The effects of cycloheximide on the biosynthesis and secretion of proteoglycans by chondrocytes in culture

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Proteoglycans synthesized by rat chondrosarcoma cells in culture are secreted into the culture medium through a pericellular matrix. The appearance of [^{35}S]sulphate in secreted proteoglycan after a 5 min pulse was rapid (half-time, $t_{1/2} < 10$ min), but that of [^3H]serine into proteoglycan measured after a 15 min pulse was much slower ($t_{1/2}$ 120 min). The incorporation of [^3H]serine into secreted protein was immediately inhibited by 1 mM-cycloheximide, but the incorporation of [^{35}S]sulphate into proteoglycans was only inhibited gradually ($t_{1/2}$ 79 min), suggesting the presence of a large intracellular pool of proteoglycan that did not carry sulphated glycosaminoglycans. Cultures were pulsed with [^3H]serine and [^{35}S]sulphate and chased for up to 6 h in the presence of 1 mM-cycloheximide. Analysis showed that cycloheximide-chased cells secreted less than 50% of the [^3H]serine in proteoglycan of control cultures and the rate of incorporation into secreted proteoglycan was decreased (from $t_{1/2}$ 120 min to $t_{1/2}$ 80 min). Under these conditions cycloheximide interfered with the flow of proteoglycan protein core along the route of intracellular synthesis leading to secretion, as well as inhibiting further protein-core synthesis. The results suggested that the newly synthesized protein core of proteoglycan passes through an intracellular pool for about 70–90 min before the chondroitin sulphate chains are synthesized on it, and it is then rapidly secreted from the cell. Proteoglycan produced by cultures incubated in the presence of cycloheximide and labelled with [^{35}S]sulphate showed an increase with time of both the average proteoglycan size and the length of the constituent chondroitin sulphate chain. However, the proportion of synthesized proteoglycans able to form stable aggregates did not alter.

Cartilage proteoglycans are complex macromolecules composed of a protein backbone to which many chondroitin sulphate and keratan sulphate chains are attached. They form multimolecular aggregates in which many proteoglycans are bound non-covalently to each hyaluronate chain. In the native aggregate a specific link protein stabilizes each proteoglycan–hyaluronate bond (Muir & Hardingham, 1975). The proteoglycan protein core consists of a globular hyaluronate-binding region that is devoid of glycosaminoglycan chains, which extends into a short region rich in keratan sulphate chains, and then further into the major chondroitin sulphate-attachment region (Heinegård, 1977).

Each preparation is very polydisperse, typically with a molecular-weight range from 1×10^6 to 4×10^6 , and the polydispersity is proposed to arise primarily from a variation in the length of the chondroitin sulphate attachment region (Hascall, 1977). This suggestion is supported by electron micrographs of proteoglycans spread in a monolayer, which show a Gaussian distribution of protein core lengths (Kimura *et al.*, 1978).

The average molecule contains 100 chondroitin sulphate chains and 50 keratan sulphate chains (Hascall, 1977), but also contains a large number of short non-sulphated oligosaccharides of the *O*-glycosidically linked and *N*-glycosylamine-linked types (De Luca *et al.*, 1980; Lohmander *et al.*, 1980). The average composition of a proteoglycan is thus 90% sulphated glycosaminoglycans, 2% oligosaccharides and 8% protein.

The biosynthesis of proteoglycans appears to follow the same general principles as that of other secretory glycoproteins, but no details of protein-core synthesis in chondrocytes have yet been reported. However, it is evident that in post-translational modifications the protein core has attached to it more than 10 times its weight of carbohydrate. The origin of the variation in length of the protein core and the control of the large structural variation observed in proteoglycans from different cartilaginous tissues has yet to be determined.

Proteoglycans from the Swarm rat chondrosarcoma are of high molecular weight, characteristic of cartilage. All their properties are similar to

those from hyaline cartilage except that they contain no fully developed keratan sulphate chains (Oegema *et al.*, 1975). In its place they contain *O*-glycosidically linked oligosaccharides that may represent the linkage region of keratan sulphate (Lohmander *et al.*, 1980). Cells isolated from the chondrosarcoma continue to synthesize and secrete proteoglycans when maintained in culture, and these were shown to aggregate extracellularly in the medium of the cultures (Kimura *et al.*, 1979).

In the present experiments we examined the effects of inhibiting protein synthesis on the rate of synthesis and secretion of proteoglycans in cultures of chondrosarcoma cells, and also the effects that this had on the structure of the completed and secreted molecule.

Materials and methods

Materials

Collagenase (CLS II) (EC 3.4.24.3) was obtained from Worthington [Millipore (U.K.) Ltd., London N.W.10, U.K.]. Papain (EC 3.4.22.2) (twice crystallized) was obtained from BDH Chemicals (Poole, Dorset, U.K.). Trypsin (EC 3.4.21.4), 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, disodium EDTA, *N*-ethylmaleimide and cycloheximide were obtained from Sigma (Poole, Dorset, U.K.). Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid) and Bes {2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid} were obtained from Hopkin and Williams (Romford, Essex, U.K.). Dulbecco's modification of Eagle's medium, Hank's medium, foetal calf serum, gentamycin and kanamycin were from Flow Laboratories (Irvine, Scotland, U.K.). Insulin (B.P.; 80 units/ml) was from Wellcome (Beckenham, Kent, U.K.). Benzamidinium hydrochloride was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). *p*-Nitrophenyl β -D-xylopyranoside was from Koch-Light (Colnbrook, Bucks., U.K.). Sepharose, Sephadex and Sephacel gels were from Pharmacia (Uppsala, Sweden). [35 S]Sulphate (carrier-free) and L-[3- 3 H]serine (11 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Lumagel was from Lumac Systems, Basle, Switzerland.

All reagents were analytical-reagent grade, except carbazole, glucuronolactone and guanidine hydrochloride, which was purified with activated charcoal and Celite (Hopkin and Williams).

Assay methods

Uronic acid was determined by an automated procedure (Heinegård, 1973) of the modified (Bitter & Muir, 1962) carbazole reaction, with glucuronolactone as a standard.

Radioactivity was monitored by mixing samples with water and adding Lumagel; d.p.m. were measured on a Searle Mark III liquid-scintillation spectrometer with automatic quench correction.

Preparation of primary monolayer cultures of Swarm rat chondrosarcoma chondrocytes

Cells were isolated by trypsin and collagenase digestion as described by Kimura *et al.* (1979). They were plated at a density of 1.5×10^6 cells per 35 mm culture plate in Dulbecco's modified Eagle's medium containing 15 mM-Hepes, 10 mM-Bes, 10 mM-Tes and 20% foetal calf serum. After 24 h, the medium was changed to a modified form, containing 2% foetal calf serum, insulin (0.016 unit/ml) and kanamycin (10 μ g/ml). The cells were maintained in this standard medium with daily changes.

To follow the incorporation of [35 S]sulphate into proteoglycan, a 5 min pulse was found to give sufficient incorporation, except in following the inhibition of synthesis, when a longer pulse (10 min) was used. With [3 H]serine at least 10 min incorporation was necessary to follow total protein synthesis, and when the incorporation was to be measured into isolated proteoglycan or chondroitin sulphate peptides, longer incorporation times were necessary (15 min and 20 min respectively). In each case the time of incorporation was reasonably short compared with the rate of change in the synthesis and secretion processes being examined.

Incorporation of [35 S]sulphate into culture fractions

Duplicate plates were pulsed for 5 min with 1 ml of fresh sulphate-free medium containing 50 μ Ci of [35 S]sulphate. They were washed and chased with 1 ml of medium containing 1 mM non-radioactive sulphate. At the end of incubation, the medium was removed and frozen and 1 ml of 4 M-guanidine hydrochloride added to the plates at 4°C for 1 h to extract a pericellular fraction. A residue fraction was then isolated by digesting the culture plates with 25 μ g of papain in 1 ml of solution containing 50 mM- Na_2EDTA , 0.1 M-sodium acetate and 10 mM-cysteine hydrochloride, pH 6.1, at 60°C overnight. Each fraction was adjusted to 4 M-guanidine hydrochloride in a total volume of 2 ml and chromatographed on a Sephadex G50 column (0.8 cm $\times$ 24 cm) eluted with 4 M-guanidine hydrochloride (pH 6–7) to separate unincorporated radioactivity. The elution of radioactivity was monitored and the void-volume fractions were then pooled.

Effect of 1 mM-cycloheximide on the secretion of [35 S]sulphate- and [3 H]serine-labelled macromolecules

Duplicate plates were incubated with and without 1 mM-cycloheximide in 1 ml of standard medium for

various times (0–240 min). A 10 min 'pulse' of 20 μ Ci of [35 S]sulphate and 50 μ Ci of [3 H]serine was then given. The plates were washed and chased in 1 ml of the appropriate medium, with and without cycloheximide, for a further 60 min. The incubations were terminated by adding 1 ml of 8 M-guanidine hydrochloride containing proteinase inhibitors (0.2 M-6-aminohexanoic acid, 20 mM- Na_2EDTA , 20 mM-benzamidine hydrochloride and 2 mM-phenylmethanesulphonyl fluoride, pH 5.8) and the cultures were extracted at 4°C for 1 h. This gave a combined medium and extract fraction which was then run on Sephadex G50 columns as described above, but in 4 M-guanidine hydrochloride containing half the concentration of the proteinase inhibitors.

Rate of appearance of [3 H]serine and [35 S]sulphate in proteoglycan

Triplicate plates were pulsed for 15 min with 20 μ Ci of [35 S]sulphate and 40 μ Ci of [3 H]serine in 1 ml of medium free of non-radioactive sulphate and serine. The plates were washed and chased in 1 ml of medium containing non-radioactive sulphate and serine for periods up to 6 h with and without cycloheximide; then 1 ml of 8 M-guanidine hydrochloride with proteinase inhibitors was added to the plates and a medium and extract fraction isolated as described above. The void-volume fractions containing proteoglycans from Sephadex G50 columns were then run on a direct dissociative density gradient in 4 M-guanidine hydrochloride with proteinase inhibitors as above, with the starting density adjusted to 1.5 g/ml with CsCl. The samples were centrifuged in a MSE 65 instrument with an angle rotor (8 $\times$ 25 ml) at 34 000 rev./min for 48 h at 10°C. The tubes were frozen in a solid- CO_2 /acetone bath and cut into four equal fractions. The most dense fraction contained over 90% of the ^{35}S radioactivity in the gradient. It was assayed for [35 S]sulphate and [3 H]serine and digested with papain as described above. Samples were run on a Sepharose 6B column (120 cm $\times$ 0.9 cm) eluted with 0.5 M-sodium acetate, pH 6.8, at 4°C. The K_d of the peak elution fraction of included material was calculated from the following formula: $K_d = (V_e - V_0)/(V_t - V_0)$, where V_e is the peak elution volume, V_0 is the excluded volume and V_t is the included volume. V_0 and V_t were determined with aggregated proteoglycan and $^3\text{H}_2\text{O}$ respectively.

Hydrodynamic size of proteoglycans and their ability to aggregate

Cells were incubated in 1 ml of standard medium with and without cycloheximide for various times (0–360 min), and [35 S]sulphate (50 μ Ci) was added for the last 30 or 60 min of culture. Samples of the

medium were taken and mixed with 2.0 mg of non-radioactive proteoglycan monomer for 4 h at 4°C. [The non-radioactive proteoglycan monomer was isolated by the method of Oegema *et al.* (1975) from a rat chondrosarcoma.] They were then chromatographed on a Sepharose 2B column (170 cm $\times$ 1.1 cm) in 0.5 M-sodium acetate, pH 6.8, at 4°C at a flow rate of 7 ml/h. The remaining plates were extracted with 1 ml of 4 M-guanidine hydrochloride and proteinase inhibitors, and samples of the extracts were chromatographed on a Sepharose CL-2B column (120 cm $\times$ 1.0 cm) eluted with 4 M-guanidine hydrochloride.

To determine the size of chondroitin sulphate chains, samples of combined medium and 4 M-guanidine hydrochloride fractions were dialysed against water, freeze-dried and digested with papain as described above.

Rate of appearance of [3 H]serine in the chondroitin sulphate region of the proteoglycan

Duplicate cultures were incubated with 100 μ Ci of [3 H]serine and 20 μ Ci of [35 S]sulphate in 1 ml of standard medium for 20 min. They were washed and chased for up to 6 h as previously described. To stop incorporation, the cells with medium were frozen on solid CO_2 and then digested with papain (24.5 μ g) added in 0.4 ml of 0.4 M- KH_2PO_4 /20 mM- Na_2EDTA containing 2.5 mM-cysteine; all other conditions were as described above. The digest was passed through glass wool, and the eluate placed on 'automated' DE52 DEAE-cellulose columns (7 cm $\times$ 1 cm) (Handley & Lowther, 1976). First 100 ml of 0.2 M-NaCl/50 mM-Tris/HCl, pH 7.2, was pumped through to remove free [35 S]sulphate, and then glycosaminoglycans were eluted with 15 ml of 2 M-NaCl/50 mM-Tris/HCl, pH 7.2. The material was dialysed against water and put on a smaller column (3.0 cm $\times$ 1.5 cm) of DEAE-Sepacel. The column was eluted with 12 ml of 0.5 M-NaCl and 12 ml of 2 M-NaCl, both containing 50 mM-Tris/HCl, pH 7.2. The fractions were monitored for [35 S]sulphate and [3 H]serine.

Results

Synthesis and secretion of ^{35}S -labelled proteoglycans

The incorporation of [35 S]sulphate by the cells in culture and the secretion of ^{35}S -labelled macromolecules into the medium was followed in pulse-chase experiments (Fig. 1). The changing distribution of incorporated radioactivity between the three culture fractions showed a rapid flow from the cell residue through the 4 M-guanidine hydrochloride extract and into the medium (Kimura *et al.*, 1979); 75% of the radioactivity moved out of the cell fraction in the first 10 min of non-radioactive chase, and after 60 min of chase incubation 55–60% of the

Fig. 1. Secretion of [^{35}S]sulphate-labelled macromolecules

Duplicate cultures were pulsed with $50\mu\text{Ci}$ of [^{35}S]sulphate for 5 min and chased in non-radioactive medium for various times up to 60 min. The appearance of radioactivity in macromolecules excluded from Sephadex G50 columns was monitored as described in the text. At each time the distribution of radioactivity is shown in the papain-digested cell residue (●), 4 M-guanidine hydrochloride extract (○) and culture medium (□).

radioactivity was secreted into the medium (Fig. 1). Newly sulphated proteoglycans were thus rapidly secreted by the cells.

The synthesis of glycosaminoglycan chains and their sulphation depends on the supply of proteoglycan protein core as an acceptor for chain synthesis.

The effects of inhibiting protein synthesis on the incorporation of [^{35}S]sulphate were thus examined.

Effect of cycloheximide on the incorporation of [^{35}S]sulphate and of [^3H]serine by chondrocytes

Cultures were incubated with and without 1 mM-cycloheximide for various times before pulse labelling for 10 min with [^3H]serine and [^{35}S]sulphate. The incorporation of [^3H]serine into macromolecules was immediately inhibited; after 20–30 min the incorporation was only 5% of the control and remained at that value even with longer preincubation (Fig. 2). The incorporation of [^{35}S]sulphate

Fig. 2. Effect of cycloheximide on the secretion of [^{35}S]sulphate- and [^3H]serine-labelled macromolecules

Chondrocyte cultures were incubated with or without 1 mM-cycloheximide for 0–240 min. A pulse of $20\mu\text{Ci}$ of [^{35}S]sulphate and $50\mu\text{Ci}$ of [^3H]serine was then given for 10 min, and the cultures were incubated in non-radioactive chase medium for a further 60 min. Cycloheximide was included in one set of cultures throughout the preincubation, pulse and chase incubations. A combined medium and extract fraction was prepared from the cultures as described in the Materials and methods section. (a) [^3H]Serine and (b) [^{35}S]sulphate incorporated into macromolecules of control (●) and 1 mM-cycloheximide-treated (○) cultures. The results of [^{35}S]sulphate incorporation in cycloheximide-treated cultures were fitted to a first-order exponential decay (correlation coefficient -0.994). This suggested the approximate $t_{1/2}$ of this process to be 79 min.

also became inhibited in the presence of cycloheximide, but whereas with protein synthesis the effect was immediate, the rate of [^{35}S]sulphate incorporation declined only slowly and fitted a

Fig. 3. Rate of appearance of $[^3\text{H}]$ serine and $[^{35}\text{S}]$ sulphate in secreted proteoglycan isolated from a direct dissociative gradient

Triplicate cultures were pulsed for 15 min with $40\mu\text{Ci}$ of $[^3\text{H}]$ serine and $20\mu\text{Ci}$ of $[^{35}\text{S}]$ sulphate, and chased with or without 1 mM-cycloheximide for up to 360 min. The appearance of radioactivity in secreted proteoglycan from a combined medium and extract fraction isolated from the bottom of a direct dissociative gradient was measured as described in the Materials and methods section. $[^{35}\text{S}]$ Sulphate in secreted proteoglycan in control (■) and cycloheximide-treated cultures (●); $[^3\text{H}]$ serine in secreted proteoglycan in control (□) and cycloheximide-treated cultures (○). These results of $[^3\text{H}]$ serine incorporation were fitted to first-order rate curves with $t_{1/2}$ 120 min (correlation coefficient 0.960) for control cultures, and $t_{1/2}$ 80 min (correlation coefficient 0.959) for cycloheximide-treated cultures.

first-order exponential decay with $t_{1/2}$ 79 min (Fig. 2b). This suggested that there was a large intracellular pool of newly synthesized proteoglycan that was only slowly depleted when proteoglycan protein-core synthesis was inhibited.

Rate of secretion of newly synthesized proteoglycan in control and cycloheximide-treated cultures

In order to obtain further evidence for the preformed proteoglycan pool, the rate of appearance of $[^3\text{H}]$ serine in secreted proteoglycans was measured. Cultures were pulse-labelled with $[^3\text{H}]$ serine and $[^{35}\text{S}]$ sulphate for 15 min and then chased for various times. Proteoglycans were isolated from a direct dissociative CsCl gradient, and after dialysis 86% of the $[^{35}\text{S}]$ sulphate incorporated and 9% of the $[^3\text{H}]$ serine incorporated were in the proteoglycan fraction (bottom 25% of the gradient). The rate of appearance of $[^{35}\text{S}]$ sulphate in secreted proteoglycans was rapid ($t_{1/2} < 10$ min), whereas the appearance of $[^3\text{H}]$ serine was much slower ($t_{1/2}$ 120 min)

(Fig. 3). This suggested that the average time taken for the newly synthesized proteoglycan to be completely glycosylated and its glycosaminoglycan chains to be sulphated was more than 100 min. As it was possible that the incorporation of $[^3\text{H}]$ serine may have continued into the chase period, owing to the presence of significant intracellular $[^3\text{H}]$ serine pools (such as $[^3\text{H}]$ seryl-tRNA), similar experiments were also carried out with the chase incubations containing cycloheximide to prevent further protein synthesis. The results showed a shorter delay ($t_{1/2}$ 80 min) in the appearance of $[^3\text{H}]$ serine in secreted proteoglycan, but the total amount of $[^3\text{H}]$ serine appearing in proteoglycan was only 30% of that in control cultures. This decrease was more than expected from limiting further protein synthesis, and it may have been caused by secondary effects of cycloheximide on the flow of nascent proteoglycan protein core to the sites of post-translational modification (Jamieson & Palade, 1968), or to an effect on secretion. These experiments were carried out on proteoglycans that were secreted by the cells and were present in the medium and in the 4M-guanidine hydrochloride-extractable fraction (Kimura *et al.*, 1979).

Rate of incorporation of $[^3\text{H}]$ serine into completed intracellular proteoglycan

In order to measure the time taken for newly synthesized protein to be formed into a proteoglycan, the appearance of $[^3\text{H}]$ serine attached to chondroitin sulphate isolated from a combined cell and medium fraction was determined. Chondroitin sulphate is attached to the protein backbone of the proteoglycan at serine residues (Muir & Hardingham, 1975), and papain digestion leaves a small peptide of serine and some adjacent amino acids attached to the chondroitin sulphate linkage region. Isolation of chondroitin sulphate by DEAE-cellulose chromatography after papain digestion of the cells and culture medium therefore permitted the determination of the appearance of $[^3\text{H}]$ serine in the chondroitin sulphate attachment region of proteoglycans, including those that were still at intracellular stages of synthesis.

Cultures were pulsed for 20 min with $[^3\text{H}]$ serine and $[^{35}\text{S}]$ sulphate. The rate of incorporation of $[^3\text{H}]$ serine into chondroitin sulphate peptide when fitted to a first-order exponential was of $t_{1/2}$ 96 min (Fig. 4). This was thus the average time taken for the proteoglycan protein core to have chondroitin sulphate attached to it intracellularly. As expected, it was somewhat shorter than the estimated average time for the completed proteoglycan to be secreted (120 min), but suggested that the chondroitin sulphate chains were synthesized only shortly before secretion rather than at an early stage of protein-core synthesis.

Fig. 4. *Appearance of radioactivity in the chondroitin sulphate region of the proteoglycan protein core*
Cell cultures were pulsed with $100\mu\text{Ci}$ of $[^3\text{H}]$ serine and $20\mu\text{Ci}$ of $[^{35}\text{S}]$ sulphate for 20 min. They were incubated with or without cycloheximide for various times up to 360 min, and the cells together with medium were digested with papain and the chondroitin sulphate chains isolated on an ion-exchange column as described in the Materials and methods section. Each experimental point is the average of duplicate cultures. $[^{35}\text{S}]$ Sulphate in control (■) and cycloheximide-treated (●) cultures; $[^3\text{H}]$ serine in control (□) and cycloheximide-treated (○) cultures. The results of $[^3\text{H}]$ serine incorporation in control cultures was fitted to a first-order rate curve of $t_{1/2}$ 96 min (correlation coefficient 0.981).

In the cultures chased with cycloheximide, the total $[^3\text{H}]$ serine incorporated into chondroitin sulphate peptide was only 50% of the control incorporation. The kinetics of the appearance were also different, as the initial rate was close to the control rate, but after 30 min the rate rapidly decreased. This again showed evidence of interference with the intracellular transport of proteoglycans, and those proteoglycans soon to have glycosaminoglycan chains attached were possibly less affected than those at earlier stages of synthesis.

Effect of cycloheximide on the structure of secreted proteoglycan

After the addition of cycloheximide to cultures, the results showed a steadily decreasing rate of chondroitin sulphate synthesis and secretion. The effect of this on the structure of the proteoglycan was investigated. Proteoglycans labelled with $[^{35}\text{S}]$ -sulphate at various times after the addition of cycloheximide were extracted and chromatographed on Sepharose CL-2B in 4 M-guanidine hydrochloride. The average size of the proteoglycan was found to increase as the rate of synthesis decreased (Fig. 5a), as over 6 h the K_d of elution

Table 1. $[^3\text{H}]$ serine incorporation into the chondroitin sulphate region of the proteoglycan protein core
 $[^{35}\text{S}]$ sulphate and $[^3\text{H}]$ serine-labelled proteoglycan monomer (as in Fig. 3) was treated with papain and chromatographed on a Sepharose 6B column as in the Materials and methods section. The proportion of the total $[^3\text{H}]$ serine eluted with chondroitin $[^{35}\text{S}]$ -sulphate chains was determined.

Time (min)	Percentage of $[^3\text{H}]$ serine in chondroitin sulphur peptides	
	Control	Cycloheximide-treated
30	42.9	38.5
90	42.5	38.8
180	44.2	45.3
360	41.5	47.1

decreased from 0.24 to 0.12 (Fig. 5b). After papain digestion of the proteoglycans, the size of the chondroitin sulphate chains measured by elution from a column of Sepharose 6B also increased as the rate of synthesis decreased (Fig. 5c). The K_d decreased from 0.52 to 0.35 over 6 h (Fig. 5d). The increase in proteoglycan monomer size was thus at least in part due to an increase in the length of the chondroitin sulphate chains attached to the proteoglycan.

Further evidence of proteoglycan structure was obtained by determining the proportion of $[^3\text{H}]$ -serine incorporated into proteoglycan that was localized in isolated chondroitin sulphate peptides after papain digestion. There was no detectable change during the chase period in control cultures, showing that the appearance of radioactivity in the chondroitin sulphate region of the proteoglycan and in the other regions followed similar kinetics. In the cycloheximide-chased cultures there was a very similar proportion of $[^3\text{H}]$ serine in chondroitin sulphate peptides, and there was only a small change as the rate of synthesis decreased, suggesting that the number of serine residues attached to chondroitin sulphate chains did not greatly alter (Table 1).

Effect of cycloheximide treatment on the aggregation of proteoglycans

Proteoglycans secreted by the cells into the medium have been shown to form aggregates in which many proteoglycans are bound to a hyaluronate chain stabilized with a link protein (Kimura *et al.*, 1980). Chromatography of samples of media from cultures incubated with $[^{35}\text{S}]$ sulphate on Sepharose 2B (Fig. 6) in the presence of excess non-radioactive proteoglycan monomer showed that more than 85% of proteoglycan formed into link-stabilized aggregates in both control and cyclo-

Fig. 5. Gel chromatography of proteoglycan monomers and chondroitin sulphate chains from cycloheximide-treated and control cultures

Cells were incubated with or without cycloheximide for 0–360 min and $50 \mu\text{Ci}$ of $[^{35}\text{S}]\text{sulphate}$ was added for the last 30 or 60 min of incubation: (a) 0–30 min, (b) 30–60 min, (c) 60–90 min, (d) 90–120 min, (e) 120–180 min, (f) 180–240 min, (g) 240–360 min. Samples of combined medium and 4 M-guanidine hydrochloride extract fractions from the cultures were chromatographed on Sepharose CL-2B columns in 4 M-guanidine hydrochloride as described in the text (V_0 , fraction 25; V_t , fraction 70). (a) The elution profiles of samples from cycloheximide-treated cultures (a), (b), (c), (e) and (g). (b) The change in the elution mode of samples from cycloheximide-treated cultures (a)–(g) (O) and control cultures (a), (c), (e) and (g) (■). Samples of combined medium and 4 M-guanidine hydrochloride extract fractions were digested with papain as described in the text. The free chondroitin sulphate chains were then chromatographed on Sepharose 6B as described in the text (V_0 , fraction 28; V_t , fraction 74). (c) The elution profiles of samples from cycloheximide-treated cultures (a), (c), (e) and (g). (d) The change in the elution mode of samples from cycloheximide treated cultures (a)–(g) (O), and control cultures (a), (c), (e) and (g) (■).

heximide-inhibited cultures. The proteoglycans completed during the decrease in synthesis thus contained intact hyaluronate-binding regions and were as fully competent to aggregate as were control proteoglycans.

Discussion

The addition of cycloheximide to cultures of chondrocytes leads to the complete inhibition of $[^{35}\text{S}]\text{sulphate}$ incorporation into proteoglycan. This is consistent with a requirement for protein syn-

Fig. 6. Gel chromatography on Sepharose 2B of proteoglycans in the culture medium

[^{35}S]Sulphate ($50\mu\text{Ci}$) was added to cultures between 240 and 360 min of incubations as described in the Materials and methods section: (a) control culture; (b) cycloheximide-treated culture. Hatched areas indicate aggregated proteoglycan. The peak fraction of monomer proteoglycan is shown with an arrow.

thesis to form the protein core, which is the substrate for the enzymes involved in glycosaminoglycan synthesis. The decrease in [^{35}S]sulphate incorporation was very slow ($t_{\frac{1}{2}}$ 79 min), suggesting that there was only a slow fall in the supply of core protein. This was comparable with the time taken for [^3H]serine-labelled protein core to reach the stage of sulphation of the glycosaminoglycan chains ($t_{\frac{1}{2}}$ 96 min). Both of the results suggested that there is a large pool of proteoglycan protein core that is intracellular and awaits the synthesis of its chondroitin sulphate. It is also clear that after sulphation the proteoglycan is quickly secreted by the cell ($t_{\frac{1}{2}} < 10$ min). The rapid secretion of newly synthesized chondroitin sulphate has also been observed with chick chondrocytes in culture (Kim & Conrad, 1980).

The possibility that decreased [^{35}S]sulphate incorporation in the presence of cycloheximide resulted from a fall in the activity of the enzymes involved in glycosaminoglycan-chain synthesis was ruled out by measuring the rate of [^{35}S]sulphate incorporation in the presence of 1 mM *p*-nitrophenyl β -D-xyloside (Robinson *et al.*, 1975). This is an initiator of protein-free chondroitin sulphate chain synthesis and stimulates the rate of [^{35}S]sulphate incorporation. In the presence of xyloside, even after 5 h with cycloheximide, the rate of [^{35}S]sulphate incorporation into glycosaminoglycans was well above the rates for inhibited cultures without xyloside (D. C. Mitchell & T. E. Hardingham, unpublished work). The capacity of the cells to synthesize chondroitin sulphate was thus not greatly suppressed by cycloheximide within several hours.

The rate of secretion of [^3H]serine-labelled proteoglycan, when analysed as a first-order process, was of $t_{\frac{1}{2}}$ 120 min in control cultures and $t_{\frac{1}{2}}$ 80 min in cycloheximide-inhibited cultures. The accuracy of this timing in the control cultures will be limited by the continued incorporation of [^3H]serine after the pulse period, owing to the precursor [^3H]serine pools. This estimate of the time for secretion is therefore an upper limit in control cultures, but in cycloheximide-treated cultures the proportion of labelled proteoglycan secreted was less, suggesting that there was some inhibition of intracellular transport of the proteoglycan protein core. The time of the measured rate of secretion in cycloheximide-treated cultures is probably therefore a minimum estimate.

The rate of proteoglycan secretion ($t_{\frac{1}{2}}$ 80–120 min) appears to be much slower than the average time for procollagen secretion. Kao *et al.* (1977) showed that 70–80% of type I procollagen was secreted as a pseudo-first-order process, with $t_{\frac{1}{2}}$ 14 min, by chick-embryo tendon cells. The results of Harwood *et al.* (1976) also suggested that the secretion of type II procollagen by chick chondro-

cytes was relatively fast. This may be related to the large number of post-translational modifications that are carried out on proteoglycans. The estimate of the average molecular weight of proteoglycan protein core is about 200 000 (Hascall, 1977). The results of cell-free translation of mRNA from chick chondrocytes suggest that the nascent proteoglycan protein core has mol.wt. 340 000 (Upholt *et al.*, 1979). The nascent protein may contain signal consequences that are likely to be cleaved intracellularly (Davis & Tai, 1980), but these are generally of low molecular weight (1000–3000). If the core protein synthesized in chondrocytes is of 340 000 mol.wt., there must be mechanisms that decrease its size and produce the variability in the length of the protein core during the intracellular processing. The constancy of the proportion of [³H]serine of completed proteoglycan occurring in the chondroitin sulphate peptides at different times after synthesis (Table 1) suggests that the core is synthesized as a single polypeptide rather than as separate units (Hardingham *et al.*, 1976).

The proteoglycans that were sulphated and secreted by the chondrocytes after cycloheximide treatment formed link-stabilized aggregates in the medium as competently as did those from control cultures. This showed that cycloheximide treatment did not lead to the formation of incomplete molecules or molecules with inactive binding regions. However, examination of the proteoglycan monomers showed their size to increase as the rate of synthesis decreased, and this was related to increased length of the constituent chondroitin sulphate chains. Previous observations have shown that the length of chondroitin sulphate chains can be inversely correlated with their rate of synthesis. Thus β -D-xylosides that stimulate chain synthesis also decrease their length (Robinson *et al.*, 1975), whereas lowering the temperature has been shown to increase the length (Hardingham & Muir, 1970). The size of proteoglycan monomers can thus be influenced by the rate of synthesis of proteoglycan protein core and the activity of the enzymes involved in chain synthesis.

During the preparation of this work further independent evidence of a large intracellular pool of proteoglycan in chondrocytes has been obtained by Kimura *et al.* (1981).

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