

Synthesis of hyaluronate in cultured bovine articular cartilage

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The synthesis and distribution of hyaluronate and proteoglycan were studied in bovine articular cartilage in short-term explant culture with [^3H]acetate and $\text{H}_2^{35}\text{SO}_4$ as precursors. The incorporation of [^3H]acetate into hyaluronate and sulphated glycosaminoglycans was linear with time, except that hyaluronate synthesis showed a marked lag at the beginning of the incubation. [^3H]Hyaluronate represented 4–7% of the total [^3H]glycosaminoglycans synthesized over a 6 h period. However, the distributions of [^3H]hyaluronate and ^3H -labelled sulphated glycosaminoglycans were different: about 50% of the newly synthesized [^3H]hyaluronate appeared in the medium, compared with less than 5% of the ^3H -labelled sulphated proteoglycans. A pulse-chase experiment revealed that the release of newly synthesized [^3H]hyaluronate from cartilage was rapid. No difference was observed in the distribution of [^3H]hyaluronate between medium and tissue by cartilage from either the superficial layer or the deep layer of articular cartilage. When articular cartilage was incubated with 0.4 mM-cycloheximide, proteoglycan synthesis was markedly inhibited, whereas the synthesis of hyaluronate was only partially inhibited and resulted in more of the newly synthesized hyaluronate being released into the medium. Analysis of the hydrodynamic size of [^3H]hyaluronate isolated from cartilage on Sephacryl-1000 revealed one population that was eluted as a broad peak ($K_{\text{av}} < 0.7$), compared with two populations ($K_{\text{av}} > 0.5$ and < 0.5) appearing in the medium of cultures. These data suggest that hyaluronate is synthesized in excess of proteoglycan synthesis and that the hyaluronate that is not complexed with proteoglycans is rapidly lost from the tissue.

INTRODUCTION

The extracellular matrix of articular cartilage is made up of collagen, proteoglycans, matrix proteins and hyaluronate. The collagen (type II collagen) meshwork provides the cartilage with its structural integrity and entraps proteoglycan aggregates, which provide cartilage with its unique ability to withstand compression (Handley *et al.*, 1985). These matrix components are not metabolically passive, but are constantly being turned over, this process being controlled and maintained by the chondrocytes (Handley *et al.*, 1986). Perturbation of this steady-state condition, such as occurs in degenerative and diseased cartilage, will result in the loss of biomechanical function of the tissue (Kempson, 1980). It is therefore important that the concentration of these macromolecules in the matrix be kept at a level that allows the articular cartilage to maintain its biomechanical role.

Proteoglycans and link proteins are synthesized within the endoplasmic reticulum of the chondrocytes before being released into the extracellular matrix (Handley *et al.*, 1985). Synthesis of hyaluronate, on the other hand, is believed to occur on plasma membrane of chondrocytes, and the enzyme responsible for chain elongation is hyaluronate synthetase, an integral plasma-membrane protein (Prehm, 1984). Newly synthesized hyaluronate remains attached to the synthetase during synthesis via a phosphodiester bond, and, upon completion of chain elongation, the bond is cleaved, releasing hyaluronate into the matrix. In articular cartilage newly released hyaluronate is probably utilized for the formation of aggregates with proteoglycans and link proteins. In the

present paper the kinetics of hyaluronate synthesis by and release from bovine articular cartilage in explant culture are investigated.

EXPERIMENTAL

Materials

$\text{H}_2^{35}\text{SO}_4$ (carrier-free), [^3H]glucosamine (100 mCi/mmol) and [^3H]acetate (100 mCi/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). Sephadex G-50 (medium grade), Sephacryl-1000 (fine grade), DEAE-Sephacel, Superose 12 (HR 10/30 column) and Mono-Q (HR 5/5) were obtained from Pharmacia (Uppsala, Sweden). Sterile plastic screw-capped vials (20 ml and 50 ml) were from Disposable Products (Melbourne, Vic., Australia). Dulbecco's modified Eagle's medium, fetal-calf serum and Eagle's non-essential amino acids were obtained from CSL (Melbourne, Vic., Australia). Urea, NH_4HCO_3 , mannitol, Triton X-100, EDTA, imidazole, LiCl, NaCl, sodium acetate and NaN_3 were all purchased from Ajax Chemicals (Auburn, N.S.W., Australia). Papain, guanidinium chloride and chondroitin ABC lyase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CHAPS was obtained from Calbiochem (Richmond, CA, U.S.A.). *Streptomyces* sp. hyaluronidase was obtained from Seikagaku Fine Biochemicals (Tokyo, Japan). [^3H]Hyaluronate was a gift from Dr. R. B. Fraser, Department of Medicine, University of Melbourne, Melbourne, Vic., Australia.

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Methods

Cartilage cultures. Bovine articular cartilage was isolated from metacarpal-phalangeal joints as previously described (Hascall *et al.*, 1983). Tissue slices (approx. 100 mg/vial) were distributed into pre-weighed sterile plastic vials and maintained at 37 °C for a predetermined period of time in 4 ml of Dulbecco's modified Eagle's medium with or without 20% (v/v) fetal-calf serum (Handley & Lowther, 1977). In some experiments where a large batch of labelled cartilage was required, 1 g of tissue was incubated in 10 ml of medium. In other experiments the superficial layer of cartilage was dissected from the deep layer adjacent to the subchondral bone; tissue (approx. 100 mg) from each layer was distributed separately into vials as described above.

Incubation of articular cartilage with radiolabelled precursors. Cartilage cultures were preincubated in fresh medium containing 20% fetal-calf serum with 0.5 mM-sodium acetate for 1 h, and this medium was replaced with 2 ml of labelling medium containing $H_2^{35}SO_4$ (20 μ Ci/ml) and/or [3H]acetate (30 μ Ci/ml). Tissue was incubated for a predetermined period of time at 37 °C in a shaking water bath before being digested with papain. In the pulse-chase experiments, tissue was incubated with [3H]acetate (30 μ Ci/ml) and [^{35}S]sulphate (20 μ Ci/ml) for 6 h, then washed with fresh medium before being incubated with medium not containing label for up to 6 h. In experiments in which cycloheximide was used, cartilage was preincubated at 37 °C for 1 h in medium containing 20% fetal-calf serum, then with fresh medium containing 20% fetal-calf serum, [3H]acetate (30 μ Ci/ml), [^{35}S]sulphate (20 μ Ci/ml) and 0.4 mM-cycloheximide (McQuillan *et al.*, 1984). The time of this addition was designated zero time. Duplicate cultures were removed at predetermined times, and the reactions were stopped by addition of 2 ml of 0.1 M-sodium acetate buffer, pH 5.5, containing 5 mM-EDTA and 5 mM-cysteine hydrochloride.

Isolation of glycosaminoglycans. Depending on the experiment, tissue and medium were digested with papain either separately or together. Papain digestion of the tissue was carried out by adding 2.0 ml of 0.1 M-sodium acetate buffer, pH 5.5, containing 5 mM-EDTA, 5 mM-cysteine hydrochloride and 1.4 μ g of papain (Handley & Lowther, 1977). Medium was adjusted to 0.1 M-sodium acetate buffer, pH 5.5, containing 5 mM-EDTA and 5 mM-cysteine hydrochloride, and 1.4 μ g of papain was added. Digestions were carried out at 60 °C for 16 h, and the resulting glycosaminoglycans were subjected to ion-exchange chromatography on a Mono-Q column.

Ion-exchange chromatography on Mono-Q. A pre-packed Mono-Q column (HR 5/5) was pre-equilibrated in 0.15 M-LiCl/0.06 M-imidazole/HCl buffer, pH 6.0, at a flow rate of 1 ml/min. Papain-digested samples were centrifuged at 10000 g for 3 min, and the resulting supernatants (1.5 ml) were applied to the column. Anionic macromolecules were eluted from the column at 1 ml/min with a biphasic linear LiCl gradient (0.15–0.45 M over 40 min, 0.45–1.5 M over 20 min) in 0.06 M-imidazole/HCl buffer, pH 6.0. Fractions (2.0 ml) were collected and assayed for radioactivity with the use of a scintillation cocktail described by Hascall *et al.* (1983).

Fractions containing the peak of [3H]hyaluronate were pooled and applied to a column (0.8 cm \times 15 cm) of Sephadex G-50 equilibrated in 50 mM-NH₄HCO₃. Fractions corresponding to radiolabelled material eluted at the excluded volume of the column were pooled and freeze-dried before being taken up in 0.15 M-NaCl/0.02 M-sodium acetate buffer, pH 6.0. Some of this sample was treated with *Streptomyces* hyaluronidase (0.1 unit). Alternatively, after being freeze-dried some of the samples were dissolved in 0.1 M-Tris/0.1 M-sodium acetate buffer, pH 7.3, and portions digested with chondroitin ABC lyase (0.5 unit) overnight at room temperature. Samples before and after treatment with the glycosidases were analysed on Superose 12 (HR 10/30), which was eluted with 0.5 M-LiCl/0.01 M-imidazole/HCl buffer, pH 6.0, at 1 ml/min.

Characterization of newly synthesized hyaluronate. Tissue was labelled for 6 h with [3H]acetate and [^{35}S]sulphate in Dulbecco's modified Eagle's medium containing 20% fetal-calf serum. The tissue and medium were then extracted with 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 6.0, containing 0.5% (v/v) Triton X-100 and proteinase inhibitors (Hascall & Kimura, 1982) together with 10 mM-mannitol and exogenous hyaluronate (2 mg/ml) for 60 h at 4 °C. Mannitol was added as an oxy radical scavenger (Bates *et al.*, 1984). Samples of the medium and tissue extracts were applied to columns (0.8 cm \times 15 cm) of Sephadex G-50 equilibrated with 7 M-urea/0.05 M-sodium acetate buffer, pH 6.0, containing 0.15 M-NaCl and 0.5% Triton X-100. Labelled macromolecules recovered in the excluded volume were separated by ion-exchange chromatography on a DEAE-Sephacel column (0.5 cm \times 8 cm) with a linear gradient of NaCl (0.15–1.4 M over 220 ml) in 7 M-urea/0.05 M-sodium acetate buffer, pH 6.0, containing 0.5% Triton X-100 (Yanagishita & Hascall, 1985).

Fractions containing 3H -labelled hyaluronate were pooled and applied to a Sephacryl-1000 column (0.4 cm \times 84 cm) equilibrated with 1.0 M-NaCl/0.05 M-sodium acetate buffer, pH 6.0, containing 0.05% (w/v) CHAPS. Each fraction was assayed for radioactivity. The molecular mass of [3H]hyaluronate eluted from the Sephacryl-1000 column was estimated from selectivity curves supplied by the manufacturer. Samples of the hyaluronate peak were applied to columns (0.8 cm \times 15 cm) of Sephadex G-50 equilibrated with 50 mM-NH₄HCO₃. The radiolabelled material eluted at the void volume of the column was pooled and freeze-dried before being made up in 0.15 M-NaCl/0.02 M-sodium acetate buffer, pH 6.0. A portion of each sample was treated with *Streptomyces* hyaluronidase (0.1 unit), before analysis on the Sephacryl-1000 column as described above or on a column (0.8 cm \times 15 cm) of Sephadex G-50 eluted with 1.0 M-NaCl/0.05 M-sodium acetate buffer, pH 6.0, containing CHAPS.

RESULTS

Separation of 3H -labelled macromolecules from papain-digested cartilage tissue by ion-exchange chromatography on Mono-Q

Samples (100 mg) of articular cartilage were pre-incubated in Dulbecco's modified Eagle's medium containing 20% fetal-calf serum for 1 h, then incubated

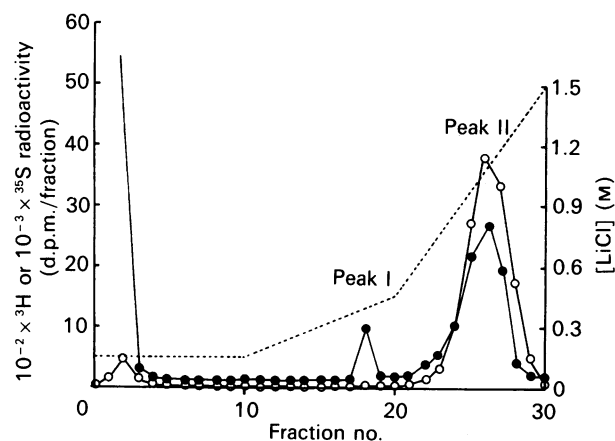


Fig. 1. Separation of [^3H]hyaluronate and [^3H]- or [^{35}S]-glycosaminoglycans by ion-exchange chromatography on Mono-Q

Bovine articular cartilage was labelled with [^3H]acetate and [^{35}S]sulphate for 6 h, then digested with papain. Digests were analysed by ion-exchange chromatography on Mono-Q with a biphasic LiCl gradient (-----) as described in the Experimental section. Fractions were analysed for ^3H (●) and ^{35}S (○) radioactivity.

with the same medium containing [^3H]acetate and [^{35}S]sulphate for 6 h. The tissue was then digested with papain and applied to a Mono-Q HR 5/5 column. Bound macromolecules were eluted from the column with a LiCl gradient as shown in Fig. 1. Two separate radiolabelled peaks were observed: the first, which contained only ^3H radioactivity, was eluted from the Mono-Q column at 0.42 M-LiCl, which corresponded exactly to the salt concentration at which standard [^3H]hyaluronate was eluted. The fractions corresponding to the [^3H]hyaluronate peak were pooled and analysed by gel filtration on Superose 12 before and after treatment with *Streptomyces* hyaluronidase as described in the Experimental section. The ^3H -labelled molecules in peak I were totally digested by this glycosidase. The second peak contained both ^3H and ^{35}S radioactivity, and was susceptible to digestion with chondroitin ABC lyase but not with *Streptomyces* hyaluronidase, demonstrating that the anionic material eluted in this peak was composed of sulphated glycosaminoglycans derived from proteoglycans.

Preliminary experiments established that the recovery of high-molecular-mass [^3H]hyaluronate from Mono-Q was poor. However, if these macromolecules were subjected to digestion with papain, then recovery from this ion-exchange column was close to 100%. Digestion brought about a decrease in the molecular size of the [^3H]hyaluronate, as assessed by gel-filtration chromatography. However, it must be pointed out that it is the EDTA and cysteine present in the buffer used for papain digestion that cause this decrease.

Synthesis and distribution of hyaluronate and proteoglycans in cartilage tissue explants

Articular cartilage (100 mg) was preincubated for 1 h in medium containing 20% fetal-calf serum and 0.5 mM-sodium acetate, then in the same medium containing [^3H]acetate and [^{35}S]sulphate for up to 10 h. Tissue and

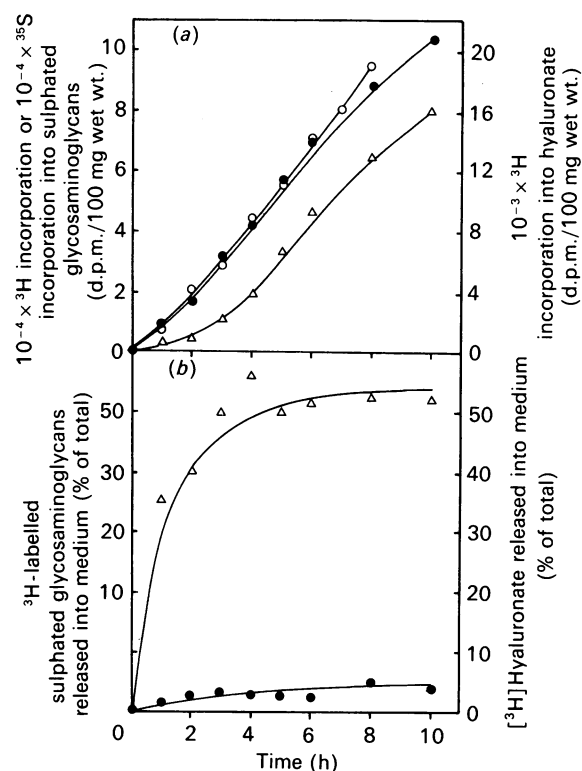


Fig. 2. Synthesis and distribution of hyaluronate and proteoglycans in bovine articular-cartilage tissue explants

Bovine articular cartilage was labelled with [^3H]acetate and [^{35}S]sulphate for up to 10 h. Tissue and medium were separately digested with papain before analysis of labelled glycosaminoglycans by ion-exchange chromatography on Mono-Q as described in the Experimental section. The syntheses of [^3H]hyaluronate (Δ), ^3H -labelled sulphated glycosaminoglycans (●) and ^{35}S -labelled sulphated glycosaminoglycans (○) are shown in panel (a). The distributions between the matrix and medium of [^3H]hyaluronate (Δ) and ^3H -labelled sulphated glycosaminoglycans (●) are shown in panel (b). The points in the plots are means from duplicate cultures.

medium were separated, digested with papain and subjected to ion-exchange chromatography on Mono-Q. The incorporation of [^3H]acetate into hyaluronate and sulphated glycosaminoglycans followed a linear relationship with time (Fig. 2a), except that hyaluronate synthesis showed a marked lag at the beginning of the incubation. About 95% of the total ^3H - or ^{35}S -labelled sulphated glycosaminoglycans were retained in the tissue, and the remainder was found in the medium (Fig. 2b). [^3H]Hyaluronate synthesized during the study period represented 4.5–7% of total [^3H]glycosaminoglycans found in tissue explants labelled with [^3H]acetate. A marked difference was observed in the distribution of labelled hyaluronate between the medium and the matrix of the tissue. About 47–52% of the total [^3H]hyaluronate was retained in tissue, and the remainder was secreted into the medium (Fig. 2b). This distribution was reached at the third hour of labelling and was maintained relatively constant thereafter. Similar kinetics and distribution of proteoglycans and hyaluronate were observed when [^3H]glucosamine was used in place of [^3H]acetate, and

Table 1. Synthesis and distribution of newly synthesized hyaluronate and proteoglycans by tissue from the superficial layer and deep layer of articular cartilage

Cartilage from the superficial and deep layers of articular cartilage were incubated with [³⁵S]sulphate and [³H]acetate for 10 h. The tissue and the medium were digested with papain and analysed for glycosaminoglycans by ion-exchange chromatography on Mono-Q as described in the Experimental section. Each value is the mean of two determinations. The values in parentheses are the percentages of the labelled glycosaminoglycan recovered from the medium.

	[³⁵ S]Sulphate incorporation into sulphated glycosamino- glycans (d.p.m./100 mg wet wt.)		[³ H]Acetate incorporation into hyaluronate (d.p.m./ 100 mg wet wt.)	
	Matrix	Medium	Matrix	Medium
Superficial layer	176 582	15 501 (8.1%)	17 900	14 974 (45.5%)
Deep layer	204 872	14 307 (6.5%)	37 978	34 769 (47.8%)
Full thickness	303 341	7 306 (5.4%)	27 676	21 420 (48.8%)

also when tissue was incubated with [³H]acetate in medium without fetal-calf serum.

A similar experiment to that described above was carried out except that tissue from the superficial layer of articular cartilage and the deep layer adjacent to the subchondral bone were separately incubated with [³H]acetate and [³⁵S]sulphate. Table 1 shows that on a tissue-wet-weight basis there was a lower rate of synthesis of hyaluronate and sulphated glycosaminoglycan by tissue from the superficial layer; however, there was no difference in the distribution between the matrix and the medium of either labelled macromolecule from that observed in whole-tissue slices.

The release of [³H]hyaluronate from the tissue into the medium was investigated in a pulse-chase experiment. After preincubation for 1 h in Dulbecco's modified Eagle's medium containing 20% fetal-calf serum plus

0.5 mM-sodium acetate, articular cartilage was incubated in the same medium with [³H]acetate and [³⁵S]sulphate for 6 h, then the medium was replaced with the same medium not containing labelled precursors. At various times during a subsequent 5 h chase period, tissue and medium were digested separately with papain and analysed for radiolabelled hyaluronate and sulphated glycosaminoglycans by ion-exchange chromatography on Mono-Q. At the end of the pulse period (*t*₀, chase time) about 50% of the [³H]hyaluronate synthesized was found in the culture medium (Fig. 3). A little more [³H]hyaluronate accumulates in the medium during the first 1 h of chase, but thereafter there is very little further loss from the tissue. Thus the fraction of [³H]hyaluronate destined for the medium is rapidly lost to the medium soon after synthesis. ³H-labelled sulphated glycosaminoglycans were found to be retained predominantly in the matrix, although there was a very slow release of sulphated glycosaminoglycans from the matrix to medium with time. ³⁵S-labelled glycosaminoglycans showed a similar distribution to ³H-labelled sulphated glycosaminoglycans.

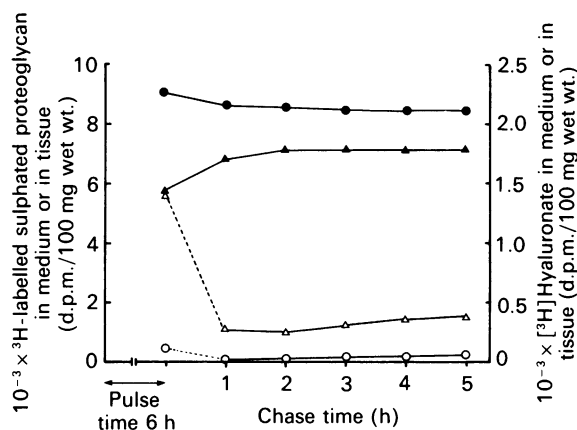


Fig. 3. Release of ³H-labelled hyaluronate and sulphated glycosaminoglycans from bovine articular-cartilage tissue explants

Bovine articular-cartilage tissue explants were incubated with [³H]acetate and [³⁵S]sulphate for 6 h and then incubated in fresh medium not containing labelled precursors. [³H]Hyaluronate in the medium (Δ) and tissue (\blacktriangle) was measured at increasing chase times. ³H-labelled sulphated proteoglycans in the medium (\circ) and tissue (\bullet) were also measured. The values in the plot are means from duplicate cultures.

Effect of cycloheximide on hyaluronate and proteoglycan synthesis

The observations described above indicate that part of the newly synthesized hyaluronate is retained within the matrix of articular cartilage, most probably in the form of complexes with proteoglycan subunits. To test whether the proportion of hyaluronate lost to the medium increases when proteoglycan synthesis is decreased, articular cartilage was preincubated in Dulbecco's modified Eagle's medium containing 20% fetal-calf serum plus 0.5 mM-sodium acetate for 1 h, then incubated for up to 6 h in the same medium containing [³H]acetate and [³⁵S]sulphate in the presence and in the absence of 0.4 mM-cycloheximide. Synthesis of ³H-labelled sulphated glycosaminoglycan was markedly decreased in cultures maintained with cycloheximide (Fig. 4a). This reflects the gradual depletion of the intracellular proteoglycan core-protein pool as the result of the inhibition of mRNA translation. The half-life of the pool was estimated to be 36 min from a semi-logarithmic plot of the data (Fig. 4a inset). The effect of cycloheximide on total hyaluronate synthesis is shown in Fig. 4(b); the synthesis of [³H]hyaluronate was linear with respect to time, but the

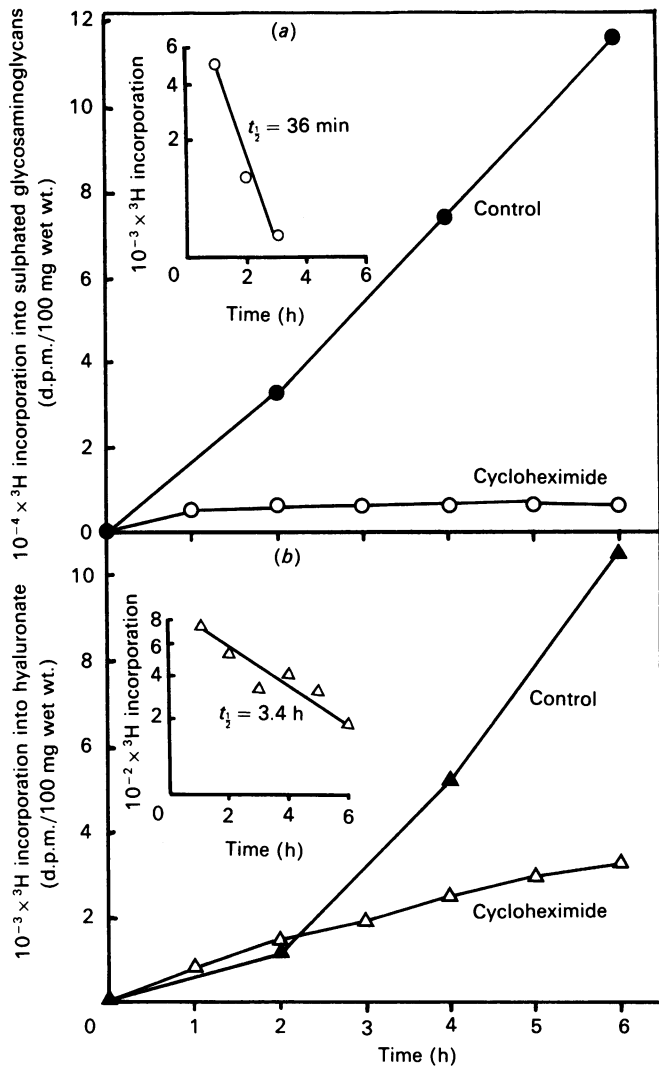


Fig. 4. Effect of cycloheximide on the synthesis of ^3H hyaluronate and ^3H -labelled sulphated glycosaminoglycans in bovine articular-cartilage tissue explants

Tissue from bovine articular cartilage was labelled for up to 6 h with ^3H acetate and ^{35}S sulphate in medium containing (○ and △) or not containing (● and ▲) 0.4 mM-cycloheximide. At various times the incorporation of ^3H acetate into total sulphated glycosaminoglycans (panel a: ● and ○) and hyaluronate (panel b: ▲ and △) was determined. The half-lives of incorporation of ^3H into total sulphated glycosaminoglycans and hyaluronate were obtained from semi-logarithmic plots of the rate of ^3H incorporated/h versus time, and are shown in the insets. The points in the plots are means from duplicate cultures.

rate of synthesis was about 70% lower than that found in control incubations. From the semi-logarithmic plot of these data, a half-life of 3.4 h was measured (Fig. 4b inset). Fig. 5 shows that more of the newly synthesized ^3H hyaluronate was released to the medium of cultures treated with cycloheximide. Since hyaluronate synthesis was relatively less inhibited than proteoglycan synthesis, this result favours the conclusion that interaction with newly synthesized proteoglycan normally immobilizes part of the pool of newly synthesized hyaluronate in the cartilage matrix.

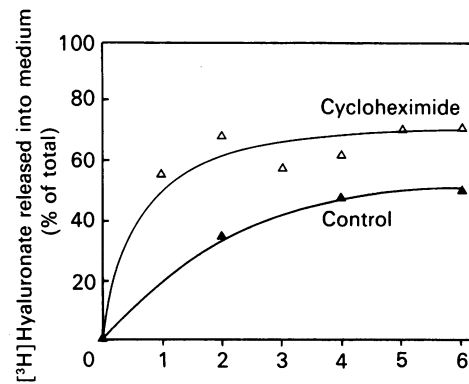


Fig. 5. Effect of cycloheximide on the distribution of ^3H -hyaluronate in bovine articular-cartilage tissue cultures

The amount of newly synthesized ^3H hyaluronate appearing in the medium of control (▲) and cycloheximide-treated (△) cultures was determined for the experiment described in Fig. 4. Each point represents the amount of ^3H hyaluronate appearing in the medium expressed as a percentage of total ^3H hyaluronate synthesized by the tissue and is a mean from duplicate cultures.

Size of ^3H hyaluronate in tissue and medium

After preincubation in Dulbecco's modified Eagle's medium containing 20% fetal-calf serum for 1 h, 1 g of articular cartilage was incubated in the same medium containing ^3H acetate for 6 h. Since papain digestion alters the size of hyaluronate, this method cannot be used to estimate the size of hyaluronate in the tissue or the medium. After labelling, tissue was extracted with 4 M-guanidinium chloride in the presence of inhibitors and mannitol as outlined in the Experimental section. Approx. 25% of the total ^3H hyaluronate was extracted from the tissue by this procedure. Extracts were then passed down a Sephadex G-50 column to exchange the guanidinium chloride-containing buffer for urea-containing buffer and to remove unincorporated radio-labelled precursors. The ^3H acetate-labelled macromolecules appearing in the void volume of this column were applied to a DEAE-Sephacel column, which was eluted with a linear salt gradient.

^3H -labelled hyaluronate fractions from tissue and medium that were eluted from DEAE-Sephacel at the same position as standard ^3H hyaluronate (0.17–0.19 M-NaCl) were analysed by gel filtration on Sephacryl-1000. Fig. 6 shows that ^3H hyaluronate from the tissue was eluted as a single broad peak (K_{av} 0.03–0.63) that was totally digested by *Streptomyces* hyaluronidase. ^3H -labelled hyaluronate was also isolated from cartilage by digestion with papain in the absence of EDTA and cysteine as described by Holmes *et al.* (1988), and when this was analysed on Sephacryl-1000 a similar profile was obtained to that for hyaluronate extracted from the tissue with guanidinium chloride. Medium ^3H -hyaluronate separated into two populations, 45% of the total ^3H -labelled material having a K_{av} of 0.5 or less (peak I) and the remaining ^3H -labelled material having a K_{av} of 0.5 or more (peak II). Fractions corresponding to the two peaks were pooled separately, and treated with *Streptomyces* hyaluronidase as described in the Experimental section before being analysed on Sephadex G-50. Both peaks were readily digested with the enzyme to yield hexa- and tetra-saccharides.

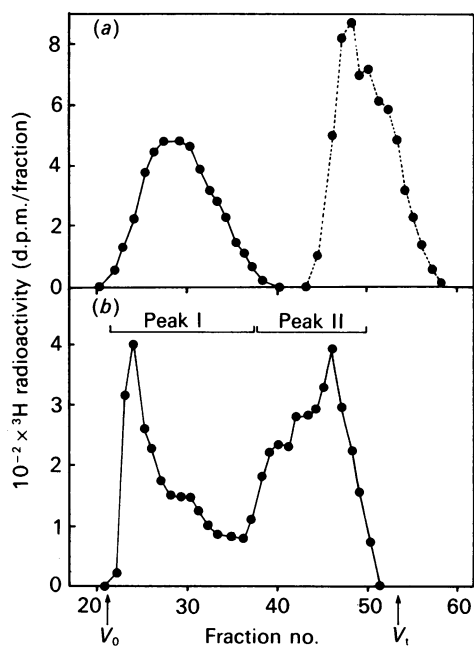


Fig. 6. Molecular size of [^3H]hyaluronate remaining in the tissue and lost to the medium of bovine articular-cartilage tissue explants

Bovine articular cartilage was extracted with 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 6.0, containing 0.5% (v/v) Triton X-100 in the presence of proteinase inhibitors and 10 mM-mannitol. [^3H]Hyaluronate in the extract was isolated by ion-exchange chromatography on DEAE-Sephacel as outlined in the Experimental section. The size of [^3H]hyaluronate isolated from the tissue (panel *a*) and the medium (panel *b*) was determined by gel filtration on Sephacryl-1000 eluted with 1.0 M-NaCl/0.05 M-sodium acetate buffer, pH 6.0, containing 0.05% (w/v) CHAPS. Some of the [^3H]hyaluronate isolated from the tissue was treated with *Streptomyces* hyaluronidase before gel filtration (-----).

DISCUSSION

The results show that the incorporation of [^3H]acetate or [^3H]glucosamine into proteoglycans or hyaluronate was linear with time, although there was an initial lag in the incorporation of radiolabelled precursors into hyaluronate. This may reflect a difference in the equilibration times of [^3H]acetate into nucleotide precursors of glycosaminoglycan synthesis, suggesting that there may be separate nucleotide sugar pools for the synthesis of hyaluronate and proteoglycans. Indeed, Kleine (1978) has presented evidence that two distinct nucleotide sugar pools exist in calf costal cartilage for hyaluronate and proteoglycan synthesis.

The data in the present paper suggest that two pools of hyaluronate exist in articular cartilage, one pool being held in the tissue, presumably complexed with the large aggregating proteoglycan of cartilage, and the other representing free hyaluronate and being rapidly lost *in vitro* from the tissue to the medium. Hyaluronate synthesis has been measured in rabbit and sheep cartilage in explant cultures, but no attempt was made in those studies to investigate the distribution of hyaluronate (Gillard *et al.*, 1975). When articular cartilage was incubated with cycloheximide, an inhibitor of protein

synthesis, proteoglycan synthesis was inhibited to a greater extent than hyaluronate synthesis, and an increased proportion of newly synthesized hyaluronate appeared in the medium of the cultures. From the decrease in the rate of incorporation of [^3H]acetate with time, the half-life of proteoglycan synthesis was calculated to be approx. 36 min. This value represents the half-life of the proteoglycan core protein, and similar values have been reported previously for bovine articular cartilage (McQuillan *et al.*, 1984). A corresponding value of 3.4 h was calculated for the half-life of hyaluronate synthesis, and this may represent the half-life for hyaluronate synthetase or enzymes generating nucleotide sugars for hyaluronate synthesis. Bansal & Mason (1986) showed that the half-life of hyaluronate synthetase in rat chondrosarcoma cells is approx. 80 min with similar concentrations of cycloheximide to that used in the present work.

Analysis of the size of the [^3H]hyaluronate lost to the medium of cartilage incubations showed two distinct populations of different size, a large-molecular-mass species of greater than 5×10^5 Da and a small-molecular-mass species of less than 5×10^5 Da. The labelled hyaluronate retained in the tissue was composed solely of high-molecular-mass chains of greater than 5×10^5 Da. The low-molecular-mass hyaluronate in the medium could have arisen either as a product of biosynthesis or by cleavage of a larger molecule after synthesis. Recent evidence suggests that the molecular size of hyaluronate synthesized by human articular cartilage undergoes a subsequent decrease, since molecules resident in the matrix are smaller than the newly synthesized hyaluronate (Holmes *et al.*, 1988). The cleavage of molecules is limited in young human cartilage, yielding resident molecules of similar size to those of the bovine peak I hyaluronate (Fig. 6). Only in very old human cartilage is cleavage sufficiently extensive to yield resident molecules of similar size to bovine peak II hyaluronate. If the latter do arise by degradation of newly synthesized larger chains, cleavage in the tissue and diffusion to the medium must be very rapid and complete, since no [^3H]hyaluronate of low hydrodynamic size was found in the cartilage (Fig. 6). No information is available for the medium of cultured human articular cartilage (Holmes *et al.*, 1988). The mechanism by which cleavage could occur is not known. Direct enzymological evidence for a hyaluronidase in articular cartilage is lacking, and extensive rapid degradation by free radicals seems unlikely, since the matrix proteoglycans and culture medium would be effective scavengers in the tissue; furthermore mannitol was added to the medium before storage.

The possibility that the two distinct populations of [^3H]hyaluronate in the medium of bovine cartilage cultures arise during biosynthesis must be considered. For example, during polymerization *in vitro*, random chains may undergo premature termination before reaching a critical length, after which all those remaining go on to become high-molecular-mass hyaluronate. This speculation would be compatible with the non-overlapping profiles of peaks I and II (Fig. 6), but is contrary to the suggestion by Prehm (1983) that termination of polymerization of all hyaluronate chains is a random process.

A lower rate of synthesis of hyaluronate and proteoglycan by the superficial layer of cartilage compared with tissue from the deep layer of cartilage was observed. However, there was no difference in the distribution of

newly synthesized hyaluronate appearing in the medium. This suggests that, in articular cartilage from mature animals, hyaluronate is synthesized in excess over proteoglycan synthesis, thereby producing a surplus of hyaluronate, which rapidly moves out of the tissue. These data support the suggestion by Morales & Hascall (1988) that the proportions of hyaluronate and proteoglycan in the tissue are maintained at constant values. This unbound hyaluronate may ensure that all the proteoglycan subunits are retained in cartilage as aggregates. Furthermore this free hyaluronate may add to the pool of hyaluronate in the synovial fluid of the diarthroidal joint.

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