Absence of covalently linked core protein from newly synthesized hyaluronate

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1. Primary cultures of chondrocytes from the Swarm rat chondrosarcoma were labelled with either [^3H]glucosamine or [^{14}C]glucosamine, and hyaluronate synthesized by the cells was isolated from the cell layer. Parallel cultures were labelled with either [^3H]serine or [^3H]lysine, and identical fractions were isolated from the cell layer. Some cultures were dual-labelled. 2. In cultures labelled with [^3H]serine for between 30 min and 24 h and extracted with 4.0 M-guanidine, a procedure that solubilizes predominantly extracellular macromolecules, small amounts of [^3H]serine-labelled molecules were found associated with the hyaluronate fraction purified from the extract by dissociative CsCl-density-gradient centrifugation and dissociative Sepharose CL-2B chromatography. About 75% of the [^3H]serine-labelled molecules in the fraction were specifically associated with hyaluronate, since they could be removed by prior treatment with proteinase-free *Streptomyces* hyaluronidase. The association of the [^3H]serine-labelled molecules with hyaluronate was non-covalent, since they could be separated from it by further centrifugation in CsCl density gradients containing 4 M-guanidinium chloride and a zwitterionic detergent. 3. In other experiments the cultures were extracted with a sequential zwitterionic-detergent/guanidinium chloride procedure that completely solubilized the cell layer and enabled fractions containing newly synthesized cell-associated hyaluronate to be isolated. Zwitterionic detergent was present throughout. No [^3H]lysine was incorporated into these fractions, irrespective of whether the cultures were pulsed concurrently with [^3H]lysine and [^{14}C]glucosamine or sequentially with [^3H]lysine to prelabel the protein pool (24 h) followed by [^{14}C]glucosamine to label hyaluronate (1 h). 4. The results show that newly synthesized hyaluronate is not associated with covalently bound protein, and suggest that chain synthesis is initiated by a mechanism other than on to a core protein. Small amounts of [^3H]serine-labelled molecules are, however, non-covalently associated with extracellular hyaluronate. Their identity is at present unknown, but they are probably of low molecular weight.

Hyaluronic acid is synthesized by chondrocytes and has an important structural role in forming proteoglycan aggregates in the extracellular matrix (Hascall, 1977), and possibly a regulatory role in controlling the extent of proteoglycan synthesis by the cells (Mason, 1981). The regulation of hyaluronate biosynthesis in chondrocytes may therefore itself

be important in the maintenance of normal cartilage function. One point where regulation could be exerted is at the initiation of synthesis of the hyaluronate chain. Other glycosaminoglycans synthesized by chondrocytes are initiated by transfer of a glycosyl residue to appropriate hydroxy amino acids in the proteoglycan core protein (Rodén & Schwartz, 1975). Purified hyaluronate from a number of sources, including hyaline cartilage (Cleland & Sherblom, 1977), is associated with small amounts of protein (Sandson & Hammerman, 1962; Hammerman *et al.*, 1966; Sandson &

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Hammerman, 1968; Swann, 1968; Laurent, 1970; Greiling *et al.*, 1970; Mathieson & Pearce, 1977; Mikuni-Takagaki & Toole, 1981). It has been proposed that the association may be covalent, and therefore indicative of a core protein, because it was maintained when hyaluronate was sedimented in CsCl density gradients containing 4.0 M-guanidinium chloride and during other dissociative procedures (Scher & Hammerman, 1972; Mikuni-Takagaki & Toole, 1981). An alkali-labile O-glycosidic linkage between hyaluronate and protein, such as exists for chondroitin sulphate chains (Muir, 1958), has not been identified, however (Varma *et al.*, 1974, 1975).

Mikuni-Takagaki & Toole (1981) proposed that a putative hyaluronate-core-protein complex may have a long intracellular existence before glycosylation. They suggested that after glycosylation the hyaluronate-protein complex is bound to the cell surface, the free glycosaminoglycan being released into the extracellular compartment by proteolytic scission. Using an experimental protocol designed to test this hypothesis, they found that [^3H]leucine-labelled protein was associated with hyaluronate extracted from the cell layer of fibroblast cultures with guanidinium chloride.

Primary cultures of chondrocytes from the Swarm rat chondrosarcoma are active in synthesizing both proteoglycans (Kimura *et al.*, 1979) and hyaluronate (Mason *et al.*, 1982). Pulse-chase experiments show that newly synthesized molecules of both types pass out of the cellular compartment into the cell-layer matrix, and finally into the medium (Kimura *et al.*, 1979; Mason *et al.*, 1982). These experiments also demonstrate that extraction of the cell layer of cultures with 4 M-guanidinium chloride effectively solubilizes extracellular, but not significant amounts of intracellular, proteoglycan and hyaluronate. In contrast, sequential treatment of the cell layer with the zwitterionic detergent ZwittergentTM 3-12 and 4 M-guanidinium chloride completely solubilizes both cell-associated and extracellular macromolecules (Kimura *et al.*, 1981). Using dual-labelling techniques, including protocols similar to those described by Mikuni-Takagaki & Toole (1981), we have employed these selective extraction techniques to investigate the association of ^3H -labelled protein with newly synthesized hyaluronate in the cell layer of chondrocyte cultures. In a first series of experiments in which cultures were labelled with [^3H]serine, small amounts of non-covalently linked ^3H -labelled molecules were shown to be specifically associated with extracellular hyaluronate. In the second series of experiments cells were labelled with high-specific-radioactivity [^3H]lysine, and the cell layer was extracted with the Zwittergent/guanidine procedure. No ^3H -labelled molecules, and therefore no core protein, could be shown to be associated with the hyaluronate fraction.

Experimental

Chondrocyte cultures

Primary cultures of chondrocytes were established from preparations of single cells obtained from the transplantable Swarm rat chondrosarcoma as described previously (Kimura *et al.*, 1979).

Chondrocytes were plated in Dulbecco's modified Eagle's medium (GIBCO Europe) containing 15% (v/v) foetal calf serum at 6×10^6 – 10×10^6 cells/25 cm² flask or 45×10^6 cells/150 cm² flask. The culture medium was changed daily.

Labelling of cultures

In the first series of experiments, day-3 cultures were labelled for periods from 30 min to 20 h with D-[6- ^3H]glucosamine hydrochloride (38 Ci/mmol; Amersham International), with L-[G- ^3H]serine (2.8 Ci/mmol; New England Nuclear Corp.) or with Na₂³⁵SO₄ (800 mCi/mmol of S; Amersham International) or dual-labelled with Na₂³⁵SO₄ and one of the ^3H -labelled compounds. Medium from which unlabelled serine and inorganic sulphate had been omitted was used for labelling, as described previously (Mason *et al.*, 1982). In some experiments duplicate cultures were labelled separately with [^3H]glucosamine and [^3H]serine.

In the second series of experiments, three different protocols were used to label cells with L-[4,5- ^3H]lysine (108 Ci/mmol; Amersham International) in lysine-free medium. In the first protocol, day-1 cultures were pulsed with [^3H]lysine and [^{14}C]glucosamine (239 mCi/mmol; Amersham International) in medium containing 15% serum. In the second protocol, day-3 cultures were dual-labelled for 24 h either with [^3H]lysine and Na₂³⁵SO₄ or with [^3H]glucosamine and Na₂³⁵SO₄. In a third protocol day-3 cultures were labelled for 24 h with [^3H]lysine, the medium was removed and the cells were pulsed with [^{14}C]glucosamine in fresh medium for a further 1 h. In the experiments in which day-3 cultures were used, the medium for maintenance and labelling was changed from day 2 to Dulbecco's modified Eagle's medium containing 2% (v/v) foetal calf serum and 100 ng of insulin/ml (d'Arville & Mason, 1981). This medium minimizes artifacts arising from the non-specific association of glucosamine with serum proteins (Mason *et al.*, 1982) while maintaining conditions for proteoglycan and hyaluronate synthesis.

Extraction of cell layers

In the first series of experiments the cell layer was extracted with 4.0 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors 6-aminohexanoic acid (0.1 M), benzamidine hydrochloride (5 mM) and disodium EDTA (10 mM) for 1 h at 4°C with gentle agitation.

In the second series of experiments, the cell layer was extracted for 30 min with 8% Zwittergent^{TM3-12} (Calbiochem-Behring Corp.), after which an equal volume of 8 M-guanidinium chloride was added and the extraction was continued for a further 30 min at 4°C with gentle agitation. The extraction was then repeated with fresh 4 M-guanidinium chloride, and the two extracts were pooled (Kimura *et al.*, 1981). In addition to the proteinase inhibitors above, extracts in the second series also contained phenylmethanesulphonyl fluoride (1 mM) and *N*-ethylmaleimide (1 mM). Any residue remaining on the plates after the extraction was dissolved in 0.5 M-NaOH (18 h, 20°C) and its radioactivity measured.

Equilibrium density-gradient centrifugation

After the addition of carrier proteoglycan (final concn. approx. 1 mg/ml) extracts were chromatographed on PD-10 columns (Pharmacia) eluted with 4 M-guanidinium chloride, to remove unincorporated radioactivity (Mason *et al.*, 1982). For the second series of experiments the columns were equilibrated and eluted with 4 M-guanidinium chloride containing 4% Zwittergent and proteinase inhibitors. Solid CsCl was added to the void-volume fraction to give a density of 1.43 g/ml and the solutions were centrifuged in either a 6 × 5 ml rotor (Beckman SW50.1) or a 3 × 6.5 ml rotor (MSE no. 43127-102) at 37000 rev./min for 48 h at 10°C. Each gradient was fractionated as described previously, and individual fractions were pooled to give fractions D1-D4 (Mason *et al.*, 1982). These were dialysed twice against an excess of 4 M-guanidinium chloride to remove CsCl. The densities of the fractions were as shown in Fig. 2, except where stated otherwise.

In one preparative-scale experiment, CsCl-density-gradient centrifugation of culture fractions in 4 M-guanidinium chloride was performed in an MSE 8 × 50 ml angle rotor with an initial density of 1.53 g/ml. After a centrifuging at 33000 rev./min for 48 h at 10°C in an MSE 65 Superspeed centrifuge, the gradients were removed from the tubes by pumping out from the meniscus downwards, and 2.5 ml fractions were collected. Fractions with densities 1.43-1.55 g/ml were pooled to give the D2 fraction, which was concentrated by ultrafiltration with an Amicon PM-10 membrane. The sample was then diluted with 4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, and solid Zwittergent^{TM3-12} was added to give a solution containing 4 M-guanidinium chloride and 4% (w/v) Zwittergent, density 1.43 g/ml. The solution was centrifuged in 6.5 ml tubes in an MSE titanium swing-out rotor at 37000 rev./min for 96 h at 10°C. The gradient (density range 1.31-1.52 g/ml) was fractionated by upward displacement (Mason *et al.*, 1982), and fractions with densities greater than

1.43 g/ml were pooled to give a D2-D2 fraction. This fraction was dialysed twice for 18 h against 350 vol. of 4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, before Sepharose CL-2B chromatography.

Gel-permeation chromatography

Analytical-scale Sepharose CL-2B (Pharmacia) chromatography was done with a column (0.65 cm × 150 cm) eluted with 4.0 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, at 2.8 ml/h. The eluent contained 0.1% Zwittergent for the second series of experiments. The void volume (V_0) and total volume (V_t) of the column were determined by loading a mixture of 0.6 mg of high-molecular-weight hyaluronic acid (type I, human umbilical cord; Sigma Chemical Co.) and 0.4 mg of glucuronolactone and collecting fractions (0.8 ml) that were then assayed manually for hexuronic acid (Bitter & Muir, 1962). The column was conditioned by pumping 2.5 mg of rat chondrosarcoma aA1 proteoglycan fraction (Faltz *et al.*, 1979) through it before use. Samples for analytical chromatography were loaded in 0.3 ml of 4.0 M-guanidinium chloride containing 1.0 mg of aA1 proteoglycan as carrier. Each eluent fraction was diluted (1:1, v/v) with 70% (v/v) ethanol, mixed with Aquasol (12.5 ml; New England Nuclear Corp.) and its radioactivity counted. Preparative-scale chromatography was performed with a column (2.5 cm × 100 cm) packed with Sepharose CL-2B in 4.0 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8. The column was conditioned with 25 mg of aA1 proteoglycan before use. Samples (5 ml) were loaded in 4.0 M-guanidinium chloride with 10 mg of aA1 proteoglycan as carrier. The column was eluted at 17 ml/h, and 4 ml fractions were collected; 0.2 ml of each was monitored for radioactivity.

Analysis of ³H-labelled hyaluronate

³H-labelled hyaluronate and ³H-labelled chondroitin sulphate were assayed by quantitative t.l.c. of their respective unsaturated disaccharide repeat units produced by digestion with chondroitinase ABC (Mason *et al.*, 1982). Total ³H-labelled chondroitin sulphate is expressed as the sum of the ³H radioactivities for the unsaturated disaccharides Δ di-6-S + Δ di-4-S + Δ di-0-S (Mason *et al.*, 1982).

Treatment of fractions with Streptomyces hyaluronidase

Carrier hyaluronate (final concn. 1 mg/ml; type I, Miles Biochemicals) was added to fractions before dialysis against 100 vol. of 50 mM-sodium acetate buffer, pH 5.0. Portions (0.5 ml) were then digested with *Streptomyces* hyaluronidase (Calbiochem-Behring Corp.; 20 turbidity-reducing units/digest, 60°C, 18 h) in the presence of 0.1 M-6-amino-hexanoic acid, 5 mM-benzamidine hydrochloride and

10 mM-disodium EDTA. In some experiments a preparation of proteinase-free hyaluronidase (Caputo *et al.*, 1980) was used, in which case the proteinase inhibitors were omitted. The incubation was terminated by the addition of an equal volume of 8 M-guanidinium chloride (containing 4% Zwittergent where appropriate).

Results and discussion

Association of [^3H]serine-labelled protein with the hyaluronate fraction extracted from cultures with guanidinium chloride

After labelling of chondrocyte cultures with [^3H]glucosamine for 30 min, about 30% of the ^3H -labelled hyaluronate synthesized is found in the cell-layer extract and medium (Mason *et al.*, 1982). Whether newly secreted hyaluronate is associated with protein was investigated by labelling duplicate

cultures (6×10^6 cells) for 30 min either with [^3H]glucosamine plus $\text{Na}_2^{35}\text{SO}_4$ (125 $\mu\text{Ci/ml}$ and 50 $\mu\text{Ci/ml}$ respectively) or with [^3H]serine plus $\text{Na}_2^{35}\text{SO}_4$ (125 $\mu\text{Ci/ml}$ and 50 $\mu\text{Ci/ml}$ respectively). After removal of the medium, the cell layers were extracted with 4 M-guanidinium chloride containing proteinase inhibitors, and the extracts were passed through a PD-10 column to remove unincorporated labelled precursor. ^3H -labelled hyaluronate formed 14.7% of the total ^3H -labelled glycosaminoglycan fraction in the cell-layer extract, as determined by chondroitinase ABC digestion and disaccharide analysis. The cell-layer extracts were centrifuged in dissociative density gradients, and a D2 fraction was isolated (Figs. 1a and 1c). Previous results (Mason *et al.*, 1982) indicated that 90% of the ^3H -labelled hyaluronate in the cell-layer extract bands in the D2 fraction under these conditions. The D2 fractions from both the culture labelled with [^3H]glucosamine

Fig. 1. Association of newly synthesized protein with hyaluronate

Dissociative CsCl-density-gradient centrifugations of cell-layer extracts from chondrocyte cultures labelled with (a) [^3H]serine plus [^{35}S]sulphate or (c) [^3H]glucosamine plus [^{35}S]sulphate are shown. Fractions 9–11 ($\rho = 1.40$ – 1.46 g/ml) were pooled for each gradient to give the respective D2 fractions. (b) Dissociative Sepharose CL-2B chromatography of the D2 fraction from the gradient in (a). (d) Dissociative Sepharose CL-2B chromatography of the D2 fraction from the gradient in (c). —, Density; ·····, [^{35}S]sulphate radioactivity; - - - - , [^3H]serine radioactivity; —, [^3H]glucosamine radioactivity. Percentages refer to the amount of [^3H]serine- or [^3H]glucosamine-labelled molecules of the respective D2 fractions that were eluted in the void volume of Sepharose CL-2B. For full experimental details see the text.

and that labelled with [^3H]serine were chromatographed on Sepharose CL-2B in 4 M-guanidinium chloride (Figs. 1b and 1d).

The D2 fractions of both cultures contained about 10% of the total ^{35}S radioactivity in the gradient, and the chromatographic profiles suggest that this was due to the presence of two partially separated proteoglycan species that were included within the column. In the D2 fraction from the culture labelled with [^3H]glucosamine, 59% of the ^3H -radioactivity was eluted in the void fraction of the column, clearly separated from the ^{35}S -labelled proteoglycan. Chondroitinase ABC digestion and disaccharide analysis of the void-volume fractions obtained by the same

methods from other cultures labelled with [^3H]glucosamine showed the presence almost solely of ^3H -labelled hyaluronate (see below). The D2 fraction from the culture labelled with [^3H]serine also showed a small ^3H -labelled peak eluted in the void fraction (Fig. 1b). It accounted for 2% of the total ^3H label in the D2 fraction and was clearly resolved from the proteoglycan. This suggested that a small amount of [^3H]serine-labelled protein may be associated with the hyaluronate.

Larger cultures were labelled either with [^3H]glucosamine (50 $\mu\text{Ci/ml}$) or with [^3H]serine (50 $\mu\text{Ci/ml}$) for either 6 h or 20 h, the media and cell-layer extracts were fractionated into D1–D4 fractions by

Fig. 2. Isolation of high-molecular-weight hyaluronate fraction from 4.0 M-guanidinium chloride extracts of the cell layers of chondrocyte cultures

Cultures were labelled with either [^3H]glucosamine or [^3H]serine for 6 h, and the cell-layer extracts were centrifuged in dissociative density gradients. (a) Hyaluronate distribution in the D1–D4 fractions was determined by disaccharide analysis, and [^3H]serine distributions by direct counting of radioactivity. (b) Fractions were chromatographed on Sepharose CL-2B in 4.0 M-guanidinium chloride. —, [^3H]Glucosamine radioactivity; ·····, [^3H]serine radioactivity. Percentages refer to the proportion of ^3H radioactivity in the fractions eluted in the void volume. For full experimental details see the text.

dissociative CsCl-density-gradient centrifugation and each fraction was chromatographed on Sepharose CL-2B in 4.0M-guanidinium chloride. The results for the cell-layer extracts from cultures labelled for 6 h with [^3H]glucosamine (—) or with [^3H]serine (.....) are indicated in Fig. 2(b). Analyses of gradients prepared from the media from cultures labelled for 6 h and from both the media and cell-layer extracts of the cultures labelled for 20 h gave nearly identical results (results not shown). The amounts of ^3H -labelled hyaluronate present in the D1–D4 fractions for the culture labelled with [^3H]glucosamine were measured, and the relative distribution is indicated in Fig. 2(a), along with the distribution of label for fractions from the culture labelled with [^3H]serine. About 1–2% of the ^3H label in the D2 fraction of the culture labelled with [^3H]serine was eluted in the Sepharose CL-2B void volume. The same proportion was eluted in this position when a portion of the D2 fraction was chromatographed in the absence of carrier aA1 proteoglycan (see the Experimental section). Thus aggregation of ^3H -labelled molecules present in the D2 fraction with the small amount of hyaluronate in the carrier is unlikely to be responsible for the ^3H radioactivity in the void fraction. As for the experiment in Fig. 1(d), more than 50% of the ^3H label in the D2 fraction from the culture labelled with [^3H]glucosamine was eluted in the void fraction of the column, and disaccharide analysis of this fraction indicated that more than 85% of the radioactivity was ^3H -labelled hyaluronate (results not shown). Therefore most of the ^3H -labelled hyaluronate synthesized by the chondrocytes is of high molecular weight and is eluted in the void volume of Sepharose CL-2B columns run in dissociative conditions.

The D3 and D4 fractions of cell-layer extracts contain most of the [^3H]serine-labelled molecules in the extract but little or no hyaluronate (Fig. 2a). Only 0.3% of D3-fraction and 3.8% of D4-fraction [^3H]serine radioactivity is eluted in the void fraction of Sepharose CL-2B (Fig. 2b). The D4-fraction profile, in particular, shows that the cells synthesize some proteins that are eluted in the void volume of Sepharose CL-2B, presumably as aggregates formed through protein–protein association, through protein–lipid micelle formation or by interaction with the hyaluronate in the carrier aA1 proteoglycan with which they were chromatographed.

Susceptibility of [^3H]serine-labelled molecules in the hyaluronate fraction to Streptomyces hyaluronidase

A portion of the [^3H]serine-labelled cell-layer extract from the 6 h culture, containing 1.0 mg of carrier aA1 proteoglycan, was chromatographed on a PD-10 column to remove unincorporated labelled precursor. It was then chromatographed on Seph-

arose CL-2B in 4M-guanidinium chloride, and the void-volume fraction was collected. This fraction was then centrifuged under dissociative conditions in a CsCl density gradient. Of the ^3H radioactivity in the gradient 19% was recovered in the D2 fraction (densities 1.42–1.53 g/ml). The initial concentration of hyaluronate in the gradient was approx. 2 $\mu\text{g/ml}$, or less. After the addition of 5 mg of carrier hyaluronate, the D2 fraction was dialysed exhaustively and concentrated in a dialysis bag against dry Sephadex G-200. A sample was mixed with an equal volume of 8M-guanidinium chloride and chromatographed on Sepharose CL-2B. Another was incubated overnight with proteinase-free *Streptomyces* hyaluronidase, then mixed with an equal volume of 8M-guanidinium chloride and chromatographed on the same column. The column profiles, together with those of controls, are shown in Fig. 3. Of the ^3H radioactivity of the untreated fraction 72% was eluted in the void fraction of the column. After digestion with hyaluronidase, only 23% of the ^3H radioactivity was found in the excluded fraction, the remainder being recovered mainly in fractions 40–60. A control composed of 2.5 mg of carrier hyaluronate mixed with a sample of relatively-low-molecular-weight ^3H -labelled hyaluronate isolated from fibroblast cultures (Caputo *et al.*, 1980) (Fig. 3c) was degraded completely by the hyaluronidase (Fig. 3d). The elution profile of a [^3H]serine-labelled D1 fraction of Sepharose CL-2B was virtually identical before (Fig. 3e) and after (Fig. 3f) incubation with hyaluronidase, indicating the absence of any proteinase activity. About 9% of the ^3H radioactivity of the untreated D1 fraction was eluted just ahead of the V_t of the column. This radioactivity probably represents a spill-over of the prominent ^3H -labelled component in the D2 fraction that is eluted in this area of the chromatograph (see Fig. 2b). After treatment of the D1 fraction with the hyaluronidase, about 13% of the ^3H radioactivity was recovered in this fraction. This small difference is probably within the experimental reproducibility of the column technique, although it may reflect the presence of a small amount of hyaluronate in D1 fractions (Fig. 2a) from which associated [^3H]serine-labelled molecules would move into the column after hyaluronidase treatment. The results of the experiment show that approx. 70% of the [^3H]serine-labelled molecules eluted in the V_0 fraction (Fig. 3a) are associated with high-molecular-weight hyaluronic acid and are no longer eluted in this fraction if the latter is degraded with the specific hyaluronidase (Fig. 3b).

Stability of [^3H]serine-labelled protein associated with hyaluronate fraction to further centrifugation

A portion of the D2 fraction from the cell-layer extract of the culture labelled for 6 h with [^3H]serine

Fig. 3. Effect of *Streptomyces hyaluronidase* on Sepharose CL-2B elution profile of [^3H]serine radioactivity associated with the hyaluronate fraction

Elution profile of the hyaluronate fraction isolated as described in the text from the cell layer of a culture labelled for 6 h with [^3H]serine, either direct (a) or after digestion with proteinase-free *Streptomyces hyaluronidase* (b). Elution profile of [^3H]labelled hyaluronate from [^3H]glucosamine-labelled fibroblast cultures, either direct (c) or after digestion with *Streptomyces hyaluronidase* (d). Elution profile of [^3H]serine-labelled D1 proteoglycan fraction, either direct (e) or after digestion with *Streptomyces hyaluronidase* (f). Percentages refer to the pro-

portion of [^3H]radioactivity of the whole fraction eluted in the peak indicated. For full experimental details see the text.

was chromatographed on a preparative Sepharose CL-2B column in 4 M-guanidinium chloride, and the high-molecular-weight hyaluronate fraction in the void-volume fraction was isolated (Fig. 4a). As indicated above, this fraction from a parallel culture labelled with [^3H]glucosamine contained more than 85% [^3H]labelled hyaluronate. Fractions 36–42 of the preparative column effluent (Fig. 4a) were pooled. CsCl was then added to a density of 1.42 g/ml, and the solution was centrifuged under dissociative conditions without further addition of carrier. It was estimated that the initial carrier hyaluronate concentration in the centrifuge tube (from addition of aA1 proteoglycan to the original cell-layer extract and to the preparative chromatography step) was less than 20 $\mu\text{g}/\text{ml}$. The results are shown in Fig. 4(b). Only 22% of the [^3H]radioactivity in the gradient (about 0.05% of the total in the original extract) formed a band in the density range 1.41–1.52 g/ml (fractions 7–11), even though this corresponded exactly to the original D2 fraction of the cell-layer extract and to the density range in which 90% of the hyaluronate bands under dissociative conditions (Fig. 2a). Therefore about 80% of the [^3H]labelled molecules originally associated with the high-molecular-weight hyaluronate fraction isolated from the cell-layer extracts of cultures labelled with [^3H]serine separate into gradient fractions with lower densities than that of hyaluronate after a further 48 h of dissociative centrifugation. These results and those of the hyaluronidase experiment suggest that small amounts of [^3H]labelled molecules are associated with extracellular hyaluronate but that the association is for the most part non-covalent. Therefore experiments were performed to determine whether extracellular hyaluronate synthesized by cultures labelled with [^3H]serine could be prepared entirely free of [^3H]radioactivity.

Cultures were labelled for 20 h on day 1 either with [^3H]serine plus [^{35}S]sulphate (50 $\mu\text{Ci}/\text{ml}$ and 25 $\mu\text{Ci}/\text{ml}$ respectively) or with [^3H]glucosamine plus [^{35}S]sulphate (50 $\mu\text{Ci}/\text{ml}$ and 25 $\mu\text{Ci}/\text{ml}$ respectively). The media were adjusted to dissociative conditions by adding solid guanidinium chloride and proteinase inhibitors, and then combined with their respective 4 M-guanidinium chloride extracts of the cell layers. After removal of unincorporated radioactivity by PD-10 column chromatography, CsCl was added and D2 fractions were prepared by preparative centrifugation as described in the Experimental section. The zwitterionic detergent ZwittergentTM3–12 was then added to each D2 fraction, the density of which was lowered to

portion of [^3H]radioactivity of the whole fraction eluted in the peak indicated. For full experimental details see the text.

Fig. 4. Preparation of hyaluronate fraction from cell-layer extract of culture labelled with $[^3\text{H}]$ serine for 6 h and re-centrifugation in a dissociative gradient

(a) Elution profile of the D2 fraction on a preparative Sepharose CL-2B column. The elution profile at the void volume is also shown on an enlarged scale (inset). (b) The void-volume fraction in (a) was re-centrifuged in a dissociative gradient with 22% of the radioactivity recovered in the hyaluronate fraction (hatched area). For full experimental details see the text.

1.43 g/ml before centrifugation for 96 h to obtain a D2–D2 fraction (see the Experimental section). The centrifugal conditions were designed to separate high-density proteoglycan and most of the protein from hyaluronate in the first run, and to band the remaining protein in the top of the gradient and hyaluronate in the bottom of the gradient in the second run.

The dissociative Sepharose CL-2B profile of D2–D2 fraction from the $[^3\text{H}]$ glucosamine-labelled culture shows a prominent peak, characteristic of ^3H -labelled hyaluronate, in the void-volume fraction of the column (— in Fig. 5a). In contrast, ^3H radioactivity recovered in the void-volume fraction of D2–D2 fraction from the $[^3\text{H}]$ serine-labelled

culture accounted for less than 0.3% of the 66 000 d.p.m. of ^3H radioactivity recovered from the column (— in Fig. 6b). Therefore after prolonged dissociative centrifugation in the presence of Zwittergent extracellular hyaluronate can be prepared free of ^3H radioactivity incorporated from $[^3\text{H}]$ serine.

Extraction of cell-layer macromolecules with Zwittergent and guanidinium chloride

Extraction of the cell layer with 4% Zwittergent and 4 M-guanidinium chloride completely solubilizes both intra- and extra-cellular macromolecules synthesized by the cell cultures (Kimura *et al.*, 1981). Since the experiment above shows that these

Fig. 5. Separation of ^3H radioactivity from a ^3H serine-labelled culture from hyaluronate

The D2 fractions were isolated from chondrocyte cultures labelled with either ^3H glucosamine plus ^{35}S sulphate or ^3H serine plus ^{35}S sulphate. After the addition of Zwittergent, the D2 fractions were centrifuged in a dissociative gradient, and D2–D2 fractions were isolated. Dissociative Sepharose CL-2B elution profiles are shown for D2–D2 fractions from (a) a culture labelled with ^3H glucosamine (—) and ^{35}S sulphate (.....), and from (b) a culture labelled with ^3H serine (—) and ^{35}S sulphate (.....). For full experimental details see the text.

reagents effectively dissociate all labelled proteins from extracellular hyaluronate, a further set of experiments was performed to investigate whether any non-dissociable protein could be detected in the cell-associated hyaluronate pool. High-specific-radioactivity ^3H lysine in lysine-free medium was used to label protein in these experiments to enhance the possibility of detecting synthesis of minor components of the total polypeptide pool.

Chondrocyte cultures were labelled for 1 h with ^3H lysine ($125\ \mu\text{Ci/ml}$) and ^{14}C glucosamine ($25\ \mu\text{Ci/ml}$), and the cell layer was extracted with the Zwittergent/guanidine procedure; 98% of the ^{14}C glucosamine-labelled macromolecules in the cell layer were extracted. The extract was centrifuged in a dissociative CsCl density gradient containing 4%

Zwittergent, and the D2 and D3 fractions of the gradient were obtained and chromatographed on a Sepharose CL-2B column equilibrated with 4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, containing 0.1% Zwittergent (Fig. 6). A ^{14}C -labelled peak was eluted in the void volume of the column for both D2 fraction and D3 fraction. Although 4.6×10^4 d.p.m. of ^3H radioactivity was recovered from the column for D2 fraction (Fig. 6a) and 3.3×10^5 d.p.m. of ^3H radioactivity for D3 fraction (Fig. 6b), none was eluted in the void volume. About 75% of the hyaluronate synthesized by chondrocyte cultures during a 30 min period is associated with the residue remaining after extraction of the cell layer with 4 M-guanidinium chloride, and pulse-chase experiments show that these mol-

Fig. 6. Zwittergent/guanidinium chloride extracts of cultures labelled for 1 h

Dissociative CL-2B chromatography of (a) D2 fraction and (b) D3 fraction isolated from the Zwittergent/guanidinium chloride extract of cell layers of cultures labelled for 1 h with [^3H]lysine (—) and [^{14}C]glucosamine (----). For full experimental details see the text.

Fig. 7. Zwittergent/guanidinium chloride extracts of cultures labelled for 24 h

Dissociative CL-2B chromatography of the D2 fraction isolated from extracts of parallel cultures labelled for 24 h with [^3H]glucosamine (—) and [^{35}S]sulphate (----) (a) and (b), or with [^3H]lysine (—) (c). The fraction shown in (b) was treated with hyaluronidase before chromatography: the stippled area corresponds to ^3H radioactivity due to ^3H -labelled hyaluronate tetra- and hexa-saccharides. The ^{35}S -radioactivity profile (not shown) was the same as for (a). For full experimental details see the text.

ecules are all cell-associated (Mason *et al.*, 1982). Even after a 2 h pulse about 40% of the hyaluronate synthesized is cell-associated (Mason *et al.*, 1982). Thus in the present experiment, where a 1 h pulse was used and the Zwittergent/guanidine procedure was employed to solubilize the cell layer completely, at least 50% of the ^{14}C -labelled hyaluronate in the extract would come from the cell-associated pool. Nevertheless, there is no evidence that any [^3H]lysine was incorporated into macromolecules that were bound to hyaluronate synthesized under these solvent conditions.

Mikuni-Takagaki & Toole (1981) have proposed that hyaluronate synthesis is initiated by glycosylation of a long-lived protein acceptor, bound to the endoplasmic-reticulum membrane, and that the completed glycosaminoglycan chains are released into the extracellular compartment by proteolytic action after the complex has moved to the cell surface. Thus in experiments with a short pulse period it is possible that labelled hyaluronate may represent chains elongated on a pre-existing unlabelled core protein. We have therefore examined hyaluronate synthesis

in cultures pulsed with [^3H]lysine (125 $\mu\text{Ci}/\text{ml}$) for 24 h to ensure that any putative hyaluronate-core-protein complex synthesized during this period would be labelled. Parallel cultures were labelled with [^3H]glucosamine and [^{35}S]sulphate. The Zwittergent/guanidine extraction solubilized more than 98% of the total [^3H]glucosamine-labelled macromolecules present in the cell layer after the 24 h label. The D2 and D3 fractions were isolated and chromatographed on a dissociative Sepharose CL-2B column. Fig. 7(a) shows the chromatographic profile for D2 fraction. A prominent ^3H -labelled peak in the void volume of the column was completely eliminated by prior treatment with *Streptomyces* hyaluronidase in the presence of proteinase inhibitors (Fig. 7b), showing that the peak is due to ^3H -labelled hyaluronate. Furthermore, the

increase in ^3H radioactivity eluted in fractions 46–58 of the column after hyaluronidase treatment (stippled area in Fig. 7b) exactly matched the loss of ^3H radioactivity in fractions 16–24. Since *Streptomyces* hyaluronidase specifically degrades hyaluronate to tetra- and hexa-saccharides (Ohya & Kaneko, 1970; Hatae & Makita, 1975), which would be eluted in the total column volume (V_t), this result indicates that fractions 16–24 contain all the ^3H -labelled hyaluronate present in D2 fraction. When the D2 fraction from the parallel culture labelled with [^3H]lysine was chromatographed on the same column, only background radioactivity was detected in fractions 16–24 (Fig. 7c). The D3 fractions from the cell-layer extracts gave similar profiles on dissociative chromatography (results not shown), with only background ^3H radioactivity in the void volume for the [^3H]lysine-labelled sample. Less than 20% of the total ^3H -labelled hyaluronate synthesized during a 24 h period remains in the cell layer at the end of the pulse (Mason *et al.*, 1982). That which does is all of very high molecular weight, and must represent molecules synthesized throughout the period that are specifically retained in the cell layer, together with molecules synthesized during the latter part of the pulse, but not yet lost to the medium. The results of this experiment show that no ^3H -labelled protein synthesized during the 24 h period is covalently associated with this pool of hyaluronate.

In another experiment cultures were labelled for 24 h with [^3H]lysine (125 $\mu\text{Ci/ml}$), after which the medium was replaced with one containing [^{14}C]-glucosamine (25 $\mu\text{Ci/ml}$) and incubation was continued for a further 1 h. This protocol is similar to that described by Mikuni-Takagaki & Toole (1981) to label putative hyaluronate–core-protein complex. After solubilization of the cell layer by the Zwittergent/guanidine procedure, the extract was fractionated by dissociative centrifugation in the presence of Zwittergent, and the D2, D3 and D4 fractions from the gradient were chromatographed on a Sepharose CL-2B column (Fig. 8). Fraction D2 had a ^{14}C -radioactivity peak in the void volume, but only background ^3H radioactivity was eluted in the same fractions (Fig. 8a). The ^{14}C -labelled void-volume peak was completely eliminated by prior treatment with hyaluronidase (results not shown). Fraction D3 also had a small ^{14}C -radioactivity peak in the void volume of the chromatogram, together with a very small ^3H -labelled peak in the same position. The profile was unchanged by prior dialysis against excess 50 mM-acetate buffer (Fig. 8b), but the ^{14}C -radioactivity peak in the void volume was eliminated by digesting the dialysed fraction with hyaluronidase (Fig. 8c). However, the ^3H -labelled void-volume peak was not affected by the enzyme (Fig. 8c, inset) and constituted the same proportion of total ^3H radioactivity (0.7%) in D3 fraction as

Fig. 8. Zwittergent/guanidinium chloride extracts of cultures labelled sequentially for 24 h and 1 h

Dissociative CL-2B chromatography of the D2, D3 and D4 fractions isolated from the extract of a culture labelled for 24 h with [^3H]lysine (—), followed by a 1 h chase with [^{14}C]glucosamine (---). (a) D2 fraction; (b) D3 fraction before treatment with hyaluronidase; (c) D3 fraction after treatment with hyaluronidase; (d) D4 fraction. Insets show the ^3H -radioactivity profile at the void volume on an enlarged scale. For full experimental details see the text.

was found before digestion. The ^3H -labelled void-volume peak in D3 fraction in this experiment was therefore not associated with hyaluronate. It may be due to a spill-over into D3 fraction of very large

^3H -labelled molecules normally present in the void-volume fractions of D4 fraction (Fig. 8*d*, inset; note the difference in scale). Only background ^{14}C radioactivity was present in the void volume of the D4-fraction elution profile (Fig. 3*d*), and in any case this fraction contains virtually no hyaluronate of any molecular weight, as shown by specific disaccharide analysis (Mason *et al.*, 1982).

^{14}C -labelled hyaluronate recovered in the void volume of Sepharose CL-2B columns in the last experiment represents synthesized high-molecular-weight molecules. A high proportion of these would be expected to be cell-associated after only a 1 h labelling period, as discussed above, and should still be covalently linked to ^3H -labelled hyaluronate-core-protein complex, according to the Mikuni-Takagaki & Toole (1981) hypothesis. Moreover, any ^3H -labelled hyaluronate-core protein complexes synthesized before the ^{14}C glucosamine pulse, but still associated with the cell layer after it, would also contribute to ^3H radioactivity in the void-volume fractions. At the beginning of the ^{14}C glucosamine pulse over 6×10^7 d.p.m. of ^3H radioactivity was present in the cell layer. If up to 95% of this radioactivity was extracellular, there would still be 3×10^6 d.p.m. of ^3H radioactivity incorporated into intracellular polypeptides. Thus, even if a hypothetical hyaluronate-core-protein complex accounted for as little as 0.1% of this intracellular radioactivity, it would still have 3000 d.p.m. of ^3H radioactivity associated with it, an amount that would have been readily detectable in our experiments. However, only background ^3H radioactivity was found in the void-volume fractions for D2 fraction, and the very small peaks of ^3H radioactivity in similar fractions from D3 and D4 fractions were not associated with hyaluronate.

General discussion

The chondrocyte cultures used for the present investigation are particularly suitable for the study of differences in intracellular and extracellular hyaluronate. The cells are active in hyaluronate synthesis, and previous experiments show that only extracellular hyaluronate (Mason *et al.*, 1982) and proteoglycans (Kimura *et al.*, 1980) are extracted from the cell layer with 4.0 M-guanidinium chloride. Radioautographic studies on cartilage after labelling with ^{35}S sulphate also support this proposal (Hardingham & Muir, 1972). In the first series of experiments, in which cells were labelled with ^3H serine, the results show that small amounts of ^3H -labelled molecules are specifically bound to extracellular high-molecular-weight hyaluronate and that they can be completely separated from it after dissociative centrifugation in the presence of 4%

Zwittergent. The ^3H -labelled molecules are therefore not bound covalently to the hyaluronate.

The nature of the ^3H serine-labelled molecules that are associated non-covalently with hyaluronate is unknown. After hyaluronidase treatment most of the ^3H radioactivity chromatographed just ahead of the V_i of a Sepharose CL-2B column, suggesting the presence of a low-molecular-weight protein. Lysozyme is bound to hyaluronate isolated from several sources, including cartilage, and is only gradually removed from the glycosaminoglycan by repeated dissociative centrifugation (K. E. Kuettner, personal communication). However, we have no evidence at present to suggest that lysozyme is the protein bound to hyaluronate in these experiments. The amino acid analyses of hyaluronate fractions that have been extensively purified resemble one another closely. For example, the analysis from fraction HAIIB (Swann, 1968), a hyaluronate preparation from rooster comb, is similar to that of hyaluronate isolated from bovine nasal-septum cartilage (Cleland & Sherblom, 1977), suggesting the hyaluronate from widely differing sources may be associated with a similar protein component.

In the second series of experiments, in which cultures were labelled with ^3H lysine, the cell layers were extracted with 4 M-guanidinium chloride and 4% Zwittergent, a procedure that completely solubilizes intracellular as well as extracellular macromolecules (Kimura *et al.*, 1981). This technique was combined with short-term, long-term or sequential labelling protocols, in experiments designed to identify cell-associated hyaluronate, synthesized either concurrently with labelled proteins or after prelabelling of the protein in the cells. ^3H -labelled protein was not detected in the hyaluronate fraction isolated from the cultures after any of these procedures. It seems unlikely that any putative hyaluronate-core-protein complex was cleaved from the glycosaminoglycan during the extraction and centrifugation procedures, since proteinase inhibitors were present at all stages. The results therefore provide evidence that hyaluronate chains are not synthesized on a covalently bound core protein.

Mikuni-Takagaki & Toole (1981) have reported the association of ^3H leucine-labelled molecules with purified hyaluronate isolated from the medium and 6 M-guanidinium chloride extracts of the cell layer of Rous-sarcoma-virus-transformed fibroblasts. The ^3H radioactivity co-migrated with hyaluronate during electrophoresis and, after incubation in 1% sodium dodecyl sulphate, it co-chromatographed with it on Bio-Gel A-15m columns run in 0.1% sodium dodecyl sulphate. These results were interpreted as favouring a covalent association between protein and hyaluronate and appear to be contradictory to the data reported above. It is notable, however, that oval-

bumin, caeruloplasmin, casein and mercapto-albumin associate with hyaluronate to form complexes that are stable under the conditions of Tiselius electrophoresis, showing that strong non-specific interactions can occur (Niedermeier & Gramling, 1970). The [^3H]leucine-labelled protein associated with hyaluronate in the experiments described by Mikuni-Takagaki & Toole (1981) was purified by Sephadex G-25 chromatography after degradation of the glycosaminoglycan with chondroitinase ABC. It bound to the gel and could only be eluted with buffers containing 1% sodium dodecyl sulphate, suggesting that it was hydrophobic. Our experiments show that the [^3H]serine-labelled molecules that are non-covalently bound to extracellular hyaluronate are only readily dissociated from it when both 4% Zwittergent and 4 M-guanidinium chloride are present, a finding consistent with there being strong hydrophobic interactions between the macromolecules. It may therefore be necessary to employ procedures using both these reagents to separate ^3H -labelled protein completely from fibroblast-culture hyaluronate. Alternatively, hyaluronate may be synthesized on a covalently bound protein core in the fibroblast system, or the putative core may have an unusual structure and lack lysine residues.

If, as our experiments suggest, a core protein is not involved in the biosynthesis of hyaluronate in the chondrocytes, a novel hypothesis is required to account for the mechanism of chain initiation. Intermediates involving dolichol phosphate do not appear to play a role (Appel *et al.*, 1979). A possible mechanism may therefore involve chain elongation on to a small carbohydrate precursor, although attempts to identify such a molecule have not yet been successful (Sugahara *et al.*, 1979; Appel *et al.*, 1979). A further possibility is that a non-covalent protein 'primer' may be involved in initiating hyaluronate synthesis, in a manner analogous to the role of α -lactalbumin (Ginsburg & Stadtman, 1970) in the synthesis of lactose and milk oligosaccharides.

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