The relation of RNA synthesis to chondroitin sulphate biosynthesis in cultured bovine cartilage

David J. McQUILLAN,* Christopher J. HANDLEY,† H. Clem ROBINSON, Ken NG and Chris TZAICOS
Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia

Addition of actinomycin D (or cordycepin, an alternative inhibitor of RNA synthesis) to cartilage cultures resulted in a first-order decrease in the rate of incorporation of [³⁵S]sulphate into proteoglycan (half-life = 7.5 ± 1.1 h). Addition of 1.0 mM-benzyl β -D-xyloside relieved the initial inhibition of glycosaminoglycan synthesis induced by actinomycin D; however, after a lag of about 10 h the rate of xyloside-initiated glycosaminoglycan synthesis also decreased with apparent first-order kinetics (half-life = 7.1 ± 1.8 h), which paralleled the decrease in the rate of core-protein-initiated glycosaminoglycan synthesis. The hydrodynamic size of the proteoglycans formed in the presence of actinomycin D remained essentially constant (K_{av} , 0.21–0.23), whereas the constituent glycosaminoglycan chains were larger than those formed by control cultures, which suggested that the core protein was substituted with fewer but larger glycosaminoglycan chains. Proteoglycans formed in the presence of β -D-xyloside were significantly smaller (K_{av} , ~ 0.33) than those synthesized by control cultures, and were further diminished in size after exposure of cultures to actinomycin D. Glycosaminoglycan chains synthesized by these same cultures on to both core-protein and xyloside acceptors were also smaller than those of control cultures. The decrease in synthesis observed after exposure to actinomycin D was not reflected by any significant decrease in the activities of several glycosyltransferases involved in chondroitin sulphate synthesis (galactosyltransferase-I, galactosyltransferase-II, N-acetylgalactosaminyltransferase and glucuronosyltransferase-II).

INTRODUCTION

Articular cartilage is a specialized tissue consisting primarily of a fibrous collagen (type II) network immersed in a hydrated gel composed of high- M_r proteoglycan aggregate (cartilage-specific chondroitin sulphate-proteoglycan). The structure of a typical cartilage-specific proteoglycan is well defined (for reviews, see Lindahl & Höök, 1978; Rodén, 1980; Hascall & Hascall, 1981), and consists of a core protein ($M_r \sim 2.0 \times 10^5$), which comprises 8–10% of the molecule (Pasternack *et al.*, 1974), to which are bound about 150 chondroitin sulphate chains ($M_r \sim 1 \times 10^4$ per chain), 60 keratan sulphate chains ($M_r \sim 5 \times 10^3$), and a variable number of O-linked and N-linked oligosaccharides.

The biosynthesis of these complex macromolecules by chondrocytes is initiated by the translation of mRNA to form the core-protein precursor ($M_r \sim 3.3 \times 10^5$; Vertel *et al.*, 1984) at the rough endoplasmic reticulum (Upholt *et al.*, 1979). This is followed by extensive post-translational modifications, including the addition of oligosaccharides and sulphated glycosaminoglycans in the Golgi apparatus before secretion from the cell (Thonar *et al.*, 1983; Fellini *et al.*, 1984).

We have previously shown (McQuillan *et al.*, 1984) that addition of cycloheximide to cultures of bovine articular cartilage is followed by a first-order decay in the rate of proteoglycan synthesis (half-life ~ 32 min). Similar observations have been reported for chondrosarcoma chondrocytes in culture (Kimura *et al.*, 1981; Mitchell & Hardingham, 1981). In contrast with the results reported

for chondrosarcoma chondrocyte cultures, we observed that addition of 1.0 mM-benzyl β -D-xyloside increased the rate of incorporation of [³⁵S]sulphate into glycosaminoglycans by the cultured articular cartilage, but this elevated rate was also diminished by cycloheximide. This suggested that there might be a close co-ordination of the activity of the glycosyltransferases of glycosaminoglycan synthesis with translation products.

In the present study, this phenomenon has been further analysed in cultures of bovine articular cartilage. The effect of inhibition of RNA synthesis on proteoglycan synthesis by the tissue is reported. These experiments demonstrate a similar pattern to that observed with cycloheximide, although the effects are manifest over a longer period of time.

EXPERIMENTAL

Materials

Actinomycin D and cordycepin were purchased from Sigma (St. Louis, MO, U.S.A.). All other materials were obtained as previously described (McQuillan *et al.*, 1984).

Cartilage cultures

Explant cultures of articular cartilage from the metacarpal-phalangeal joints of 1–2-year-old steers were prepared as described by Hascall *et al.* (1983). Tissue was maintained in batch culture for 6 days in the presence of 20% (v/v) foetal-calf serum before further treatment (McQuillan *et al.*, 1984).

* Present address: Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892, U.S.A.

† To whom all correspondence should be addressed.

Incubation of cultures with [³⁵S]sulphate

For each experiment, a large batch of labelling medium was prepared so that each culture was incubated in medium of constant specific radioactivity. Before labelling, each culture was preincubated for 1 h in fresh medium either with or without 1.0 mM-benzyl β -D-xyloside. After preincubation, the medium was replaced with 2 ml of medium containing 20–30 μ Ci of [³⁵S]sulphate/ml either with or without 1.0 mM-benzyl β -D-xyloside. Cultures were incubated with [³⁵S]sulphate for 1 h in loosely capped culture vials in a shaking water bath at 37 °C. Control experiments showed that [³⁵S]sulphate was incorporated linearly with respect to time up to 4 h, with no apparent lag time.

Actinomycin D and cordycepin were dissolved in medium at stock concentrations of 1 mg/ml and 10 mg/ml respectively, sterilized by passage through a 0.22 μ m microporous membrane and finally added to appropriate cultures to give the required concentration. In a typical experiment, in which actinomycin D was used, control cultures were incubated with [³⁵S]sulphate in the presence and absence of benzyl β -D-xyloside. Actinomycin D (final concn. 10 μ g/ml) was then added to the remaining cultures; the time of this addition was designated zero time. After the addition of actinomycin D, cultures were incubated with [³⁵S]sulphate at the times indicated in the Results section, with or without benzyl β -D-xyloside as appropriate. All points shown represent the mean of duplicate determinations.

Extraction procedures

Labelling was terminated either by the direct addition of 5.0 M-NaOH to give a final concentration of 0.5 M-NaOH or by the direct addition of 8 M-guanidinium chloride containing proteinase inhibitors (Hascall & Kimura, 1982) to give a final concentration of 4 M-guanidinium chloride. In each method the tissue was extracted for 2 days at 4 °C to solubilize macromolecules. Samples of the alkali extracts were fractionated on Sephadex G-25 columns (1.5 cm $\times$ 5.5 cm) equilibrated with 4 M-guanidinium chloride/0.2 M-Na₂SO₄/0.1 M-sodium acetate/0.5% (v/v) Triton X-100, pH 6.0, to determine the amount of incorporated radioactivity (Kimura *et al.*, 1979). Samples of guanidinium chloride extracts were subjected to gel chromatography as described below.

Column chromatography

Columns (0.8 cm $\times$ 100 cm) of Sepharose CL-6B and CL-2B were eluted with 4 M-guanidinium chloride/0.2 M-Na₂SO₄/0.5% Triton X-100/0.1 M-sodium acetate, pH 6.0, at a flow rate of 6 ml/h. Fractions (1.4 ml) were collected and assayed for radioactivity (Hascall *et al.*, 1983).

Enzyme assays

The preparation of cultures for assay of glycosyltransferases has been described previously (McQuillan *et al.*, 1984). Briefly, batch cultures (2–3 g wet wt. of tissue) were maintained in 30 ml of medium for 7 days, and then incubated for up to 24 h with 10 μ g of actinomycin D/ml. After the incubation period, the medium was removed and the tissue blotted dry and frozen in a plastic tube with solid CO₂. Frozen tissue was pulverized under liquid N₂ (Spex mill; Spex Industries, Metuchen, NJ, U.S.A.) and

further homogenized by hand in 3–6 ml of enzyme buffer [50 mM-KCl/15 mM-MgCl₂/1.25 mM-EDTA/0.05 mM-Phenol Red/0.5% (v/v) Nonidet P-40 detergent, pH 7.0].

The following glycosyltransferase activities were assayed as previously described (McQuillan *et al.*, 1984): galactosyltransferase-I (UDP-galactose: β -D-xylopyranoside galactosyltransferase), *N*-acetylgalactosaminyltransferase (UDP-*N*-acetylgalactosamine:chondroitin *N*-acetylgalactosaminyltransferase), glucuronosyltransferase-II (UDP-D-glucuronic acid:chondroitin-D-glucuronosyltransferase) and UDP-galactose: β -*N*-acetylglucosaminide galactosyltransferase. Galactosyltransferase-II (UDP-galactose:D-galactoside galactosyltransferase) was assayed as described by Robinson & Robinson (1985).

Determination of ATP

The ATP content of cartilage was determined as described by McQuillan *et al.*, (1984).

Estimation of chondrocyte viability

Cartilage slices (approx. 0.5 g) were washed three times with medium alone before being stirred with 8 ml of 0.25% (w/v) trypsin in phosphate-buffered saline (Dulbecco & Vogt, 1954) for 30 min at 37 °C. The trypsin solution was replaced with the same volume of 0.25% (w/v) collagenase in serum-free medium and incubated with gentle stirring at 37 °C. After 2.5 h the tissue had dispersed, and the digest was then filtered through four layers of gauze and the filtrate centrifuged for 10 min at 1500 *g*. The cell pellet was then resuspended in 2 ml of medium containing 20% foetal-calf serum, and then 0.1 ml of the cell suspension was mixed with 0.1 ml of 0.4% (w/v) Trypan Blue in phosphate-buffered saline. Cells were counted in a haemocytometer, and non-viable cells were assessed by inclusion of the dye (Girardi *et al.*, 1956).

RESULTS

Effect of actinomycin D on the rate of proteoglycan biosynthesis

Actinomycin D is a commonly used inhibitor of RNA synthesis, and this reflects the high efficiency of inhibition. Actinomycin D binds strongly to DNA on a site in the DNA helix which is assumed to be the specific template site for the RNA polymerase (Reich, 1964). At a concentration of 10 μ g/ml, actinomycin D has been shown to inhibit the incorporation of [³H]uridine by rat chondrosarcoma chondrocytes in culture (Mitchell & Hardingham, 1982), and this concentration of actinomycin D was therefore employed in the experiments described in this study.

Articular cartilage from 1–2-year-old steers was maintained in batch culture for 6 days in the presence of 20% foetal-calf serum. Tissue was distributed into individual cultures and, at zero time on day 7, cultures were exposed to 10 μ g of actinomycin D/ml in fresh medium for up to 32 h. At the times shown in Fig. 1, cultures were incubated with [³⁵S]sulphate for 1 h, and the rate of proteoglycan synthesis was determined. A parallel set of cultures were treated in the same manner, except that they were preincubated with 1 mM-benzyl β -D-xyloside before labelling with [³⁵S]sulphate in the

Fig. 1. Effect of actinomycin D on glycosaminoglycan synthesis in the absence and presence of benzyl β -D-xyloside in bovine articular cartilage

Bovine articular cartilage was maintained in explant culture as described in the Experimental section. At zero time, individual cultures were exposed to $10 \mu\text{g}$ of actinomycin D/ml for up to 32 h. At the times shown, duplicate cultures were incubated with [^{35}S]sulphate for 1 h with (●) or without (○) 1 mM-benzyl β -D-xyloside. The bars represent the average deviation from the mean of duplicates on each curve. The half-life was obtained for each curve from the slope of a semi-logarithmic plot of the experimental results, shown in the inset, and was 6.4 h and 6.3 h for core-protein- and xyloside-initiated glycosaminoglycan synthesis respectively.

presence of the same concentration of β -D-xyloside (Fig. 1, ●).

Fig. 1 shows the rate of [^{35}S]sulphate incorporation into core-protein-initiated and xyloside-initiated glycosaminoglycans decreasing as a function of time of incubation with actinomycin D. Sulphate incorporation into proteoglycan (Fig. 1, ○) exhibited an exponential decay which presumably reflected the depletion of the available pool of mRNA coding for core protein, coupled with the depletion of the pool of core protein. As previously described for experiments using cycloheximide (McQuillan *et al.*, 1984), a half-life for this decay could be determined from the slope of a plot of the logarithm of the incorporation rate versus time (Fig. 1, insert). For the experiment shown, the half-life was 6.4 h, and the mean apparent half-life over four separate experiments ($\pm$ S.D.) with separate batches of tissue maintained with serum was 7.5 ± 1.1 h.

Zero-time control cultures incubated and pulse-labelled with [^{35}S]sulphate in the presence of benzyl β -D-xyloside (Fig. 1, ●) exhibited a marked increase in glycosaminoglycan synthesis, as previously reported for embryonic chicken cartilage by Robinson *et al.*, (1975), thus confirming that core-protein or xylosyltransferase synthesis is a rate-limiting step. After addition of actinomycin D, the rate of glycosaminoglycan synthesis in the presence of β -D-xyloside remained constant for 8–12 h, after which there was once more an exponential decay in the synthetic rate. In four separate experiments, the time required for the mean rate of xyloside-initiated chondroitin sulphate synthesis to reach a value half that

of the zero-time control was 17.7 ± 1.2 h (mean $\pm$ S.D.). The lag time over these same four experiments was 9.6 ± 0.6 h (mean $\pm$ S.D.), which represents the time taken before there was any detectable decrease in xyloside-initiated chondroitin sulphate synthesis after exposure to actinomycin D. The half-time of xyloside-initiated chondroitin sulphate synthesis after the lag time was 6.3 h for the results shown in Fig. 1 (insert), and the mean value over four separate experiments ($\pm$ S.D.) was 7.1 ± 1.8 h. This value is similar to the half-life for the decrease in core-protein-initiated chondroitin sulphate biosynthesis.

Actinomycin D may inhibit protein synthesis by mechanisms other than those described above (for review, see Lodish, 1976). The possibility that the observations described above were due to some action other than by depletion of mRNA was investigated by the use of an alternative inhibitor of RNA synthesis. Cordycepin (3'-deoxyadenosine) has been shown to inhibit the activity of DNA-dependent RNA polymerase (Kenow & Frederiksen, 1964) and is growth-inhibitory at a concentration of $100 \mu\text{g}/\text{ml}$ (Rich *et al.*, 1965). An experimental protocol identical with that described for Fig. 1 was followed, except that $100 \mu\text{g}$ of cordycepin/ml was substituted for actinomycin D. A similar result to that shown in Fig. 1 was obtained, with a half-life of the pool of core-protein mRNA of 7.5 h. Since cordycepin and actinomycin D inhibit RNA synthesis by different mechanisms, it is suggested that the observations described above were most likely due to inhibition of RNA synthesis.

To assess the effect of actinomycin D on the metabo-

lism of chondrocytes over a 24 h period, cultures were assayed for ATP content and for chondrocyte viability measured by exclusion of the dye Trypan Blue. Articular cartilage was maintained in culture for 5 days in medium containing 20% foetal-calf serum. On day 6 some of the tissue was placed in medium containing 10 μ g of actinomycin D/ml, and after 12 h and 25 h the ATP content of the tissue was measured. Control cultures contained 141.0 ± 17.4 ($n = 4$) pmol of ATP/mg dry wt., and after 12 h and 25 h exposure to actinomycin D the tissue contents of ATP were 141.4 ± 30.7 ($n = 3$) and 152.2 ± 7.1 ($n = 3$) pmol/mg dry wt. respectively. In a similar experiment, chondrocytes were isolated by enzymes from cartilage explants which had been treated with actinomycin D for 25 h. In control cultures the recovery of chondrocytes from the tissue was 1.643 (± 0.016 , $n = 3$) $\times 10^6$ cells/100 mg wet wt. of tissue, of which > 99% were viable; in actinomycin D-treated cultures, the recovery of chondrocytes was 1.645 (± 0.098 ; $n = 3$) $\times 10^6$ cells/100 mg wet wt., and > 99% of the cells were viable.

Hydrodynamic size of the proteoglycans synthesized in the presence of actinomycin D

Samples of 4 M-guanidinium chloride extracts from cultures incubated with actinomycin D and labelled in the presence or absence of excess β -D-xyloside were analysed by gel chromatography on Sepharose CL-2B. The elution profile of the zero-time control (Fig. 2a) shows the two labelled proteoglycan peaks characteristic of this tissue (Hascall *et al.*, 1983): a major peak with K_{av} 0.23 (peak 1), and a minor peak with K_{av} 0.71 (peak 2). After 10 or 24 h exposure to actinomycin D, cultures synthesized proteoglycans that were eluted at the same positions, the major proteoglycan being eluted with a peak K_{av} of 0.21–0.23.

In the presence of benzyl β -D-xyloside, the incorporation of [35 S]sulphate into proteoglycan is markedly decreased (Schwartz, 1977; Robinson & Robinson, 1984), and the size of the proteoglycan formed is smaller than that of control tissue, as indicated by the profile of the zero-time 4 M-guanidinium chloride extract from cultures incubated in the presence of β -D-xyloside (Fig. 2d). This profile shows the presence of a proteoglycan peak ($K_{av} \approx 0.33$), which was eluted well ahead of a larger peak of radioactivity, presumed to consist of xyloside-initiated chondroitin sulphate chains together with the smaller non-aggregating proteoglycan (Heinegård & Hascall, 1979; Campbell *et al.*, 1984) and unincorporated [35 S]sulphate at the total volume of the column. The peak at K_{av} 0.33, which made up only 4.8% of the total 35 S label incorporated into macromolecules, probably represented the large species of cartilage proteoglycan, which was diminished in size because it contained shorter glycosaminoglycan chains (see below). After incubation of cultures with actinomycin D, the proteoglycans synthesized in the presence of β -D-xyloside were eluted later than the zero-time control, with a peak K_{av} of 0.35 at 10 h (Fig. 2e) and 0.39 at 24 h (Fig. 2f). The proportion of [35 S]sulphate incorporated into these proteoglycans was 6.3% and 1.6% respectively of the total 35 S incorporation into macromolecules.

Fig. 2. Elution from Sepharose CL-2B of proteoglycans synthesized by bovine articular cartilage in the absence and presence of 1 mM-benzyl β -D-xyloside with and without actinomycin D

Guanidinium chloride extracts of explant cultures incubated with 10 μ g of actinomycin D/ml (Fig. 1), were applied to a column of Sepharose CL-2B eluted with 4 M-guanidinium chloride (V_0 , fraction 29; V_t , fraction 62). Profiles are shown for 35 S-labelled proteoglycans from cartilage incubated: (a) without additions; (b) with 10 μ g of actinomycin D/ml for 10 h; (c) with 10 μ g of actinomycin D/ml for 24 h; (d) incubated with 1.0 mM-benzyl β -D-xyloside; (e) with 10 μ g of actinomycin D/ml for 10 h and labelled in the presence of 1.0 mM-benzyl β -D-xyloside; (f) with 10 μ g of actinomycin D/ml for 24 h and labelled in the presence of 1.0 mM-benzyl β -D-xyloside.

Hydrodynamic size of the glycosaminoglycans synthesized in the presence of actinomycin D

The sizes of the glycosaminoglycan chains synthesized by the cartilage cultures, in the presence and absence of actinomycin D, on to endogenous and exogenous

Fig. 3. Elution from Sepharose CL-6B of glycosaminoglycans synthesized by bovine articular cartilage in the absence and presence of 1 mM-benzyl β -D-xyloside with and without actinomycin D

Alkali extracts of explant cultures identical with those analysed in Fig. 2 were applied to a column of Sepharose CL-6B eluted with 4 M-guanidinium chloride (V_0 , fraction 19; V_t , fraction 50). K_{av} values for the peak elution volume of zero-time cultures are indicated. The treatments were the same as those described in the legend to Fig. 2.

acceptor (Fig. 3a) were eluted with a peak K_{av} of 0.63 ($M_r \sim 10.0 \times 10^3$; Wasteson, 1971), whereas glycosaminoglycans synthesized in the presence of xyloside (Fig. 3d) were eluted with a peak K_{av} of 0.69 ($M_r \sim 7.2 \times 10^3$). That smaller chains are synthesized in the presence of β -D-xyloside is consistent with data presented previously (McQuillan *et al.*, 1984) and confirms observations by other workers (Kato *et al.*, 1978; Lohmander *et al.*, 1979a; Mitchell & Hardingham, 1982).

Glycosaminoglycans synthesized on to endogenous core-protein acceptor after exposure to actinomycin D increased in hydrodynamic size with increasing time of exposure. After 10 h (Fig. 3b), the glycosaminoglycans were eluted with a peak K_{av} of 0.58 ($M_r \sim 12.5 \times 10^3$), and after 24 h (Fig. 3c) with a peak K_{av} of 0.52 ($M_r \sim 18.0 \times 10^3$). Since the size of the proteoglycan subunit remained constant despite the significant increase in chain size, it seems likely that there were fewer glycosaminoglycan chains synthesized per core protein. In contrast with this, chondroitin sulphate chains synthesized in the presence of benzyl β -D-xyloside exhibited a marked decrease in size after exposure to actinomycin D. After 10 h (Fig. 3e) and 24 h (Fig. 3f), the xyloside-initiated chondroitin sulphate chains were eluted with peak K_{av} values of 0.81 ($M_r \sim 3.8 \times 10^3$) and 0.85 ($M_r \sim 2.4 \times 10^3$) respectively. If the sizes of the chondroitin sulphate chains synthesized on to endogenous and exogenous acceptor in the presence of xyloside were identical, then this might explain the smaller size of the proteoglycan subunit synthesized in the presence of xyloside. The further decrease in chain size with time of exposure to actinomycin D is consistent with a decrease in the size of the proteoglycan subunit with time of exposure to this inhibitor.

Effect of actinomycin D on glycosyltransferase activity in bovine articular cartilage

The observed decrease in β -xyloside-initiated glycosaminoglycan synthesis after addition of actinomycin D may have arisen through depleted enzymic activity caused by the inhibition of RNA synthesis. This possibility was investigated by the measurement of several glycosyltransferase enzymes involved in chondroitin sulphate synthesis.

Four separate batches of articular cartilage were maintained in culture with 20% foetal-calf serum for 7 days before incubation with actinomycin D for up to 24 h. After exposure to actinomycin D, tissue homogenates were prepared and assayed for activity, as described in the Experimental section. Separate cultures were maintained for determination of the rate of proteoglycan synthesis by incubation with [35 S]sulphate for 1 h. Table 1 shows the enzymic activity present in cultures of articular cartilage after 0 and 24 h exposure to actinomycin D. By 24 h after RNA synthesis was inhibited, the rate of proteoglycan synthesis had decreased to 18% of that of the zero-time control cultures. However, the greatest decrease in chondroitin sulphate glycosyltransferase enzyme activity was exhibited by galactosyltransferase-I (Table 1, 'Gal-T-I'), which decreased to 74% of the zero-time control value. The activities of the other enzymes assayed did not fall below 80% of their respective zero-time control values after 24 h in the presence of actinomycin D.

acceptors were examined by gel chromatography on Sepharose CL-6B. Alkali extracts of the cultures analysed in Fig. 2 were used, and K_{av} values of the peak elution volumes were calculated. Glycosaminoglycans synthesized at zero time on to endogenous core-protein

Table 1. Effect of actinomycin D on the activity of glycosyltransferases in articular cartilage

Cultures of bovine cartilage were incubated in the presence of 10 μ g of actinomycin D/ml for 24 h. Cultures were homogenized in enzyme buffer and assayed for glycosyltransferase activity. Enzyme activity is expressed per mg dry wt. of tissue. Each result is the mean $\pm$ S.D. for three determinations. Separate cultures were labelled with [35 S]sulphate to determine the rate of proteoglycan synthesis. Values in parentheses are expressed as a percentage of those of the zero-time control cultures. Abbreviations: Gal-T-I, galactosyltransferase-I; Gal-T-II, galactosyltransferase-II; GalNAc-T, *N*-acetylgalactosaminyltransferase; GlcA-T-II, glucuronosyltransferase-II; GlcNAc-Gal-T, UDP-galactose: *N*-acetylglucosaminide galactosyltransferase.

Time in the presence of actinomycin D (h)	[35 S] Sulphate incorporation (d.p.m./h per mg wet wt.)	10 ⁻² $\times$ Activity (nmol of substrate transferred/h)				
		Gal-T-I	Gal-T-II	GalNAc-T	GlcA-T-II	GlcNAc-Gal-T
0	3374 $\pm$ 47	14.8 $\pm$ 0.3	13.6 $\pm$ 0.3	7.5 $\pm$ 0.1	11.0 $\pm$ 0.1	31.9 $\pm$ 1.2
24	617 $\pm$ 28 (18)	10.9 $\pm$ 0.2 (74)	13.1 $\pm$ 0.1 (96)	6.3 $\pm$ 0.2 (84)	8.8 $\pm$ 0.2 (80)	23.6 $\pm$ 1.5 (74)

DISCUSSION

Addition of actinomycin D to cultures of bovine articular cartilage resulted in a first-order decline in the rate of proteoglycan synthesis by the tissue. The inclusion of benzyl β -D-xyloside in the labelling medium of cartilage cultures showed that the inhibition of chondroitin sulphate synthesis resulting from treatment with actinomycin D could be relieved for a period of approx. 10 h. This indicates that during this period neither the supply of nucleotide sugars precursors for glycosaminoglycan synthesis, nor the activity of the glycosyltransferase enzymes, is a limiting factor. In confirmation of this, the activity of several glycosyltransferases involved in chondroitin sulphate synthesis was measured in articular-cartilage cultures treated with actinomycin D for 24 h; only slight decreases in the activity of these enzymes could be detected, despite a marked inhibition of proteoglycan synthesis. This result confirms previous observations that, with the exception of xylosyltransferase, the other glycosyltransferases of chondroitin sulphate have long cellular half-lives (Schwartz, 1976; McQuillan *et al.*, 1984). We therefore propose that addition of actinomycin D to articular-cartilage cultures inhibits primarily the synthesis of mRNA coding for the proteoglycan core protein, or possibly for xylosyltransferase, the first glycosyltransferase involved in chondroitin sulphate synthesis. Schwartz (1976) has reported that xylosyltransferase has a short half-life in cultured avian chondrocytes, which suggested a regulatory function for this enzyme. Studies by Sandy *et al.* (1980), however, failed to show any marked change in xylosyltransferase activity in culture bovine articular cartilage, despite a more than 2-fold change in proteoglycan synthesis over a period of 3 days.

After 10 h exposure of articular cartilage cultures to actinomycin D, the rate of xyloside-initiated chondroitin sulphate synthesis declined with the same half-life as that measured for core-protein-initiated glycosaminoglycans inhibited by actinomycin D. A number of possible causes could underlie this delayed decline in xyloside-initiated chondroitin sulphate synthesis. Actinomycin D might have inhibited the synthesis of proteins, of short half-life, that are crucial in the metabolism of chondrocytes. This in turn would cause a fall in phosphoadenosine phosphosulphate or nucleotide sugar concentrations, or disruption of intracellular structures such as the endoplasmic reticulum or the Golgi body. Over a 25 h

period of exposure to actinomycin D this inhibitor did not affect the concentration of ATP in cartilage or alter the viability of the chondrocytes isolated from the tissue. Since little is known about the rate of turnover of enzymes involved in the formation of nucleotide precursors for chondroitin sulphate synthesis, an indirect effect of actinomycin D and cordycepin on the cellular concentrations of phosphoadenosine phosphosulphate or nucleotide sugar cannot be excluded.

The results are consistent with our previous proposal (McQuillan *et al.*, 1984) that a translation product might regulate the concerted action of the glycosyltransferases of chondroitin sulphate synthesis. In this model, the translation product, which could be the nascent proteoglycan core, a membrane structural protein, an independent regulatory protein (Fitting & Kabat, 1982) or even the xylosyltransferase, which is known to interact specifically with at least one of the other glycosyltransferases of chondroitin sulphate biosynthesis (Dorfman, 1981), would only become limiting in amount after 10 h, when its concentration fell below a critical value. We suggest that it may not be fortuitous that the rate of decay of xyloside-initiated chondroitin sulphate synthesis is the same as that for core-protein-initiated synthesis in the presence of actinomycin D. We propose that a common factor may be involved in each decay.

After 24 h in the presence of actinomycin D the chondrocytes still formed small quantities of proteoglycan subunit of normal size, even though this appeared to contain longer glycosaminoglycan chains than those synthesized by control cultures. If the xylosyltransferase was a limiting factor, this might explain the postulated decrease in substitution of the proteoglycan core. Using the same tissue incubated with cycloheximide, McQuillan *et al.*, (1984) reported a similar increase in the size of core-protein-initiated glycosaminoglycan chains. In that study, no measure of proteoglycan size was undertaken, thus preventing any conclusions about the degree of substitution of the core protein, but in other studies Kimura *et al.* (1981) and Mitchell & Hardingham (1982) reported an increase in the size of both proteoglycan subunits and glycosaminoglycan chains after inhibition of proteoglycan biosynthesis by cycloheximide in chondrocytes from Swarm-rat chondrosarcoma. Addition of benzyl β -D-xyloside to articular-cartilage cultures produced a marked decrease in the amount and in the size of the proteoglycans synthesized. This effect of β -D-

xyloside on proteoglycan size has been reported previously (Kato *et al.*, 1978; Lohmander *et al.*, 1979b) and has been attributed both to an overall switch to the synthesis of shorter chondroitin sulphate chains and to the formation of proteoglycans with fewer chondroitin sulphate chains.

We thank Mrs. M. T. Scott and Mrs. V. E. Milway for expert technical assistance, Ferntree Gully Abattoirs for the kind donation of bovine metacarpal-phalangeal joints, and the Australian National Health and Medical Research Council for financial assistance. We also acknowledge the interest and support of Professor D. A. Lowther.

REFERENCES

- Campbell, M. A., Handley, C. J., Hascall, V. C., Campbell, R. A. & Lowther, D. A. (1984) *Arch. Biochem. Biophys.* **234**, 275–289
- Dorfman, A. (1981) in *Cell Biology of Extracellular Matrix* (Hay, E. D., ed.), pp. 115–138, Plenum, New York
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
- Fellini, S. A., Hascall, V. C. & Kimura, J. H. (1984) *J. Biol. Chem.* **259**, 4634–4641
- Fitting, T. & Kabat, D. (1982) *J. Biol. Chem.* **257**, 14011–14017
- Girardi, H. J., McMichael, H. & Henle, W. (1956) *Virology* **2**, 532–544
- Hascall, V. C. & Hascall, G. K. (1981) in *Cell Biology of Extracellular Matrix* (Hay, E. D., ed.), pp. 39–63, Plenum Press, New York
- Hascall, V. C. & Kimura, J. H. (1982) *Methods Enzymol.* **82**, 769–800
- Hascall, V. C., Handley, C. J., McQuillan, D. J., Hascall, G. K., Robinson, H. C. & Lowther, D. A. (1983) *Arch. Biochem. Biophys.* **224**, 206–223
- Heinegård, D. & Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 927–934
- Kato, Y., Kimata, D., Ho, K., Karasawa, K. & Suzuki, S. (1978) *J. Biol. Chem.* **253**, 2784–2789
- Kenow, H. & Frederiksen, S. (1964) *Biochim. Biophys. Acta* **87**, 495–498
- Kimura, J. H., Hardingham, T. E., Hascall, V. C. & Solursh, (1979) *J. Biol. Chem.* **254**, 2600–2609
- Kimura, J. H., Caputo, C. B. & Hascall, V. C. (1981) *J. Biol. Chem.* **256**, 4368–4376
- Lindahl, U. & Höök, M. (1978) *Annu. Rev. Biochem.* **47**, 385–417
- Lodish, H. F. (1976) *Annu. Rev. Biochem.* **45**, 38–72
- Lohmander, L. S., Madsen, K. & Hinek, A. (1979a) *Arch. Biochem. Biophys.* **192**, 148–157
- Lohmander, L. S., Hascall, V. C. & Caplan, A. I. (1979b) *J. Biol. Chem.* **254**, 10551–10561
- McQuillan, D. J., Handley, C. J., Robinson, H. C., Ng, K., Tzaicos, C., Brooks, P. R. & Lowther, D. A. (1984) *Biochem. J.* **224**, 977–988
- Mitchell, D. & Hardingham, T. (1981) *Biochem. J.* **196**, 521–529
- Mitchell, D. & Hardingham, T. (1982) *Biochem. J.* **202**, 387–395
- Pasternack, S. G., Veis, A. & Breen, M. (1974) *J. Biol. Chem.* **249**, 2206–2211
- Reich, E. (1964) *Science* **143**, 684–689
- Rich, M. A., Meyers, P., Weinbaum, G., Cory, J. G. & Suhadolnik, R. J. (1965) *Biochim. Biophys. Acta* **95**, 194–204
- Robinson, H. C., Brett, M. J., Tralaggan, P. J., Lowther, D. A. & Okayama, M. (1975) *Biochem. J.* **148**, 25–34
- Robinson, J. A. & Robinson, H. C. (1984) *Biochem. Int.* **9**, 501–509
- Robinson, J. A. & Robinson, H. C. (1985) *Biochem. J.* **227**, 805–814
- Rodén, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
- Sandy, J. D., Brown, H. L. & Lowther, D. A. (1980) *Biochem. J.* **188**, 119–130
- Schwartz, N. B. (1976) *J. Biol. Chem.* **251**, 3346–3351
- Schwartz, N. B. (1977) *J. Biol. Chem.* **252**, 6316–6321
- Thonar, E. J.-M., Lohmander, L. S., Kimura, J. H., Fellini, S. A., Yanagishita, M. & Hascall, V. C. (1983) *J. Biol. Chem.* **258**, 11564–11570
- Upholt, W. B., Vertel, B. M. & Dorfman, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4847–4851
- Vertel, B. M., Upholt, W. B. & Dorfman, A. (1984) *Biochem. J.* **217**, 259–263
- Wasteson, Å. (1971) *J. Chromatogr.* **59**, 87–97

Received 21 May 1985/11 November 1985; accepted 13 December 1985