; Uppsala, Sweden) and, in the case of tendon, after papain digestion of freeze-dried specimens (27). For the purpose of comparison with centrifugate concentrations measured per gram wet weight, the dry weight tendon concentrations were converted to micrograms per gram wet weight based on an initial water content of 0.62 (1), with correction for net water loss or gain during the centrifugation sequence.

Molecular Weight of HA

The molecular weight of HA in early and late tendon fluid samples obtained with centrifugation rates of 500 or 14,000 rpm was estimated by gel filtration on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) using 0.15 M phosphate-buffered saline (pH 7.4) as eluent and a high-molecular-weight Gel Filtration Calibration Kit (Pharmacia Biotech) for standards. Fractions of 1 ml were collected at a flow rate of 0.3 ml/min, and the HA concentration in each fraction was measured as in the eluate.

Statistics

Results are presented as means ± SE. Statistical significance of differences was estimated using Student’s t-test.

RESULTS

Analysis of Elution Data

As described above, each experiment gave information on 1) the concentration of HA in centrifuged and uncentrifuged tendon samples, 2) the total amount of Alb and HA recovered and in the centrifugate, and 3) the HA and Alb concentrations in successive centrifugates. The analysis of this information is illustrated in Fig. 1, A and B, which shows an experiment initiated by three 10-min centrifugations of a 0.53-g tendon sample at 3,000, 6,000, and 14,000 rpm, followed by 30 consecutive centrifugations at 14,000 rpm, each preceded by the addition of 0.15 M NaCl solution, 15% of initial tendon weight, i.e., 0.080 ml.

Concentration of HA in centrifuged and uncentrifuged tendon samples. In this experiment, the HA concentration in the centrifuged tendon sample was 459 µg/g dry wt compared with 653 µg/g dry wt in the uncentrifuged sample from the same tendon. Conversion to contents per gram wet weight, with correction for a weight loss of 11%, gave a net HA loss of 71 µg/g initial wet wt, or 33% of initial content.

Total amount of Alb and HA recovered in centrifugate. The accumulated eluted amount of Alb (ΣAlb) and HA (ΣHA) is depicted in Fig. 1A as a function of accumulated eluted volume, showing ΣAlb rising rapidly and then flattening out after 10–15 centrifugations, whereas appreciable amounts of HA were still being eluted after 30 centrifugations. (The fewer steps on the HA curve versus on the Alb curve reflect that two subsequent samples were mixed and analyzed for HA together to economize with the HA reagent.) The total amount of HA removed was 87 µg/g wet wt, i.e., in the same range as the 71 µg/g wet wt lost from the tendon.

HA and Alb concentrations in successive centrifugates. Analysis of the eluate concentration pattern was based on the assumption that Alb and HA should reflect the concentration in the elutable tendon space. If so, the concentration should fall exponentially toward zero. With some irregularities, especially for HA, this seemed to be confirmed in the 30 centrifugation experiment shown in Fig. 1, A and B. (The flat portion at the end of the Alb curve reflects concentrations close to the minimum detectability.) Concentrations are plotted on a logarithmic scale on the y-axis versus the accumulated eluate volume (ΣTFV) on the linear x-axis.

The fractional rate of concentration decline (k) was estimated from the accumulated elution volume needed to reduce the concentration by 50% (ΣTFV0.5) as 

\[
\ln 2 / \Sigma TFV
\]

where ΣTFV is the accumulated eluate volume, (ml/g wet wt)−1, giving a five times greater elution rate for Alb than for HA (Table 1). Assuming that elution would have continued at the same pace beyond 30 centrifugations, the total elutable amount was calculated by integrating to infinity

\[
\int_0^\infty C_1 e^{-k \Sigma TFV} \, d \Sigma TFV = C_1 / k
\]

where \( C_1 \) is the zero intercept concentration. Similarly, the amount of HA and Alb actually eluted up to the final centrifugation (n = 30) was estimated as

\[
\int_0^{C_30} e^{-k \Sigma TFV} \, d \Sigma TFV - \int_0^\infty C_30 e^{-k \Sigma TFV} \, d \Sigma TFV = (C_1 - C_30) / k
\]

where \( C_{30} \) is the eluate concentration at the end of 30 centrifugations. Finally, the difference between the elutable and actually eluted amounts (\( C_{30} / k \)) is referred to as “rest,” making up 2.5 and 20% for Alb and HA, respectively (Table 1).

The apparent distribution space (“tendon fluid equivalent space”) was calculated as \( (C_i / k) / C_0 \), where \( C_0 \) is the intercept concentration of the initial centrifugations at 3, 6, and 14,000 rpm without addition of saline, as shown on an expanded x-axis in Fig. 1B.

| k, Fractional rate of concentration decline. | Elutable, mg/g wet wt | Rest, fraction of elutable, space, ml/g wet wt |
|____|____|____|
| Albumin | 1.73 | 3.65 | 0.025 | 0.34 |
| Hyaluronan | 0.37 | 0.111 | 0.200 | 1.74 |

Table 1. Numerical data from experiment shown in Fig. 1.
The elutable fraction of total tendon HA was estimated as

$$\frac{(C/k)}{m_t}$$

(3)

where $m_t$ is the amount of HA per gram uncentrifuged tendon sample from the same tail. (Tendon and tendon fluid density were assumed to be equal.) In this experiment, the elutable fraction was 0.46. A practically important piece of information from the sequence of 30 centrifugations in Fig. 1 was that the number of centrifugations could be reduced to 15 without appreciable loss of accuracy. Thus the duration of the experimental procedure was reduced from 8–9 to 4–5 h.

**Determinants of Elution Patterns**

To test the determinants of the elution pattern, we varied the centrifugation rate as well as the composition of the fluid added before each centrifugation. When not otherwise stated, centrifugations were repeated 14–16 times with intervening additions of 0.15 M NaCl, corresponding to 15% of initial tendon wet wt.

**Centrifugation rate.** Figure 2 shows an example of centrifugations at 500 rpm (17 g) with the addition of saline (15% of sample wt) before each centrifugation. No saline was added before the initial centrifugation used to calculate $C_0$. The most striking difference from that obtained with 14,000 rpm (13,000 g) is the rapid decline in HA concentration; in fact, it approached that of Alb, which did not deviate radically from that with 14,000 rpm. Whereas the steep fall in HA concentration could give the impression of rapid elution of HA, closer analysis shows the opposite result: because of the low and rapidly falling HA concentration in the eluate, a much smaller amount is eluted.

As shown by the averaged values in Table 2, the HA distribution space at 14,000 rpm was four to five times greater than the Alb space and, in fact, exceeded the tendon volume by 23%. This clearly indicates that the estimated space is a “$C_0$-equivalent space” and not a physically defined volume within the tendon. At 500 rpm, the HA space was much lower, 0.73 ml/g wet wt, whereas the Alb space remained at $0.30\text{ ml/g wet wt}$. Elution from that space was practically complete for Alb, with only 3.6 and 9.7% left at 14,000 and 500 rpm, respectively, whereas the elutable HA remaining in the tendon sample fell from 32 to 19%.

With centrifugations at 4,000 and 6,000 rpm (1,100–2,400 g), the pattern was not different from that of 14,000 rpm, whereas the results with 1,000 to 2,500 rpm (67–420 g) were in between that obtained with 500 and 14,000 rpm. However, as shown in Fig. 3, both the washout rates for Alb and HA ($k_{Alb}$ and $k_{HA}$, respectively) and the HA space tended to fall at increasing revolutions per minute, whereas the Alb space remained practically unchanged.

**Table 2. Elution with 0.15 M NaCl and 15 centrifugations at 500 or 14,000 rpm**

<table>
<thead>
<tr>
<th>Analysis of elution</th>
<th>Albumin</th>
<th>Hyaluronan</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k$, (ml/g wet wt)$^{-1}$</td>
<td>$1.99 \pm 0.07$</td>
<td>$1.76 \pm 0.26$</td>
</tr>
<tr>
<td>Space, ml/g wet wt</td>
<td>$0.29 \pm 0.03$</td>
<td>$0.24 \pm 0.03$</td>
</tr>
<tr>
<td>Elutable, µg/g wet wt</td>
<td>$4,250 \pm 560$</td>
<td>$3,490 \pm 420$</td>
</tr>
<tr>
<td>Eluted, µg/g wet wt</td>
<td>$3,970 \pm 0.45$</td>
<td>$3,740 \pm 0.30$</td>
</tr>
<tr>
<td>Rest, fraction of elutable amount</td>
<td>$0.036 \pm 0.001$</td>
<td>$0.07 \pm 0.008$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analysis of tendon</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Uneluted tendon, mg/g wet wt</td>
<td>$247 \pm 28$</td>
</tr>
<tr>
<td>Removed from tendon, mg/g wet wt</td>
<td>$51 \pm 11$</td>
</tr>
<tr>
<td>Elutable fraction</td>
<td>$0.45 \pm 0.06$</td>
</tr>
</tbody>
</table>

**Values are means ± SE; n = no. of tails.**
Analysis of centrifuged and uncentrifuged samples from the same tendons showed an average HA loss of 25 (n = 8) and 51 mg/g wet wt (n = 11) with centrifugations sequences at 500 and 14,000 rpm, respectively, statistically not different from the estimated eluted amounts of 36 (n = 3) and 67 mg/g wet wt (n = 11). Of special interest is the estimate of the elutable fraction of HA, increasing from 22 to 40% when centrifugation was increased from 500 to 14,000 rpm (Fig. 4).

The molecular weight of HA was 2.1–2.3 \times 10^6 in early and late samples eluted with 500 or 14,000 rpm.

Composition of Elution Fluid

Salt concentration. In eight experiments, a volume of 2 M NaCl (corresponding to 15% of tendon wet wt) was added to the tendon sample before centrifugations at 14,000 rpm to eliminate possible electrostatic binding of HA. The elution data for Alb were practically identical to those obtained with 0.15 M NaCl. \( k_{HA} \) was 0.44 compared with 0.67 (not significant). The elutable fraction of HA showed great scatter with an average of 0.60, higher than in any other experimental group (Fig. 4), but the difference from the control group did not reach statistical significance (\( P > 0.05 \)).

A single experiment with 0.05 M NaCl gave values close to that obtained with 0.15 M.

Addition of plasma. Plasma or serum was added to the elution fluid in three experiments because serum factors participating in the binding of HA (e.g., see Refs. 6, 8, and 33) might be eluted similar to the observed washout of endogenous Alb. We therefore replaced the protein loss by adding one part of rat serum, rat plasma, or fetal calf serum to two parts of 0.15 M NaCl. In all three experiments, \( k_{HA} \) as well as the elutable fraction were close to average control values (Fig. 4).

Metabolic inhibitors. In three experiments, sodium azide (0.02 M) and iodoacetate (0.02 M) were added to the elution fluid (0.15 M NaCl) to prevent de novo synthesis of HA during the 4-h centrifugation proce-
procedure. Alb space and $k_{Alb}$ were similar to those in control experiments. On average, $k_{HA}$ was slightly higher than in controls, whereas both the distribution space and the elutable fraction of HA were lower than in controls, without reaching statistically significant difference (Fig. 4) ($P > 0.1$).

**DISCUSSION**

The main new observations may be summarized and interpreted as follows. Alb and HA are distributed in three different compartments: *Compartment I* contains all tendon Alb and ~23% of total HA content, both substances being elutable with centrifugation rates from 500 to 14,000 rpm. *Compartment II* contains another 22% of HA and can be eluted with centrifugation rates of 4,000 rpm or higher. *Compartment III* contains the remaining 55% of HA, which is not elutable at any centrifugation rate but is readily extracted by maceration and proteolytic treatment of the tendon.

This distribution was not significantly influenced by the addition of plasma or metabolic inhibitors to the elution fluid or by increasing its saline content from 0.15 to 2 M. Before attempting to interpret this descriptive summary, it should be emphasized that the term “compartment” is used here to designate any population of molecules with a similar response to the experimental procedures and may therefore reflect a bound fraction as well as an anatomic distribution volume.

Furthermore, before turning to the interpretation of the distribution volumes, some comments on the analytic methods are pertinent. Whereas the analysis of HA and Alb has high specificity and reasonably good accuracy, biological variations may explain much of the scatter expressed as SE. Especially, the tissue concentration of HA showed considerable individual variations, which may well have been exaggerated by the use of mature rats of different ages and sex and by performing experiments through all seasons. Also, uncertainties in data analysis, e.g., in estimating elutable HA from a rather flat elution curve or in estimating HA loss from the tendon as the small difference between two different tendon samples, added to the scatter. Still, the marked effect of centrifugation rate on elutable HA demonstrated by the eluted amount and by tissue analysis (Fig. 5) is beyond reasonable doubt ($P < 0.05$). On the other hand, the overall scatter might conceal possible small differences, caused, e.g., by altering the elution fluid composition.

*Compartment I*

*Compartment I* containing all Alb, is readily localized in the extracellular space, which makes up 0.57 (1) or 0.51 ml/g wet wt (32), measured as $^{51}$Cr-labeled EDTA ($[^{51}$Cr]EDTA) space. Because of their size, Alb molecules are totally excluded from the collagenous intrafibrillar fluid volume of ~0.20 ml/g wet wt and from a considerable part of the extrafibrillar fluid by proteoglycans and collagen. This explains the average Alb spaces of 0.23 and 0.22 ml/g wet wt for endogenous and radiolabeled Alb, respectively (1, 32). The somewhat larger Alb space obtained in the present study, 0.24 ml/g wet wt at 500 rpm and 0.29 ml/g wet wt at

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**Fig. 5.** A: tendon HA concentration (top) before and after 15 elutions with centrifugation at 500 and 14,000 rpm compared with the maximal elutable amount (bottom). Note the practically complete recovery of the HA elutable at 500 rpm, similar to the recovery of Alb (B) at both rates.
higher resolutions per minute (Fig. 3), probably reflects retention of some of the added fluid, resulting in a 10−15% increase in total tissue weight during the first three to six elutions.

The estimated space for HA obtained in the same experiments (500 rpm) was larger than expected, 0.73 ml/g wet wt. This might in part result from a smaller excluded volume for HA than for Alb in the extracellular-extrafibrillar space, as discussed in a previous study (3). It might also reflect a too low estimate of the initial HA concentration ($C_0$), resulting from some degree of sieving of HA in the compressed tissue (5). Finally, it might represent some “contamination” from the Alb-free compartment II. However, only 20−25% of total tendon HA was elutable with centrifugation at 500 rpm.

The most interesting and unexpected finding in the present study was that compartment II, representing a sequestered HA population of ~20% of total HA content, required a centrifugation of 4,000 rpm or more to become elutable. However, it seems advantageous to first consider the binding or sequestration of the 55% of total tendon HA (compartment III), making it unelutable at any centrifugation rate tested here.

**Compartment III**

*Intracellular HA.* In contrast with the previous concept, several recent studies (7, 10) have described the presence of HA and HA-binding proteins in cultured cells from various organs. As suggested by Evanko and Wight (7), the failure to detect intracellular HA in intact tissue may simply be that “it may have been overlooked because of the overwhelming amount in the extracellular matrix.” If the distribution is similar in the tail tendon with a cell volume of only ~5% (21), the cells would certainly not contribute appreciably to the large HA content of compartment III.

*Binding to cell surfaces, matrix proteoglycans, or collagen.* During the last 15 years, several specific HA-binding substances (“hyaladherins”) have been demonstrated, including cell surface HA receptors as well as serum-derived binding substances (see Refs. 6, 8, 10, 14, and 33). Especially impressive is the HA-containing pericellular matrix or cell coat, as demonstrated by exclusion of erythrocytes (8). The tendon cell surface may be quite large despite the low cell volume, because of the stellate forms of the cells with long thin longitudinal and transverse extensions bound together by gap junctions, creating a network around the collagen fibrils (19). HA has been shown to bind to type VI collagen (13, 18) but apparently not to type I collagen, which dominates in the rat tail tendon.

It appears reasonable to attribute the large unelutable fraction (compartment III) to one or more of the aforementioned binding types, but we found no evidence as to what extent they would resist the compressive and shear forces induced by centrifugation. Anyhow, breakage of some sort of molecular binding could possibly explain the additional 20% of HA eluted at 4,000 rpm or higher compared with that obtained with 500 rpm (compartment II). Another possibility might be a disruption of the HA network formation in the extracellular space, as described by Scott and Heatley (30) as likely to be broken by mechanical force. The possibility that low- and high-molecular-weight HA might need different centrifugation rates to be eluted, or that centrifugation per se might break down HA molecules, was excluded by the finding of equal molecular weights in early and late eluates obtained with 500 or 14,000 rpm. However, the exponential elution pattern maintained at all centrifugation rates seems more likely to result from opening up an additional anatomic distribution space, containing HA but no Alb, by prehydration and a high centrifugation rate.

**Compartment II**

These considerations and a previous study (3) of excluded fluid spaces lead us to the hypothesis that the HA in compartment II is derived from the collagen extracellular space, containing ~0.2 ml fluid/g normally hydrated tendon, or ~40% of the total extracellular water. Apparently, this is contradicted by our previous finding (3) that successive centrifugations at 3, 6, and 14,000 rpm without fluid addition gave centrifugates with increasing amounts of fluid from which both Alb and HA was excluded, which we attributed to increasing amounts of intrafibrillar fluid. To reconcile these observations, we have to conclude that mobilization of HA-containing extracellular fluid requires prehydration of the collagen molecules as well as high rpm. The best evidence for such fluid movement has been provided by Maroudas and co-workers (17), who showed by X-ray diffraction that collagen fibrils in cartilage could be compressed by mechanical or osmotic force. At low force, fluid was squeezed out mainly from the extracellular space, whereas the low-compliant intrafibrillar space required higher pressure.

The equivalent in the present study would be that, at low centrifugation rates (500–1,000 rpm), fluid was forced out mainly from the extracellular space, and, when fluid was added before the next centrifugation, it was taken up in the extracellular space and to a little extent in the collagen fibrils. Also, with high centrifugation rates (~4,000), the fibrils were compressed and then filled up again when fluid was added. This tide and ebb in the intrafibrillar space would then provide an exponential washout of HA from the extracellular space.

Admittedly, several objections may be raised against this scheme. First, molecular reconstruction of collagen fibrils, mainly based on X-ray diffraction studies, have provided little space for intrafibrillar entry of HA. Thus intermolecular distances of 1.2−1.5 nm and collagen molecules with a diameter of 1.3−1.4 nm (20), or an average gap between the collagen molecules of 0.2−0.6 nm “in most areas” (11, 12), would not admit HA molecules with a chain diameter of 0.7 (15) or 1.1 nm (22). Moreover, the passage of HA into and out from the intrafibrillar space might be impeded by the length of the “stiff segments” (persistence length), variously es-
timated at 0.4–1.6 nm (4, 29). True enough, Katz and Li (11) estimated an almost six times greater intrafibrillar water content in “fully hydrated” steer skin collagen, but the gap between the collagen molecules would presumably not exceed 0.5 nm. Still, the water content seems compatible with our own measurements of the distribution space for $^{[51}\text{Cr}]$EDTA, which has a hydrodynamic diameter of 0.94 nm (23), similar to the HA chain diameter. In the normally hydrated rat tail tendon, Aukland et al. (1) found a $^{[51}\text{Cr}]$EDTA space of 0.57 ml/g wet wt compared with a water content of 0.62 ml/g, and Wiig et al. (32) reported corresponding numbers of 0.51 and 0.61 ml/g wet wt, i.e., 92 and 82% of total water volume in the two studies, respectively. Considering that the extrafibrillar-extracellular space makes up only $\sim$0.35 ml/g wet wt (3), these findings strongly indicate a large $^{[51}\text{Cr}]$EDTA space within the compartments I and II, one might expect similar HA spaces and concentrations inside and outside the fibrils. Clearly, that estimate would be highly misleading if many of the long-stranded HA molecules were “trapped” with long portions of the molecule extending out from the interior of the fibrils.

To reconcile these estimates with the molecular studies quoted above, we have to assume that both HA chains and $^{[51}\text{Cr}]$EDTA would have to enter the intrafibrillar space through the 1.2- to 2.1-nm “gaps” or “pores” resulting from the staggering in the collagen molecule assembly (11, 20). These openings, which occupy $\sim$10% of the length of collagen, would also add distribution space to the true intrafibrillar volume. A proteoglycan belt has been described to surround the tail tendon fibrils in the gap region (31), but there seems to be no direct evidence for HA accumulation in this site.

Attempts at direct demonstration of intrafibrillar HA have been negative. True enough, Pease and Bou- tellle (25) described a regular pattern of phosphotung- stic acid-stained intrafibrillar structures in the aortic intima believed to represent HA, but other histochem- ists (9) have questioned the specificity of the technique. Exposure of tissue slices to biotinylated HA-binding protein followed by avidin peroxidase clearly demonstrates the gross distribution of HA (8, 16), but the resolution seems inadequate to relate to the level of collagen fibrils. Anyhow, the large binding protein (mol wt, 90,000) would probably be effectively prevented from entering the intrafibrillar space and binding to HA in that location. More promising for that purpose, Brown and Fraser [quoted by Fraser and Laurent (8)] located HA by autoradiography of mouse skin exposed to $^3\text{H}]$HA for 12 h. They found HA surrounding, but not inside, the “collagen bundles.” (According to the magnification shown in their Fig. 5A, these “bundles” would contain a large number of fibrils.) With our hypothesis removing HA from the intrafibrillar space in mind, one might wonder whether repeated hydration-dehydration could have provided for uptake in the fibrils.

The preceding discussion on intrafibrillar entry of HA may be misleading. What we observed is an exit of HA from a pool requiring both prehydration and centrifugation at high revolutions per minute. Maybe HA was incorporated during fibril growth, resulting from apposition of new collagen molecules or “microfibrils?”

As summarized by Parry and Craig (24), collagen fibrils in the newborn rat have a uniform diameter of 49 nm. At 8 wk and older, the fibril population has become greatly heterogeneous, with a mass average diameter of 320 nm, while still $\sim$50% of the fibrils have a diameter $<$150 nm. In the same period, the tail HA content falls from 9.1 to 0.7 mg/g dry wt, most steeply during the first 2–3 wk. Clearly, plenty of HA would be available for incorporation during the period of rapid growth.

What could be the consequences of intrafibrillar HA? At normal hydration, intrafibrillar HA seems to be trapped and might therefore exert an osmotic pressure acting to pull water into the fibril. It might thereby contribute to balance the osmotic pressure of plasma proteins, proteoglycans, and HA in the extracellular-extrafibrillar fluid, in fact a counterpart to the implication of extrafibrillar proteoglycans in the regulation of intrafibrillar fluid volume in cartilage demonstrated by Maroudas et al. (17). In dehydration of the tendon, intrafibrillar HA would resist removal of the intrafibrillar space, adding to the intermolecular forces keeping the collagen molecules apart. During overhydration and reduced extrafibrillar oncotic pressure, it would act to increase the intrafibrillar space. In this situation, the expansion would be limited by HA dilution and possibly a reduced reflection coefficient and by the inherent stiffness of the fibrils.

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