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The control of chondroitin sulphate biosynthesis and its influence on the structure of cartilage proteoglycans

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Chondroitin sulphate synthesis on proteoglycans was decreased in rat chondrosarcoma cell cultures in the presence of cycloheximide (0.1–1.0 μM) or *p*-nitrophenyl β-D-xyloside (50 μM). In the presence of cycloheximide the proteoglycan monomer was of larger size, the chondroitin sulphate chains were increased in length, but a similar number of chains was attached to each proteoglycan and the size of the core protein was unaltered. In the presence of *p*-nitrophenyl β-D-xyloside (50 μM), chondroitin sulphate synthesis was increased (by 60–80%), but the incorporation into proteoglycans was decreased (by 70%). The chondroitin sulphate chains were of shorter length than in control cultures and the number of chains attached to each proteoglycan was decreased. In cultures with cycloheximide or actinomycin D the synthesis of chondroitin sulphate was less inhibited on β-xyloside than on endogenous proteoglycan. When the rate of chondroitin sulphate synthesis was decreased by lowering the temperature of cultures, the chains synthesized at 22 and 4°C were much longer than at 37°C, but in the presence of *p*-nitrophenyl β-D-xyloside the chains were of the same length at all three temperatures. A model of chain elongation is thus proposed in which the rate of chain synthesis is determined by the concentration of xylosyl acceptor and the length of the chains is determined by the ratio of elongation activity to xylosyl-acceptor concentration.

Cartilage proteoglycan consists of a protein core (mol.wt. 2×10^5–3×10^5) to which is added up to 100 chondroitin sulphate chains (mol.wt. 2×10^4) and a variable number of keratan sulphate chains during intracellular post-translational synthesis (see Muir & Hardingham, 1975). All preparations of cartilage proteoglycan are polydisperse and this results from variation in the number and length of chondroitin sulphate chains and keratan sulphate chains and also possible variation in the length of the protein core (Hascall, 1977; Hardingham, 1981). The average size and composition of preparations vary from cartilage of different age and from different anatomical sites and species and it remains to be determined how the fine structure is controlled and how it is related to the properties of the tissue in which it is found.

Swarm rat chondrosarcoma contains proteoglycans that are similar to those of cartilage but contain no keratan sulphate, and chondrocytes isolated from the tissue continue to synthesize these proteoglycans in culture (Kimura *et al.*, 1979). Previous experiments showed that, after synthesis, the protein core of the proteoglycan remained within these chondrocytes for 60–90 min before secretion (Mitchell & Hardingham, 1981; Kimura *et al.*, 1981), and it was only within 10 min of secretion that the chondroitin sulphate chains were synthesized on the protein core.

Chondroitin sulphate contains a neutral trisaccharide of xylose and two galactose residues at the reducing end and is attached to the protein core at a xylosye–serine linkage. The main part of the chain consists of repeating disaccharide units of glucuronate and 4- or 6-sulphated *N*-acetylgalactosamine. The entire chain appears to be synthesized by the successive addition of single sugar residues and requires six glycosyltransferases and one or two sulphotransferases (Rodén & Schwartz, 1975). The length of the chains is not uniform but forms an approximately Gaussian distribution about a mean that varies with source from 10000 to 60000 mol.wt.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Bes, 2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

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(20–120 disaccharide units). Previous studies showed that, in the presence of β-D-xylosides, total chondroitin sulphate synthesis was stimulated (Schwartz *et al.*, 1974; Robinson *et al.*, 1975) as the xyloside was an acceptor for the first galactosyltransferase (Schwartz, 1977) and chondroitin sulphate chains were thus synthesized on it. The average size of the chondroitin sulphate chains was at the same time reduced. Proteoglycan protein core synthesis was uninhibited in chick chondrocytes in the presence of β-xylosides (Schwartz, 1977), although fewer, shorter, chondroitin sulphate chains were attached to the proteoglycans (Schwartz, 1977; Lohmander *et al.*, 1979*a*). It was also found that when protein synthesis was partially inhibited in chick chondrocytes (Kato *et al.*, 1978) or completely inhibited in rat chondrosarcoma cells (Mitchell & Hardingham, 1981; Kimura *et al.*, 1981), chondroitin sulphate synthesis was gradually inhibited and the size of proteoglycans and the length of their chondroitin sulphate chains then increased.

In the present study the effects of partial inhibition of protein synthesis and the presence of *p*-nitrophenyl β-D-xyloside on the structure of newly synthesized proteoglycan were examined. In view of the slow turnover of intracellular protein core, several hours' exposure to the agents was given in order to give sufficient time to approach a new steady state of synthesis.

Materials and methods

Materials

The sources of material were as previously published (Mitchell & Hardingham, 1981). In addition, chondroitinase ABC was obtained from Worthington [Millipore (U.K.) Ltd., London NW10, U.K.]. [5,6-^{3}H]Uridine (45 Ci/mmol) and NaB^3H_4 (422 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Cellulose thin-layer plates were obtained from Merck (Darmstadt, Germany) and standard chondroitin sulphate disaccharides from Miles Laboratories Ltd. (Stoke Poges, Slough, Berks., U.K.).

Assay methods

Hexuronate and protein were determined by automated procedures (Heinegård, 1973) with glucuronolactone and bovine serum albumin respectively as standards. Radioactivity was measured with a Searle Mark-III liquid-scintillation spectrometer, as previously described (Mitchell & Hardingham, 1981).

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-diameter culture plate in 2 ml of Dulbecco's modified Eagle's medium containing 15 mM-Hepes, 10 mM-Bes, 10 mM-Tes and 20% (v/v) foetal-calf serum. After 24 h the medium was changed to 2 ml of a modified form containing 2% foetal-calf serum, insulin (0.016 unit/ml) and Kanamycin (10 μg/ml). The modified medium was changed daily and experiments were performed after 2 or 3 days in culture with duplicate plates for each measurement. Radioactive incubations up to 10 h were in 1 ml of medium and for longer experiments in 2 ml of medium unless noted otherwise.

Measurement of the incorporation of [^{35}S]sulphate or [^{3}H]serine into macromolecules

Cultures were incubated in medium containing [^{35}S]sulphate or [^{3}H]serine. At the end of the incubation an equal volume of 4 M- or 8 M-guanidine hydrochloride with 0.05 M-sodium acetate, pH 5.8, containing proteinase inhibitors (0.2 M-6-aminohexanoic acid, 20 mM-disodium EDTA, 20 mM-benzamidine hydrochloride and 2 mM-phenylmethanesulphonyl fluoride) was added to the cultures. After extraction at 4°C for 1 h, up to 2 ml of the combined medium/extract fraction was chromatographed on a column (24 cm × 0.8 cm) of Sephadex G-50 eluted with 4 M-guanidine hydrochloride (pH 6–7) to separate unincorporated radioactivity. Fractions (1 ml) were collected and the elution of radioactivity was monitored. The void-volume fractions were pooled when required for further analysis. Both 2 M- and 4 M-guanidine hydrochloride extracted more than 95% of the ^{35}S labelled macromolecules.

Isolation and analysis of proteoglycans from the cultures

Extracts from cultures incubated with [^{35}S]-sulphate or [^{3}H]serine were chromatographed on Sephadex G-50 columns and the void-volume fractions were centrifuged in 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. After centrifugation in an MSE 65 instrument with an angle rotor (8 × 25 ml, $r = 6.87$ cm) at 34000 rev./min (100000 ***g***) 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 (D1) contained over 90% of the ^{35}S radioactivity in the gradient and was stored at −20°C after being dialysed against water and freeze-dried.

(*a*) To determine the size of proteoglycan monomer, samples from the D1 fraction were dissolved in 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8, and chromatographed on a column of Sepharose CL-2B (120 cm × 1 cm), eluted with 4 M-guanidine hydrochloride/0.05 M-sodium acetate/1 mM-disodium EDTA, pH 5.8, at 22°C. Fractions

(1.2ml) were collected and monitored for radioactivity. The K_d of the peak elution volume 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 hyaluronate of high molecular weight and ^3H_2O respectively.

(*b*) To investigate the size of the protein core, samples from the D1 fraction were mixed with carrier proteoglycan monomer (A1D1) from pig laryngeal cartilage (Hardingham *et al.*, 1976) dissolved in 0.05M-Tris/acetate buffer, pH7.3, and 0.04 unit of chondroitinase ABC per mg of hexuronic acid was added (Heinegård & Hascall, 1974). After digestion for 5h at 37°C, the sample was chromatographed on a column (170cm × 0.9cm) of Sepharose 4B, eluted with 0.5M-sodium acetate, pH6.8, at 4°C. Fractions (1.3ml) were collected and were monitored for radioactivity.

(*c*) To determine the size of chondroitin sulphate chains, samples from the D1 fraction were dissolved in 0.1M-sodium acetate/10mM-disodium EDTA/10mM-cysteine hydrochloride, pH6.1, and digested with papain (25μg/mg of hexuronate) for 18h at 60°C. The sample was chromatographed on a column (120cm × 0.9cm) of Sepharose 6B or (140cm × 1.0cm) of Sepharose CL-6B, eluted in 0.5M sodium acetate, pH6.8, at 4°C. Fractions (1.0ml or 1.3ml respectively) were collected and were monitored for radioactivity.

Determination of the proportion of [^{3}H]serine attached to chondroitin sulphate in proteoglycan

Cultures were preincubated for 4h with cycloheximide (1μM) or β-D-xyloside (100μM) and [^{35}S]sulphate (20μCi/ml) or [^{3}H]serine (40μCi/ml) was added for 4h incubation. Cultures were extracted and samples from the D1 fraction were digested with papain, as described above. For xyloside-treated cultures, the digests were chromatographed on Sepharose 6B as in Fig. 1(*d*) (below) and the proportion of ^{3}H radioactivity co-eluting with chondroitin sulphate and that eluting close to the included volume were determined. For cycloheximide-inhibited cultures the digests were applied to a column (3.0cm × 1.5cm) of DEAE-Sephacel and eluted with 12ml each of 0.1M-NaCl, 0.2M-NaCl, 0.5M-NaCl and 1.0M-NaCl, all in 50mM-Tris/HCl, pH7.2. The [^{3}H]serine attached to chondroitin sulphate was eluted in the 0.5M-NaCl and 1.0M-NaCl fractions (Mitchell, 1981). Both methods gave similar results with control cultures.

Determination of the molecular weight of chondroitin sulphate chains

A column of Sepharose 6B was calibrated with chondroitin sulphate chains radioactively labelled by reduction with NaB^3H_4. The method of Hopwood & Robinson (1973) was used, except that the standard used for reduction was D-xylose. The molecular-weight range of chondroitin sulphate eluted from a column (140cm × 0.9cm) of Sepharose 6B in 0.5M sodium acetate, pH6.8, at 4°C was determined from the analyses of hexuronate and radioactivity in the column fractions, by assuming each chain to be labelled with the same specific radioactivity as the standard [^{3}H]xylitol (1.89×10^6d.p.m./mol) (Hopwood & Robinson, 1973). There was a linear correlation between log(molecular weight) and K_d betweem 0.3 and 0.6 that fitted the formula:

$$\log(\text{molecular weight}) = (-K_d \times 2.3188) + 5.4494$$

A Sepharose CL-6B column was used in later experiments for some comparison of chondroitin sulphate chain lengths. It was not as fully characterized, but chromatography of three samples also run on Sepharose 6B produced a parallel calibration line:

$$\log(\text{molecular weight}) = (-K_d \times 2.3188) + 5.2894$$

Determination of total protein synthesis

Cultures were preincubated for 4h with cycloheximide (1μM) or xyloside (50μM), and [^{3}H]serine (40μCi/ml) and [^{35}S]sulphate (5μCi/ml) were added for a further 4h incubation. The cultures were extracted in 2M-guanidine hydrochloride/1% (w/v) Zwittergent and fractionated in a direct dissociative gradient. The total non-dialysable ^{3}H and ^{35}S radioactivity in all the gradient fractions was determined.

Determination of [^{3}H]uridine incorporation

In experiments on the effects of actinomycin D, after incubation with [^{3}H]uridine and [^{35}S]sulphate and removal of the combined medium and 4M-guanidine hydrochloride extract, the cells were washed twice on the plate with phosphate-buffered saline (Dulbecco A, pH7.3; Oxoid Ltd., Basingstoke, Hants., U.K.) and then removed with a 'rubber policeman' in 1ml water. An equal volume of cold 0.4M-perchloric acid was added to the cells and the mixture left for 1h at 4°C. The precipitate was collected by centrifugation and washed in cold 0.2M-perchloric acid. After recentrifugation, the pellet was heated at 70°C for 20min in 0.5ml of 0.4M-perchloric acid, centrifuged again and the supernatant counted for [^{3}H]radioactivity. The pellet was dissolved in 0.5M-NaOH and the protein content was determined by using an automated Folin procedure (Heinegård, 1973).

Results

Effect of cycloheximide on proteoglycan synthesis

Previous experiments with high concentrations of cycloheximide (0.3–1.0mM) showed there to be a slow onset of inhibition of [^{35}S]sulphate incor-

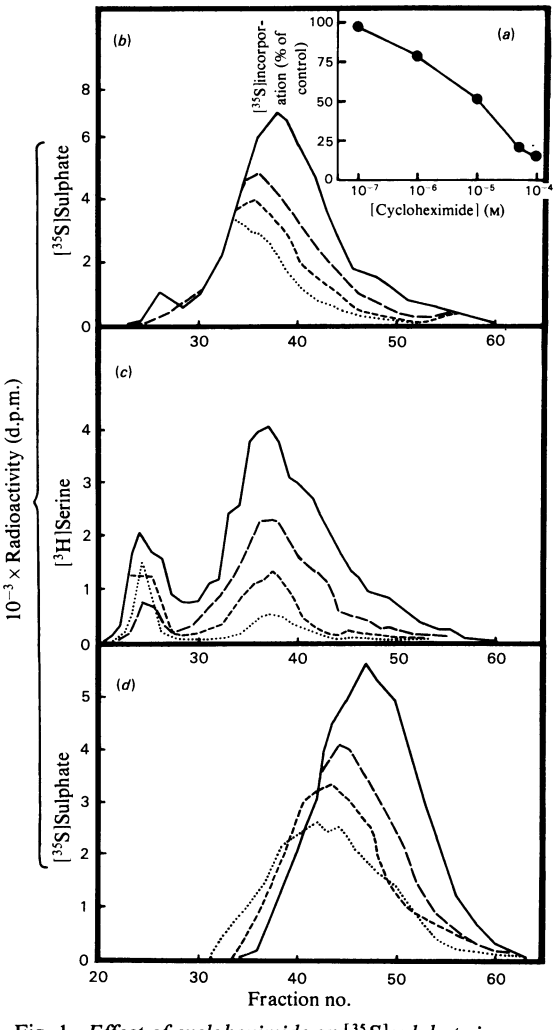

Fig. 1. *Effect of cycloheximide on [35S]sulphate incorporation (a), and the structure of proteoglycans (b, monomer size; c, chondroitinase ABC-digested protein core size; and d, chondroitin sulphate chain length)*

(*a*) Cultures were preincubated for 4h with cycloheximide (0.1 μM–0.1 mM), and [^{35}S]sulphate (2 μCi/ml) was then added to each culture. After 3h incubation, the total radioactive incorporation into macromolecules was determined by extraction in 2M-guanidine hydrochloride and isolation on Sephadex G-50 columns as described in the Materials and methods section. (*b*, *c*, *d*). Cultures were preincubated for 4h with control medium (——) and with cycloheximide [0.1 μM (— — — —), 0.5 μM (– – – –) and 1.0 μM (• • • •)], and [^{3}H]serine (40 μCi/ml) and [^{35}S]sulphate (20 μCi/ml) were added for 4h. Cultures were extracted in 4M-guanidine hydrochloride with proteinase inhibitors for 1h at 4°C and ^{35}S-labelled proteoglycan was isolated by Sephadex G-50 chromatography and equilibrium density-gradient centrifugation in 4M-guanidine hydrochloride, as described in the Materials and methods section. (*b*) Samples from the density gradient (D1 fraction) were dissolved in 4M-guanidine hydrochloride containing proteinase inhibitors and chromatographed on Sepharose CL-2B eluted with 4M-guanidine hydrochloride/0.05M-sodium acetate/1mM-disodium EDTA, pH6.1, at 22°C, as described in the Materials and methods section. (V_0, fraction 25; V_t, fraction 70). (*c*) Samples from the density gradient (D1 fraction) were mixed with carrier proteoglycan (A1D1) and digested with chondroitinase ABC and chromatographed on Sepharose 4B, as described in the Materials and methods section (V_0, fraction 23; V_t, fraction 65). (*d*) Samples of the D1 fraction were digested with papain and then chromatographed on Sepharose 6B, also as described in the Materials and methods section (V_0, fraction 24; V_t fraction 78).

poration in chondrocytes because of the presence of a large intracellular pool of proteoglycan protein core (Mitchell & Hardingham, 1981; Kimura *et al.*, 1981). In order to study the effects of partial inhibition of protein synthesis, the cells were therefore preincubated for 4h in various concentrations of cycloheximide before adding [^{35}S]sulphate in order to deplete the intracellular pool of protein core. This gave a constant rate of [^{35}S]sulphate incorporation for at least 4h after the preincubation period (Mitchell, 1981). The inhibition of [^{35}S]sulphate incorporation in chondrocytes was found to be proportional to the concentration of cycloheximide, and 1 μM gave about 50% inhibition (Fig. 1*a*). The structure of the proteoglycans was investigated in cultures incubated with [^{3}H]serine and/or [^{35}S]-sulphate. The size of the purified proteoglycan monomers were determined by gel chromatography on Sepharose CL-2B in 4M-guanidine hydrochloride (Fig. 1*b*) and showed an increase in K_d from 0.273 to 0.182 as the incorporation of [^{35}S]sulphate decreased. In contrast, there was no alteration in the size of the chondroitinase ABC-digested protein core chromatographed on Sepharose 4B (Fig. 1*c*). Samples of the proteoglycan were digested with papain and the chondroitin sulphate chains released were chromatographed on Sepharose 6B (Fig. 1*d*). Compared with chondroitin sulphate standards, the size increased from 16000 mol.wt. in control cultures to 27500 mol.wt. of 1.0 μM-cycloheximide. The increase in monomer size therefore appeared to result from a 66% increase in the length of chondroitin sulphate chains with no apparent change in the protein core structure. The number of chondroitin sulphate chains attached to the proteoglycan was determined by measuring the [^{3}H]serine radioactivity that was bound to chondroitin sulphate after exhaustive digestion with papain (Mitchell & Hardingham, 1981). With 1 μM-cycloheximide, 36.2% of the [^{3}H]serine was associated with chondroitin sulphate chains compared with 36.0% in control cultures. So the number of chains attached

to each protein core did not alter. As the total [^{35}S]sulphate incorporation was reduced to 49.3%, and allowing for the increase in chain length, the number of completed proteoglycan molecules was only 29% of that in control cultures.

Effect of p-nitrophenyl β-D-xyloside on proteoglycan synthesis

β-Xylosides stimulate [^{35}S]sulphate incorporation in chondrocytes as they act as acceptors for protein-free chondroitin sulphate synthesis (Robinson *et al.*, 1975). Incubation with β-xyloside showed maximal stimulation at 0.1 mM, although the degree of stimulation (185–220% of control) varied from one chondrocyte preparation to another (Mitchell, 1981). The effect of *p*-nitrophenyl β-D-xyloside on the structure of proteoglycans was determined in cultures incubated with [^{35}S]sulphate and/or [^{3}H]serine. The size of proteoglycan synthesized in the presence of 50 μM-xyloside was much smaller than normal (Fig. 2*a*). This resulted in part from a reduction in the size of the chondroitin sulphate chains from 21000 to 11600 mol.wt. (Fig. 2*b*), and separate experiments showed that chondroitin sulphate chains formed on proteoglycans were of the same size as those synthesized on the xyloside (Mitchell, 1981). The degree of substitution of the protein core with chondroitin sulphate was also found to be much less than in normal or cycloheximide-treated cultures, as only 23.2% of the [^{3}H]serine radioactivity was associated with chondroitin sulphate. This suggested that 38% fewer chains were attached to the proteoglycan. Gel chromatography on Sepharose 4B after digestion with chondroitinase ABC of [^{3}H]serine-labelled proteoglycan showed no evidence of any reduction in size in the protein core in the presence of xyloside (result not shown). Each proteoglycan synthesized and secreted in the presence of β-xylosides thus contained on average 38% fewer chondroitin sulphate chains, which were 42% shorter than the controls. As the incorporation of [^{35}S]-sulphate into proteoglycan was only 18.9% of the total incorporation into chondroitin sulphate chains, it was therefore 18.9 × (145/100)% of the incorporation into proteoglycan in control cultures, the number of proteoglycans secreted from xyloside treated cultures was:

$$\frac{18.9 \times 145}{100 \times 0.62 \times 0.58} = 77\%$$

of that in control cultures.

What factors limit the rate of chondroitin sulphate synthesis?

With inhibitors of protein synthesis present, it was important to establish whether falling levels of the synthesizing enzymes or their nucleotide-sugar

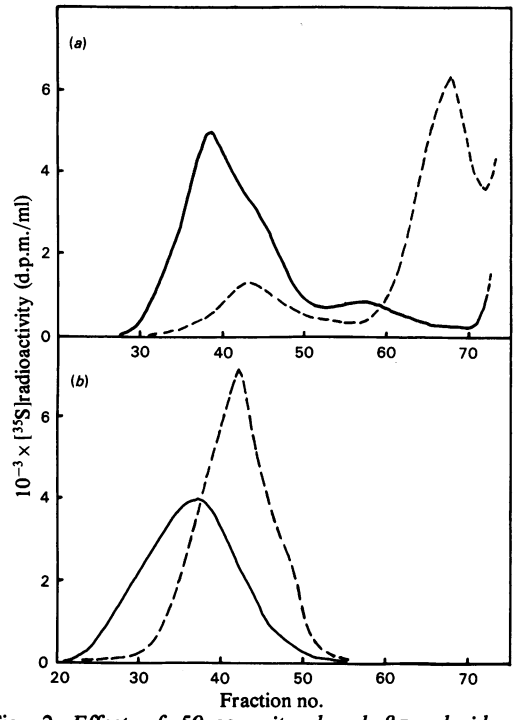

Fig. 2. *Effect of 50 μM-p-nitrophenyl β-D-xyloside on proteoglycan structure: assessment of the size of (a) proteoglycan monomer and (b) chondroitin sulphate chains*

(*a*) Cultures were preincubated for 4 h with control medium (——) or with 50 μM-*p*-nitrophenyl β-D-xyloside (– – –), and [^{35}S]sulphate (50 μCi/ml) was then added to each plate for 15 min. The medium was then removed and the plate washed with 1 ml of non-radioactive medium and replaced with a further 1 ml of medium for a non-radioactive chase incubation of 3 h. Cultures were extracted in 2 M-guanidine hydrochloride/1% (w/v) Zwittergent/0.05 M-sodium acetate, pH 5.8, for 1 h at 4°C [Zwittergent was included in this experiment to ensure the extraction of any completed proteoglycans that were still intracellular (Kimura *et al.*, 1981) and 1% (w/v) of Zwittergent 3-14 was found optimal for this purpose (Mitchell, 1981)]. The combined medium and extract were adjusted to 4 M by adding solid guanidine hydrochloride. The samples were warmed to 37°C to ensure dissociation of all components and then chromatographed on Sepharose CL-2B as described for Fig. 1(*b*) (V_0, fraction 29; V_t, fraction 79). (*b*) Cultures were preincubated for 4 h with control medium (——) or with 50 μM-*p*-nitrophenyl β-D-xyloside (– – –), and [^{35}S]sulphate (20 μCi/ml) was then added for a further 4 h incubation. Proteoglycans were extracted from the cultures and isolated as described in Fig. 1. Samples of the D1 fraction were digested with papain (see Fig. 1*d*) and chromatographed on a column (130 cm × 0.9 cm) of Sepharose CL-6B in 0.5 M-sodium acetate, pH 6.8, at 4°C. Fractions (1.6 ml) were collected and the contents of radioactivity and uronic acid (not shown) were determined (V_0, fraction 22; V_t, fraction 60).

substrates contributed to the observed changes in chondroitin sulphate synthesis. However, the addition of xyloside (100 μM) to cultures inhibited with cycloheximide (100 μM) stimulated chondroitin sulphate synthesis from 20 to 174% of the uninhibited rate in a 3 h incubation after 1 h exposure to the agents and was only slightly below the xyloside-stimulated rate of uninhibited cultures (217% of control). The availability of enzymes and substrates for chondroitin sulphate synthesis was not therefore limiting synthesis in the cycloheximide-inhibited cultures.

Effect of temperature on chondroitin sulphate synthesis (Fig. 3)

As the length of chondroitin sulphate chains appeared to be related to their rate of synthesis, the effect of temperature on the rate of synthesis and on the chain length was examined. Normal cultures showed a large inhibition of chondroitin sulphate synthesis at 22°C and more inhibition at 4°C. In the presence of 100 μM-*p*-nitrophenyl β-D-xyloside, however, the rate of synthesis was much less inhibited, being 35% of control at 22°C and 3.0% at 4°C, and it did not differ greatly from the decrease predicted for the effect of temperature on an enzyme-catalysed reaction, assuming a halving of the rate for each 10°C fall in temperature (----, Fig. 3). The size of the chondroitin sulphate chains synthesized in the control cultures increased from 17400 mol.wt. average at 37°C to 36000 mol.wt. average at 22°C and to 50000 mol.wt. average at 4°C (Fig. 4*a*). However, in the xyloside-treated cultures, the size of the chains was constant at all temperatures, namely 13000 mol.wt. average (Fig. 4*b*). These results suggested that it was only the concentration of acceptor that influenced the length of each chain and not the rate of synthesis.

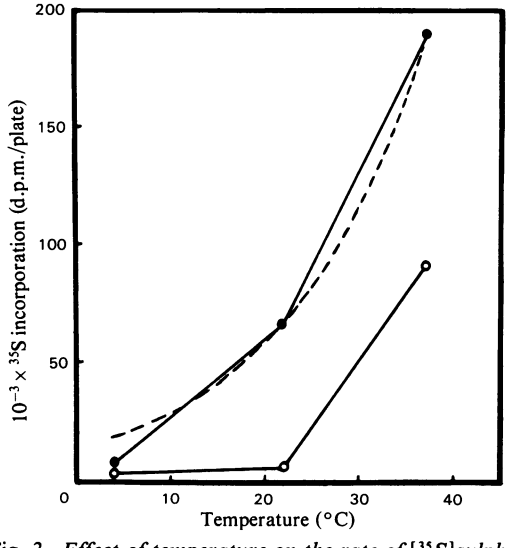

Fig. 3. *Effect of temperature on the rate of [^{35}S]sulphate incorporation in control and xyloside-stimulated cultures*

Cultures were preincubated for 1 h with (●) or without (○) 100 μM-*p*-nitrophenyl β-D-xyloside in 1 ml of medium in equilibrium with air at 37, 22 and 4°C. [^{35}S]Sulphate (10 μCi/ml) was added to each plate for 5 h incubation and the cultures were then frozen. The cultures were digested by adding 125 μg of papain in 0.4 ml of 0.4 M-KH_2PO_4/20 mM-disodium EDTA/10 mM-cysteine hydrochloride at 60°C for 18 h. The digest was adjusted to 2 ml with 8 M-guanidine hydrochloride and the samples chromatographed on Sephadex G-50 to remove unincorporated radioisotope. The total incorporation of [^{35}S]sulphate into macromolecules was determined from the radioactivity in the V_0 peak fractions.

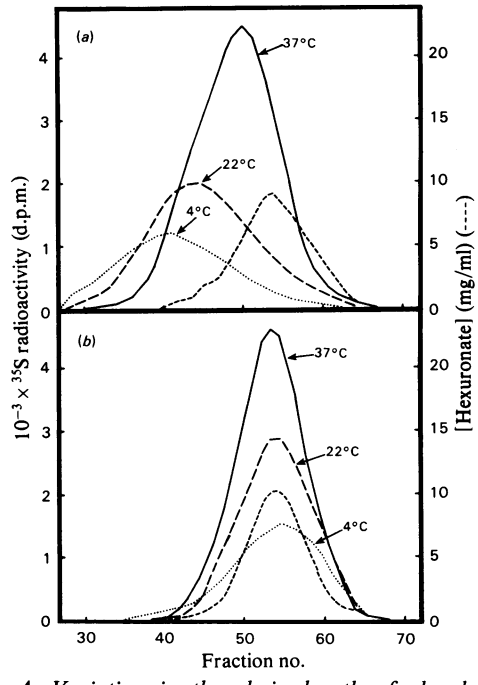

Fig. 4. *Variation in the chain length of chondroitin sulphate synthesized at different temperatures with and without p-nitrophenyl β-D-xyloside*

Cultures were as described for Fig. 3. To samples of the ^{35}S-labelled macromolecules (Sephadex G-50 V_0 peak fractions) was added 1 mg of carrier chondroitin sulphate and after dialysis against water they were freeze-dried, dissolved in 0.5 M-sodium acetate, pH 6.8, and chromatographed on a column of Sepharose CL-6B as described in the Materials and methods section (V_0, fraction 28; V_t, fraction 73).

Effect of actinomycin D on chondroitin sulphate synthesis

Actinomycin D inhibits the transcription of DNA into RNA and thus blocks the formation of mRNA for protein synthesis. With the chondrocyte cultures a concentration of 10μg of actinomycin D/ml was found to give no significant incorporation of [^{3}H]-uridine. Under these conditions, the incorporation of [^{35}S]sulphate into proteoglycan showed a steady fall that fitted an apparent first-order decay with a half-time of 5.4h (Fig. 5). However, when the synthesis of chondroitin sulphate on xylosides was determined, the rate of incorporation only fell to the control unstimulated rate after more than 10h (Mitchell, 1981). The enzymes of chondroitin sulphate synthesis on xylosides were thus active for many hours after chondroitin sulphate synthesis on proteoglycan was greatly decreased. The apparent half-life of 5.4h would thus appear to reflect the formation of xylosyl core protein, but it may be either the supply of protein core or the activity of the xylosyltransferase that is the limiting factor. If it is the supply of protein core that is limited by its mRNA production, the half-life of 5.4h would correspond to the combined half-life of intracellular protein core and the mRNA. As it was previously shown that the protein core within the cell had a half-life of 1.1–1.5h when protein synthesis was completely inhibited with cycloheximide (Mitchell & Hardingham, 1981), the half-life of proteoglycan mRNA would be 3.9–4.3h. However, caution is required in interpreting this result, since actinomycin D may inhibit protein synthesis by other mechanisms (Lodish, 1976).

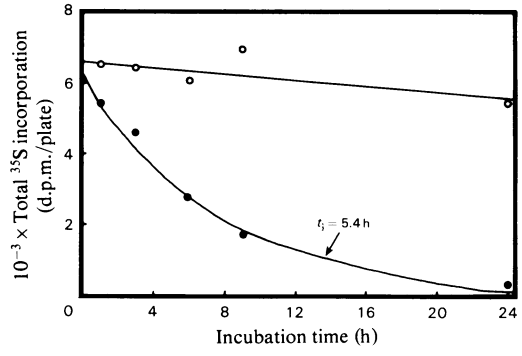

Fig. 5. *Effect of actinomycin D on the incorporation of [^{35}S]sulphate*

Cultures were incubated in control medium (○) or with actinomycin D (10μg/ml) (●) for 0–24h and the medium was changed after 10h. [^{3}H]Uridine (1.8μCi/ml) and [^{35}S]sulphate (4.8μCi/ml) were added to each plate for 1h at the end of incubation. The pericellular matrix and medium were then extracted in 4M-guanidine hydrochloride and the total incorporation of [^{35}S]sulphate into macromolecules was determined after gel chromatography on Sephadex G-50 as described in the Materials and methods section. The cell layer was scraped off the plates, washed, and the [^{3}H]uridine incorporation and the total protein content were determined as described in the Materials and methods section (results not shown).

Discussion

The changes in the structure of proteoglycan produced after several hours partial inhibition of protein synthesis with low concentrations of cycloheximide are similar to those observed as the rate of synthesis fell after complete inhibition with very high concentrations (Mitchell & Hardingham, 1981; Kimura *et al.*, 1981). The present results permit a more quantitative assessment of these changes (Table 1) and show the changes neither to result from adverse side effects of high concentrations of cycloheximide, nor to depend upon the complete inhibition of protein synthesis. They also confirm and extend the results of Kato *et al.* (1978), who showed the size of proteoglycans and their chondroitin sulphate chains to increase after a short exposure (2h) of chick chondrocytes to low concentrations of cycloheximide.

In the present study 1μM-cycloheximide gave 28.5% inhibition of protein synthesis, 52% inhibition of [^{35}S]sulphate incorporation, but 71% inhibition of proteoglycan completion, although the proteoglycans synthesized under these conditions were approx. 58% larger (Table 1). In contrast, the results with *p*-nitrophenyl β-D-xyloside (50μM) showed a large decrease (46%) in the molecular weight of proteoglycan (Table 1). This is also comparable with the changes observed in proteoglycan structure when chick chondrocytes were exposed to *p*-nitrophenyl β-D-xyloside (Schwartz, 1977, 1979; Kato *et al.*, 1978). With cycloheximide or β-xyloside there was no evidence of changes in proteoglycan protein core, either in its size or in its ability to aggregate (Mitchell, 1981). The changes in structure thus only concerned chondroitin sulphate synthesis and suggested that its synthesis in rat chondrosarcoma cells showed very similar characteristics to that in embryonic-chick chondrocytes.

The changes observed in the amount and length of chondroitin sulphate chains synthesized appeared to be related to the availability of xylosyl acceptor for the chondroitin sulphate-synthesizing enzymes (Kato *et al.*, 1978; Lohmander *et al.*, 1979*b*). Thus cycloheximide decreases the supply of xylosyl-protein core and, as a consequence, total synthesis is decreased, but chains are made longer, whereas with β-xyloside there is excess xylosyl acceptor and

Table 1. *Changes in the structure of proteoglycan synthesized by chondrosarcoma cells in the presence of cycloheximide (1 μM) or p-nitrophenyl β-D-xyloside (50 μM)*

	Chondroitin sulphate chain length (% of control)	Number of chains per proteoglycan (% of control)	Proteoglycan size (% of control)	Proteoglycan size Molecular-weight estimate*
Control	100	100	100	2×10^6
Cycloheximide (1 μM)	166	100	158	3.2×10^6
p-Nitrophenyl β-D-xyloside (50 μM)	58	62	54	1.1×10^6

* The molecular weights of proteoglycans were calculated by assuming the control to be of mol.wt. 2×10^6 and to contain 100 chondroitin sulphate chains of mol.wt. 17000, a protein core of mol.wt. 260000 and 2% oligosaccharides (Hascall, 1977; Hardingham, 1981). (Rat chondrosarcoma proteoglycans contain no keratan sulphate; Hascall, 1977).

more chains of shorter average length are produced. The evidence from the investigation of chondroitin sulphate synthesis using microsomal preparations from chick chondrocytes (Richmond *et al.*, 1973*a*,*b*; Silbert & Reppucci, 1976; Silbert, 1978) suggests that the extension of a chain occurs rapidly, as very short chains and long chains were observed but not intermediate sizes. Longer oligosaccharides were also found to be better substrates for chain extension than very short oligosaccharides (Rodén & Schwartz, 1975). From these observations it may be argued that chain elongation is a fairly concerted mechanism, possibly with the substrate remaining tightly bound to the enzymes. However, the sensitivity of chain length to the concentration of acceptor would suggest that the mechanism is not concerted during the later stages of chain synthesis. This would be explained if the affinity of the acceptor for the enzyme is very high over the major part of chain extension, but decreases when the chain exceeds a certain length. This would combine the features of a concerted mechanism in the first part of chain synthesis, but make chain termination dependent on acceptor concentration.

In considering the mechanisms that produce the observed pattern of chain synthesis, it is necessary to take into account the intracellular location of these events. If the proteoglycan protein core is initially synthesized into the cisternal space of the endoplasmic reticulum, in common with other secretory proteins, it must meet the chondroitin sulphate-synthesizing enzymes only when it is moved further towards the Golgi apparatus, and the factors that determine chain length may thus operate in only a very restricted region of the cell (Mitchell & Hardingham, 1981). Previous results showed it to form a large intracellular pool and that chondroitin sulphate was synthesized on it only just before secretion (Mitchell & Hardingham, 1981; Kimura *et al.*, 1981). This suggests that the chondroitin sulphate-synthesizing enzymes are not present within the same compartment as most of the protein core. The proteoglycans may also be exposed to the synthetic machinery for a limited time as they are moved through the compartment for chondroitin sulphate synthesis. This may account for the failure in the presence of xylosides to synthesize chondroitin sulphate on all the acceptor sites on proteoglycan, because it was moved away from the enzyme before some of the chains were begun. Conversely, increased time spent with the enzymes chain synthesis may account for the extremely long chains synthesized on proteoglycan at low temperature by chondrocytes and also by cartilage slices (Hardingham & Muir, 1970), as the intracellular movement of proteoglycan may be very much decreased at low temperature if it is membrane-dependent, whereas the low-molecular-weight xyloside may not be similarly constrained.

It has been proposed that the rate of chondroitin sulphate synthesis in the presence of β-D-xyloside is a measure of a cell's total capacity for chondroitin sulphate synthesis (comparable with a measurement of $V_{max.}$), whereas the rate of synthesis in the absence of xylosides is a measure of the rate permitted by the availability of acceptor protein (Schwartz *et al.*, 1974). The ratio of the xyloside stimulated rate to the normal rate thus reflects to what extent the normal rate is substrate-limited. This ratio is found to be much higher in cells with low rates of chondroitin sulphate synthesis than in those with high rates (Schwartz *et al.*, 1974). With prechondrogenic chick mesenchymal cells, where chondroitin sulphate synthesis is low, stimulation with xyloside is 10-fold (Schwartz *et al.*, 1974), which shows the normal rate to be strongly, substrate-limited, and the chains synthesized are long, whereas after differentiation the rate of chondroitin sulphate synthesis is high, but the chondrocytes show only 2-fold stimulation with xyloside; the normal rate is thus less substrate-limited and the chains synthesized are much shorter (De Luca *et al.*, 1978). This is entirely in keeping with a common mechanism of chain elongation in which the chain length is deter-

mined by how far the normal rate of synthesis is substrate-limited. The enzymes of chondroitin sulphate synthesis have mainly been studied from chondrocytes, but limited work on enzymes from other sources has shown no differences in their properties (Rodén & Schwartz, 1975). The characteristics of chain elongation and termination reported here may thus not be exclusive to chondrocytes, but may apply in general to chondroitin sulphate synthesis in other cell types.

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References

De Luca, S., Caplan, A. L. & Hascall, V. C. (1978) *J. Biol. Chem.* **253**, 1536–1542
Hardingham, T. E. (1981) *Biochem. Soc. Trans.* **9**, 489–497
Hardingham, T. E. & Muir, H. (1970) *FEBS Lett.* **9**, 145–148
Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127–143
Hascall, V. C. (1977) *J. Supramol. Struct.* **7**, 101–120
Heinegård, D. (1973) *Chem. Scr.* **4**, 199–201
Heinegård, D. & Hascall, V. C. (1974) *J. Biol. Chem.* **249**, 4250–4256
Hopwood, J. J. & Robinson, H. C. (1973) *Biochem. J.* **135**, 631–637
Kato, Y., Kimata, D., Ito, K., Karasawa, K. & Suzuki, S. (1978) *J. Biol. Chem.* **253**, 2784–2789
Kimura, J. H., Hardingham, T. E., Hascall, V. C. & Solursh, M. (1979) *J. Biol. Chem.* **254**, 2600–2609
Kimura, J. H., Caputo, C. B. & Hascall, V. C. (1981) *J. Biol. Chem.* **256**, 4368–4376
Lodish, H. F. (1976) *Annu. Rev. Biochem.* **45**, 38–72
Lohmander, L. S., Hascall, V. C. & Caplan, A. I. (1979*a*) *J. Biol. Chem.* **254**, 10551–10561
Lohmander, L. S., Madsen, K. & Hinek, A. (1979*b*) *Arch. Biochem. Biophys.* **192**, 148–157
Mitchell, D. C. (1981) Ph.D. Thesis, University of London
Mitchell, D. C. & Hardingham, T. E. (1981) *Biochem. J.* **196**, 521–529
Muir, H. & Hardingham, T. E. (1975) *MTP. Int. Rev. Sci. Biochem. Ser. One* **5**, 153–222
Richmond, M. E., De Luca, S. & Silbert, J. E. (1973*a*) *Biochemistry* **12**, 3898–3904
Richmond, M. E., De Luca, S. & Silbert, J. E. (1973*b*) *Biochemistry* **12**, 3904–3910
Robinson, H. C., Brett, M. H., Tralaggan, P. J., Lowther, D. A. & Okayama, M. (1975) *Biochem. J.* **148**, 25–34
Rodén, L. & Schwartz, N. B. (1975) *MTP. Int. Rev. Sci. Biochem. Ser. One* **5**, 95–152
Schwartz, N. B. (1977) *J. Biol. Chem.* **252**, 6316–6321
Schwartz, N. B. (1979) *J. Biol. Chem.* **254**, 2271–2277
Schwartz, N. B.. Galligani, L., Ho, P.-L. & Dorfman, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4047–4051
Silbert, J. E. (1978) *J. Biol. Chem.* **253**, 6888–6892
Silbert, J. E. & Reppucci, A. C. (1976) *J. Biol. Chem.* **251**, 3942–3947