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

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The effect of cycloheximide on chondroitin sulphate biosynthesis was studied in bovine articular cartilage maintained in culture. Addition of 0.4mM-cycloheximide to the culture medium was followed, over the next 4h, by a first-order decrease in the rate of incorporation of [^{35}S]sulphate into glycosaminoglycan (half-life, $t_{\frac{1}{2}} = 32$min), which is consistent with the depletion of a pool of proteoglycan core protein. Addition of 1.0mM-benzyl β-D-xyloside increased the rate of incorporation of [^{35}S]sulphate and [^{3}H]acetate into glycosaminoglycan, but this elevated rate was also diminished by cycloheximide. It was concluded that cycloheximide exerted two effects on the tissue; not only did it inhibit the synthesis of the core protein, but it also lowered the tissue's capacity for chondroitin sulphate chain synthesis. Similar results were obtained with chick chondrocytes grown in high-density cultures. Although the exact mechanism of this secondary effect of cycloheximide is not known, it was shown that there was no detectable change in cellular ATP concentration or in the amount of three glycosyltransferases (galactosyltransferase-I, *N*-acetylgalactosaminyltransferase and glucuronosyltransferase-II) involved in chondroitin sulphate chain synthesis. The sizes of the glycosaminoglycan chains formed in the presence of cycloheximide were larger than those formed in control cultures, whereas those synthesized in the presence of benzyl β-D-xyloside were consistently smaller, irrespective of the presence of cycloheximide. These results suggest that β-D-xylosides must be used with caution to study chondroitin sulphate biosynthesis as an event entirely independent of proteoglycan core-protein synthesis, and they also indicate a possible involvement of the core protein in the activation of the enzymes of chondroitin sulphate synthesis.

Chondrocytes of articular cartilage are specialized cells which synthesize chondroitin sulphate-proteoglycan and cartilage-specific collagen, these being the major constituents of the extracellular matrix. The structure of cartilage chondroitin sulphate-proteoglycan is relatively well characterized (for review, see Hascall & Hascall, 1981), whereas the biosynthesis and the control of biosynthesis of these important macromolecules is not so well understood.

Cartilage proteoglycan consists of a core protein of high M_r to which are added up to 150 chondroitin sulphate chains ($M_r \sim 1 \times 10^4$ per chain), 50 keratan sulphate chains ($M_r \sim 5 \times 10^3$ per chain), and about 100 *N*- and *O*-linked oligosaccharides. The biosynthesis of the proteoglycan molecule is dependent on the synthesis of the core protein at the rough endoplasmic reticulum (Upholt *et al.*, 1979). This is followed by extensive post-translational modifications, which include the addition of oligosaccharides and glycosaminoglycans at the Golgi apparatus before secretion from the cell into the extracellular matrix (Thonar *et al.*, 1983; Fellini *et al.*, 1984).

The synthesis of chondroitin sulphate, the predominant glycosaminoglycan of cartilage proteoglycan, involves six specific glycosyltransferases and two sulphate transferases. Apart from xylosyltransferase, which transfers xylose from UDP-xylose to the hydroxy groups of serine residues in the core protein, the glycosyltransferases are membrane-bound in the Golgi apparatus, possibly as an enzyme complex (Horwitz & Dorfman, 1968; Stoolmiller *et al.*, 1972;

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Dorfman, 1981). Synthesis of chondroitin sulphate chains is dependent on the presence of a xylosylated core protein, so that if protein synthesis is inhibited then chondroitin sulphate synthesis is also inhibited. It is possible to alleviate the requirement for xylosylated core protein by introducing exogenous xylose acceptors (Robinson *et al.*, 1975) and in this way to dissociate core-protein synthesis from chondroitin sulphate chain synthesis.

In the present study two well-characterized, but quite distinct, systems of tissue culture have been utilized to examine the biosynthesis of macromolecules by chondrocytes *in vitro*. It has previously been shown in this laboratory (Hascall *et al.*, 1983) that slices from the metacarpal-phalangeal articular cartilage of 1–2-year-old steers can be maintained in culture for up to 3 weeks. In the presence of foetal-calf serum, these cultures achieve a steady state within 2–3 days with an essentially constant rate of proteoglycan synthesis while still maintaining a constant content of proteoglycan in the matrix. Since the tissue is in a steady state, it becomes possible to examine synthesis of proteoglycan and glycosaminoglycan over relatively short periods of time after perturbation of the cell's normal metabolism. Observations in this system were verified by similar experiments on monolayer cultures of chondrocytes isolated from embryonic chicks (Handley *et al.*, 1975).

Previous studies have shown that, in the presence of β-D-xylosides, chondroitin sulphate synthesis is stimulated (Robinson *et al.*, 1975; Galligani *et al.*, 1975), suggesting that the synthesis of core protein is a rate-limiting step. It has been presumed that the presence of β-D-xylosides allows the glycosyltransferases to act independent of the presence of protein synthesis (Schwartz *et al.*, 1974; Dorfman, 1981). In the present paper we describe experiments which suggest a link between protein synthesis and the activity of the enzymes of chondroitin sulphate synthesis. We postulate that there exists a mechanism within the chondrocyte that limits the concerted activity of the glycosyltransferases in response to a decrease in protein synthesis.

Experimental

Materials

Radiolabelled precursors were obtained as follows: [^{35}S]sulphate (>5mCi/μg), UDP-[U-^{14}C]-glucuronic acid (260Ci/mol) and UDP-[6-^{3}H]-galactose (15.7Ci/mmol) from Amersham International (Amersham, Bucks., U.K.); sodium [^{3}H]acetate (1Ci/mol) and L-[4,5-^{3}H(*n*)]leucine (5Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.). Benzyl β-D-xyloside was prepared as described by Robinson *et al.* (1975). Sephadex G-25 in prepacked PD-10 columns and Sepharose CL-6B were from Pharmacia (Uppsala, Sweden). Sterile plastic (screw-capped) vials (20ml) were obtained from Disposable Products (Melbourne, Vic., Australia). Sterile culture flasks (200ml) and plastic Leighton tubes (5.5cm^2) were obtained from Nunc (Roskilde, Denmark). Foetal-calf serum was obtained from Gibco (Auckland, New Zealand). Papain, cycloheximide, UDP-glucuronic acid, UDP-galactose and β-glucuronidase (EC 3.2.1.31) were purchased from Sigma (St. Louis, MO, U.S.A.), and 2-(4-aminophenyl)-ethylamine was purchased from Aldrich (Milwaukee, WI, U.S.A.). Dulbecco's modified Eagle's medium and a preparation of Eagle's non-essential amino acids were purchased from the Commonwealth Serum Laboratories (Melbourne, Vic., Australia). Dowex 50WX2 (H^+ form; 100–200 mesh) was from Sigma and Amberlite CG 120 (H^+ form; 200–400 mesh) was from Mallinckrodt (St. Louis, MO, U.S.A.). Pyridine 3-*O*-β-*N*-acetyl-D-glucosaminide was prepared by condensing 3-hydroxypyridine with acetochloroglucosamine as described by Leaback (1963). The product was de-*O*-acetylated by treatment with sodium methoxide as described by Thompson *et al.* (1963). All other materials were obtained as previously described (Hascall *et al.*, 1983).

Cartilage cultures

Cultures of articular cartilage from metacarpal–phalangeal joints of 1–2-year-old steers were set up as previously described (Hascall *et al.*, 1983), except that initially about 2g of tissue was distributed into 30ml of medium supplemented with 20% (v/v) foetal-calf serum (Handley & Lowther, 1977). Cultures were incubated at 37°C with daily changes of medium for 5 days. After 6 days in culture, tissue was distributed into preweighed, sterile, plastic vials containing 4ml of medium. Each vial was reweighed to determine the wet weight of tissue. In all experiments described, 50–120mg wet wt. of tissue was used for each culture, unless otherwise indicated. Cultures were then maintained for 24h before further use.

Chick chondrocyte cultures

Chondrocytes were prepared from epiphyses of the femora and tibiae of 13-day-embryonic White Leghorn chicks as described by Handley *et al.* (1975). Primary cultures were inoculated at a density of about 2×10^6 cells in 5ml of medium containing 20% (v/v) foetal-calf serum (Handley & Lowther, 1977) into flat-bottomed Leighton culture tubes (surface area 5.5cm^2). Cultures were maintained for 14 days with daily changes of medium before use. Within each experiment all

cultures were derived from one chondrocyte preparation.

Incubation of cultures with [^{35}S]sulphate and [^{3}H]acetate

For each separate experiment a large batch of labelling medium was prepared, such that each culture was incubated in an identical batch. Before labelling, each culture was preincubated for 1 h in fresh medium either with or without 1 mM-benzyl β-D-xyloside.

After preincubation, the medium was replaced with 2ml of medium containing 20–30μCi of [^{35}S]sulphate/ml or 6.7μCi of [^{35}S]sulphate and 6.7μCi of [^{3}H]acetate/ml as required for the particular experiment. Cultures were incubated with [^{35}S]sulphate for 20min or 1h. Where dual label was used, the labelling period was 1h. Incubation of explant cultures with label was performed at 37°C in a shaking water bath in loose-capped culture vials. Incubation of chick chondrocytes was carried out by replacing the culture medium with 3ml of medium containing the labelled precursors and incubating for either 20min or 1h. Experiments showed that both [^{35}S]sulphate and [^{3}H]acetate were incorporated linearly with respect to time up to 4h, with no apparent lag time.

Cycloheximide was dissolved in medium at a concentration of 0.05M, sterilized by passage through a 0.22μm microporous membrane, and added to the cultures to give the required final concentration. In a typical experiment, where cycloheximide was used, control cultures were incubated with labelled precursors both with and without benzyl β-D-xyloside. Cycloheximide was then added to the remaining cultures; the time of this addition was designated as zero time. After the addition of cycloheximide, the cultures were incubated with label at 20min intervals in the presence of cycloheximide, both with and without benzyl β-D-xyloside. Unless otherwise indicated, all points shown represent the means of duplicate determinations.

Extraction procedures

For experiments with [^{35}S]sulphate as the precursor, labelling was terminated by the direct addition of 5.0M-NaOH to give a final concentration of 0.5M. The tissue was extracted for 1–2 days at room temperature to solubilize glycosaminoglycans. Samples of the extracts were eluted on Sephadex G-25 columns (1.5cm × 5.5cm) equilibrated with 4M-guanidinium chloride/0.2M-Na_2SO_4/0.1M-sodium acetate/0.5% (v/v) Triton X-100, pH6, to determine the amount of incorporated radioactivity (Kimura *et al.*, 1979).

Termination of incubations with [^{35}S]sulphate and/or [^{3}H]acetate was accomplished by freezing at −20°C, followed by digestion of the entire medium and tissue with papain. The labelled glycosaminoglycans were isolated by ion-exchange chromatography on DEAE-cellulose columns as described by Handley & Lowther (1976). The incorporation of [^{35}S]sulphate and [^{3}H]acetate into glycosaminoglycans was then determined.

Incorporation of [^{3}H]leucine into total protein

Explant cultures were prepared and maintained in culture as described above. Medium was prepared containing [^{3}H]leucine to give a final radioactivity of about 20μCi/ml (final specific radioactivity of about 1.7Ci/mmol). Individual cultures were preincubated for 1h in fresh medium, followed by incubation in 2ml of medium containing [^{3}H]leucine for 20min. Incubations were stopped by replacement of the medium with 2ml of ice-cold 5% (w/v) trichloroacetic acid containing 20mM-leucine. Acid-insoluble protein was isolated as described by Hascall *et al.* (1983) and the rate of incorporation of [^{3}H]leucine into protein determined. A duplicate set of cultures was incubated with [^{35}S]sulphate.

Column chromatography

A column (0.8 × 100cm) of Sepharose CL-6B was eluted with 4M-guanidinium chloride/0.2M-Na_2SO_4/0.5% Triton X-100/0.1M-sodium acetate, pH6, at a flow rate of 6ml/h. Fractions (approx. 1.4ml) were collected and assayed for radioactivity (Hascall *et al.*, 1983).

Preparation of cultures for assay of ATP and enzyme activity

Explant cultures were set up and maintained in batches of 2–3g per 30ml of medium. For a single experiment the cultures were derived from the cartilage of two joints, which were pooled and then divided into equal portions. On day 6 each culture was incubated with 0.5mM-cycloheximide, and after the incubation period the medium was removed, and the tissue blotted dry and then frozen in a plastic tube with solid CO_2. The frozen tissue was pulverized in a Spex mill (Spex Industries, Metuchen, NJ, U.S.A.) in liquid N_2. The powdered tissue was transferred to a glass homogenizer (Duall 22; Kontes Glass Co., Vineland, NJ, U.S.A.) and homogenized in either 3ml of 5% trichloroacetic acid for ATP assay or 3ml of enzyme buffer [50mM-KCl, 15mM-$MgCl_2$, 1.25mM-EDTA, 0.05mM-Phenol Red, 0.5% (v/v) Nonidet P-40 detergent, pH7.0] for enzyme assays until a homogeneous suspension was attained.

Chick chondrocyte cultures were set up as described and incubated with cycloheximide for the appropriate time. After the incubation, each

cell layer was scraped into a Duall glass homogenizer containing either 1 ml of 5% trichloroacetic acid or enzyme buffer (see above).

Determination of ATP

Acid-insoluble material of cartilage explants and chick chondrocyte homogenates was removed by centrifugation for 20 min at 4000 rev./min. The supernatants were removed, their volumes determined, and a portion was assayed for ATP as described by Sutton & Pollak (1978). Total ATP content was determined for each culture and expressed either as nmol of ATP/mg dry wt. of the cartilage residues, or nmol of ATP per culture flask of chick chondrocytes. The dry weight of the acid-insoluble cartilage residues was determined after washing twice with 5% trichloroacetic acid, then resuspension in a small volume of 2M-NH_3, followed by freeze-drying in preweighed tubes. Dry weights were determined by direct weighing.

Enzyme assays

The following glycosyltransferase activities were determined in whole homogenates of cartilage or chondrocyte cultures with cationic substrates, which enabled the products of glycosyl transfer to be isolated by a simple ion-exchange procedure (Robinson & Robinson, 1981). All assays described were verified by exhibiting linearity of reaction velocity versus enzyme concentration (H. C. Robinson, J. A. Robinson, K. Ng & C. Tzaicos, unpublished work).

Galactosyltransferase-I (*UDP-galactose:β-D-xylopyranoside galactosyltransferase*). This was assayed by incubating tissue homogenate (0.04 ml) for 8 h at 37°C with 20 mM-pyridine 2-*S*-β-D-thioxylopyranoside (Robinson & Robinson, 1981) and 5.0 mM-UDP-[6-^{3}H]galactose (1.0 Ci/mol) in a final volume of 0.05 ml of solution containing (final concns.) 50 mM-Mes (4-morpholine-ethanesulphonic acid)/KOH buffer, pH 6.0, 0.4 M-KCl, 12 mM-$MgCl_2$, 10 mM-$MnCl_2$, 1.0 mM-EDTA, 0.04 mM-Phenol Red and 0.4% Nonidet P-40. Each incubation was stopped by the addition of 0.5 ml of 6% trichloroacetic acid, and the product of galactose transfer was isolated on a column of Amberlite CG 120 (H^+ form) and assayed for ^{3}H radioactivity as described previously (Robinson & Robinson, 1981).

N-Acetylgalactosaminyltransferase (*UDP-N-acetylgalactosamine:chondroitin N-acetylgalactosaminyltransferase*). This was assayed by measuring the transfer of *N*-acetylgalactosamine to cationic acceptors prepared by condensing non-sulphated tetrasaccharide derived from chondroitin by hyaluronidase digestion (Telser *et al.*, 1966) with 2-(4-aminophenyl)ethylamine (Zopf *et al.*, 1978). UDP-*N*-[1-^{14}C]acetylgalactosamine was prepared as described by Telser *et al.* (1965). The enzymic activity was assayed by incubating tissue homogenate (0.08 ml) for 6 h at 37°C with 2.2 mM-UDP-*N*-[1-^{14}C]acetylgalactosamine (0.069 Ci/mol) and 10 mM-tetrasaccharide acceptor in a final volume of 0.10 ml of solution containing (final concn.) 50 mM-Mes/NaOH buffer, pH 6.5, 10 mM-$MnCl_2$, 12 mM-$MgCl_2$, 1.0 mM-EDTA, 40 mM-KCl, 0.04 mM-Phenol Red and 0.4% Nonidet P-40 detergent. The reaction was stopped by addition of trichloroacetic acid (final concn. 5%). The reaction mixture was diluted 10-fold with distilled water and the product was isolated by absorption on to columns (4.0 cm × 0.5 cm diameter) of Dowex 50 WX2 (H^+ form, 100–200 mesh) in 0.5% trichloroacetic acid. The columns were washed thoroughly with 0.5% trichloroacetic acid and the labelled product was eluted with 2M-NH_3, evaporated to dryness and assayed for radioactivity.

Glucuronosyltransferase-II (*UDP-D-glucuronic acid:chondroitin D-glucuronosyltransferase*). This was assayed by measuring transfer of D-[^{14}C]-glucuronic acid to a cationic trisaccharide acceptor prepared from the tetrasaccharide acceptor described above for assay of *N*-acetylgalactosaminyltransferase, by digestion with β-glucuronidase (Telser *et al.*, 1966). The assay procedure was the same as for the assay of *N*-acetylgalactosaminyltransferase, except that 12.5 mM trisaccharide acceptor and 2.6 mM-UDP-D-[U-^{14}C]glucuronic acid (0.32 Ci/mol) were used.

UDP-galactose:β-N-acetylglucosaminide galactosyltransferase. This was assayed by incubating tissue homogenate (0.03 ml) for 4 h at 37°C with 10 mM-pyridine 3-*O*-β-*N*-acetyl-D-glucosaminide and 5.0 mM-UDP-[6-^{3}H]galactose (1.0 Ci/mol) in a final volume of 0.05 ml of solution containing (final concns.) 50 mM-glycylglycine/NaOH buffer, pH 8.2, 30 mM-KCl, 9 mM-$MgCl_2$, 0.75 mM-EDTA, 0.03 mM-Phenol Red, 10 mM-$MnCl_2$ and 0.3% Nonidet P-40. The reaction was stopped with 1.0 ml of 5% trichloroacetic acid, and the product was isolated on a column of Amberlite CG 120 (H^+ form) as described above.

Results and discussion

Effect of cycloheximide on explant cultures and isolated chick chondrocytes

Fig. 1 describes the rate of synthesis of sulphated glycosaminoglycans in the presence of increasing concentrations of cycloheximide. When the concentration of cycloheximide exceeded 0.05 mM, proteoglycan synthesis was inhibited by more than 90%. The measurable synthesis of proteoglycan that persists after 100 min in the presence of high concentrations of cycloheximide presumably reflects the presence of a low amount of core

protein in an intracellular pool. A similar dose–response curve for cycloheximide was established for chick chondrocytes in culture (results not shown).

Cycloheximide inhibits peptide-chain initiation as well as chain elongation by interfering with the function of the 60S ribosomal subunit; it is reported that the inhibition of protein synthesis can be reversed on removal of cycloheximide (Pestka, 1971). Cycloheximide was shown not to be toxic to chondrocytes over 24h by reversal of the depression of proteoglycan synthesis. Cultures of chick chondrocytes and cartilage explants were exposed to cycloheximide for 24h and then maintained in cycloheximide-free medium for a further 48h (Table 1). [^{35}S]Sulphate incorporation into proteoglycan was determined at various times both before and after exposure to cycloheximide. Cultures maintained in the presence of cycloheximide for 24h regained their original glycosaminoglycan synthesis after 48h in cycloheximide-free medium.

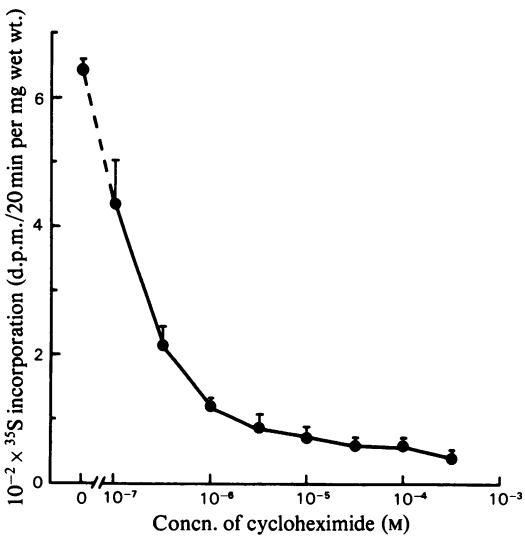

Fig. 1. *Effect of cycloheximide concentration on the rate of glycosaminoglycan synthesis in bovine articular cartilage*
Bovine articular cartilage was maintained in explant culture for 7 days and then incubated in the presence of cycloheximide concentrations ranging from 0.1 μM to 0.5mM for 100min. The rate of proteoglycan synthesis was determined by incubation in the presence of [^{35}S]sulphate for 20min, followed by extraction and quantification of the ^{35}S-labelled glycosaminoglycans. Each result is the mean of two determinations, with the range of each set of duplicates shown by the error bars.

Chondrocytes have been shown to possess an intracellular pool of core protein which initiates proteoglycan synthesis (Kimura *et al.*, 1981; Mitchell & Hardingham, 1981). The presence of this intracellular pool of core protein can be demonstrated by measuring synthesis of both proteoglycan and total protein in the presence of cycloheximide. Explant cultures of articular cartilage were set up and, after 7 days in culture, cartilage was transferred into fresh medium containing cycloheximide. At 20min intervals thereafter, cultures were incubated with either [^{35}S]sulphate or [^{3}H]leucine for 20min.

Fig. 2 shows the rate of incorporation of [^{35}S]sulphate into glycosaminoglycans and [^{3}H]-leucine into protein decreasing as a function of time of incubation with cycloheximide. Total protein synthesis was decreased to a negligible value within 20min of exposure to 0.4mM-cycloheximide, suggesting a rapid inhibition of protein synthesis. Sulphate incorporation into proteoglycan, however, exhibited an exponential decay, which reflected the depletion of the available pool of proteoglycan core protein. It was assumed that the depletion of the proteoglycan core protein

Table 1. *Reversibility of cycloheximide inhibition of glycosaminoglycan synthesis*
Monolayer cultures of chick chondrocytes and cartilage explant cultures were exposed to 0.1mM- and 0.4mM-cycloheximide respectively for up to 72h. After 24h some cultures were transferred to cycloheximide-free medium. At the times shown cultures were incubated for 1h with [^{35}S]sulphate, and the rate of incorporation of [^{35}S]sulphate into glycosaminoglycans was determined. Each result is the mean of two determinations ± range: ND, not determined.

	[^{35}S]Sulphate incorporation into glycosaminoglycans	
Treatment with cycloheximide	Cartilage explants (10^{-2} × d.p.m./h per mg wet wt.)	Chick chondrocytes (10^{-3} × d.p.m./h per culture)
Zero-time control	18.13 ± 1.32 (100%)*	25.8 ± 2.9 (100%)*
72h control	17.82 ± 2.00 (98%)	ND
Cycloheximide for 24h	0.05 ± 0.001 (0.5%)	3.36 ± 0.07 (13%)
Cycloheximide for 24h followed by 48h with no cycloheximide	16.89 ± 0.99 (93%)	35.7 ± 4.3 (138%)
Cycloheximide for 72h	0 (0%)	6.33 ± 0.69 (25%)

* Zero-time controls arbitrarily designated 100% relative to other values in the same column.

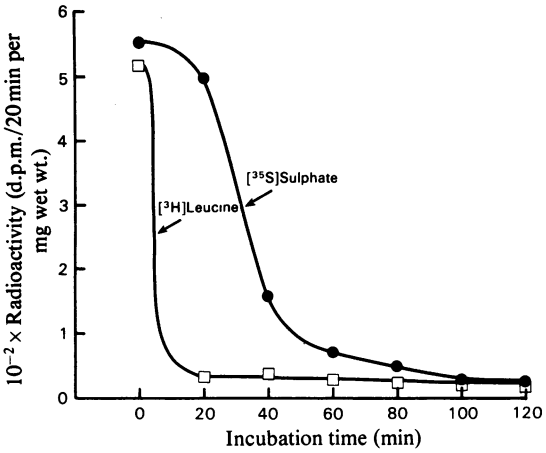

Fig. 2. *Effect of cycloheximide on the rate of glycosaminoglycan and total protein synthesis in bovine articular cartilage*

Bovine articular cartilage was maintained in explant culture for 7 days. At zero time (on day 7), 0.4mM-cycloheximide was introduced to individual cultures and, at 20min intervals, cultures were incubated with either [^{35}S]sulphate or [^{3}H]leucine for 20min. ^{35}S-labelled glycosaminoglycans were isolated from cartilage plus medium by alkali extraction followed by gel chromatography on PD-10 columns. Acid-insoluble protein was separated from unincorporated [^{3}H]leucine by extensive washing in the presence of 20mM-leucine, followed by papain digestion (see the Experimental section). The rate of [^{35}S]sulphate incorporation into proteoglycan (●) and [^{3}H]leucine incorporation into acid-insoluble protein (□) was calculated for each 20min interval.

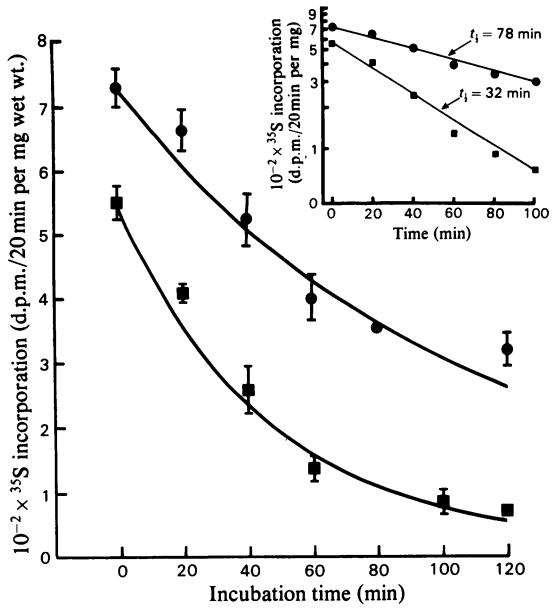

Fig. 3. *Effect of cycloheximide on the rate of synthesis of glycosaminoglycans in the absence and presence of benzyl β-D-xyloside in bovine articular cartilage*

Bovine articular cartilage was maintained in explant culture as described in the Experimental section. At zero time, individual cultures were exposed to 0.4mM-cycloheximide for up to 120min. At 20min intervals, duplicate cultures were incubated with [^{35}S]sulphate for 20min with (●) or without (■) a 1h preincubation in the presence of 1mM-benzyl β-D-xyloside. Incubation was stopped by the direct addition of concentrated NaOH, followed by solubilization of the glycosaminoglycans in the tissue and medium. Incorporation of [^{35}S]sulphate into glycosaminoglycans was quantified by gel chromatography on PD-10 columns and expressed per mg wet wt. of tissue. The experimental points are shown with the range of each set of duplicates indicated by the error bars. The continuous lines describe the theoretical curves for a first-order decay with a $t_{\frac{1}{2}}$ of 32min (lower curve) and a $t_{\frac{1}{2}}$ of 78min (upper curve). The $t_{\frac{1}{2}}$ values were obtained from semi-logarithmic plots of the experimental results, and these are shown in the insert.

followed first-order kinetics, so that an apparent half-life for the process could be determined, as described by Kimura *et al.* (1981). The half-life represents the time required for the incorporation rate to fall to one-half of the initial rate without cycloheximide and was determined from the gradient of a plot of the logarithm of the incorporation rate versus time (Fig. 3). The apparent half-life measured over five different experiments with separate batches of tissues was 32 ± 1.1min (mean ± S.D.); this value is shorter than the value of 96min reported by Kimura *et al.* (1981) for rat chondrosarcoma cells in cell cultures. The reason for this difference is unknown.

D-Xylose and β-D-xylosides are able to function as exogenous initiators of chondroitin sulphate synthesis and have been used to determine the capacity of chondrocytes to synthesize chondroitin sulphate. The purpose of the following experiments was to investigate the effect of cycloheximide on the capacity of chondrocytes to synthesize chondroitin sulphate initiated by β-D-xylosides. Benzyl β-D-xyloside was selected because it is most effective as an initiator of chondroitin sulphate synthesis and, over a 2h period, exhibits no toxicity (Robinson & Robinson, 1981).

Explant cultures of adult articular cartilage were incubated with 0.4mM-cyclohexmide for up to 2h, and at 20min intervals during this period cultures were incubated with [^{35}S]sulphate for 20min (Fig. 3, lower curve). A parallel set of cultures was treated in the same way, except that

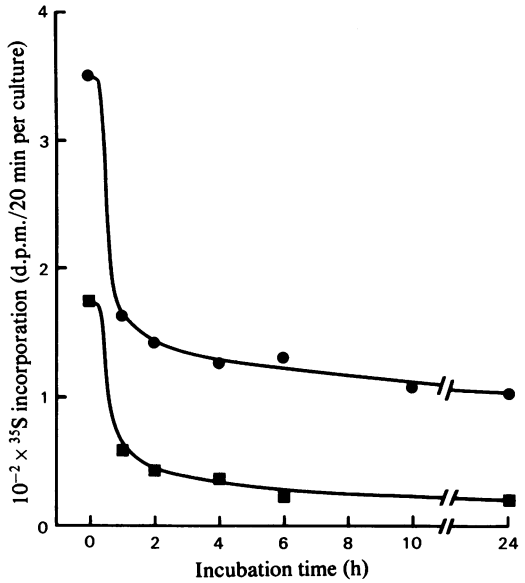

Fig. 4. *Effect of cycloheximide on glycosaminoglycan synthesis by chick chondrocyte monolayers in the absence and presence of benzyl β-D-xyloside*

Chick chondrocytes were isolated and inoculated into culture flasks at a density of about 2×10^6 cells per flask, as described in the Experimental section. Cultures were exposed to 0.1 mM-cycloheximide at zero time and maintained for up to 24 h. At the times shown, cultures were incubated with [^{35}S]sulphate for 20 min with (●) or without (■) a 1 h preincubation in the presence of 1 mM-benzyl β-D-xyloside. Incubations were stopped by the addition of alkali, and the glycosaminoglycans in the cells and medium were solubilized. [^{35}S]Sulphate incorporation was determined by isolation of the macromolecular fraction of the extracts on PD-10 columns. The rate of incorporation of [^{35}S]sulphate into glycosaminoglycans is expressed per culture flask, since the cell density in each culture was assumed to be the same.

they were preincubated for 1 h with 1 mM-benzyl β-D-xyloside before incubation with [^{35}S]sulphate (Fig. 3, upper curve). Glycosaminoglycan synthesis in the presence of core protein acceptor decreased in an exponential manner, with a $t_{\frac{1}{2}}$ for the pool of proteoglycan core protein of 32 min. Cultures incubated and pulse-labelled in the presence of an excess of benzyl β-D-xyloside exhibited a marked increase in glycosaminoglycan synthesis, confirming that the availability of core protein is a rate-limiting step in proteoglycan synthesis. Although the rate of chondroitin sulphate synthesis by cartilage was increased in the presence of benzyl β-D-xyloside, there was nevertheless a decrease in this rate on addition of cycloheximide to the cultures. The rate of synthesis of xyloside-initiated chondroitin sulphate also decreased with first-order kinetics with a $t_{\frac{1}{2}}$ of 78 min. This suggests that addition of β-D-xylosides to the cultures only partially relieved the inhibition of synthesis induced by cycloheximide.

The possibility that the observations reported above were exclusive to bovine articular cartilage in culture was investigated by examining the effect of cycloheximide on cultures of chick chondrocytes. On day 15 of culture, cycloheximide was added to a concentration of 0.1 mM and the cultures were maintained for up to 24 h. At the times shown (Fig. 4), cultures were incubated with [^{35}S]sulphate for 20 min, either with or without a 1 h preincubation with benzyl β-D-xyloside. As in the explant system, the rate of glycosaminoglycan synthesis in the presence of endogenous core protein and exogenous xyloside decreased after the addition of cycloheximide.

Since the extent of sulphation of chondroitin sulphate and the ratio of 4- to 6-sulphation is known to change in cells under different conditions of culture (Speight *et al.*, 1981), the observed inhibition by cycloheximide of [^{35}S]sulphate incorporation into xyloside-initiated chondroitin sulphate could have come about by an inhibition of sulphation alone. To eliminate this possibility, [^{3}H]acetate was used as an alternative precursor to [^{35}S]sulphate for chondroitin sulphate biosynthesis.

Explant cultures of articular cartilage were set up and incubated in the presence of cycloheximide for up to 4 h (Fig. 5). At the times shown, cultures were incubated in the presence of [^{35}S]sulphate and [^{3}H]acetate for 1 h. A parallel set of cultures was also exposed to 1 mM-benzyl β-D-xyloside. The incubations were stopped by freezing the entire medium and tissue, and the glycosaminoglycans were isolated. The rates of incorporation of [^{3}H]acetate and [^{35}S]sulphate into glycosaminoglycans with and without excess benzyl β-D-xyloside are shown in Fig. 5. After addition of cycloheximide, the incorporation of [^{3}H]acetate into glycosaminoglycans synthesized in the presence of endogenous or exogenous initiators decreased in a similar manner to that observed for the incorporation of [^{35}S]sulphate.

A similar protocol was carried out on chick chondrocytes, which were exposed to cycloheximide for up to 24 h (Fig. 6). At the times shown, cultures were incubated with [^{3}H]acetate. A similar pattern was obtained to that seen for [^{35}S]sulphate incorporation in the presence of cycloheximide. On the basis of these observations, it was presumed that neither the sulphotransferase activity nor the degree of sulphation was responsible for the observed changes in the rate of [^{35}S]sulphate incorporation.

We concluded that there was a secondary effect

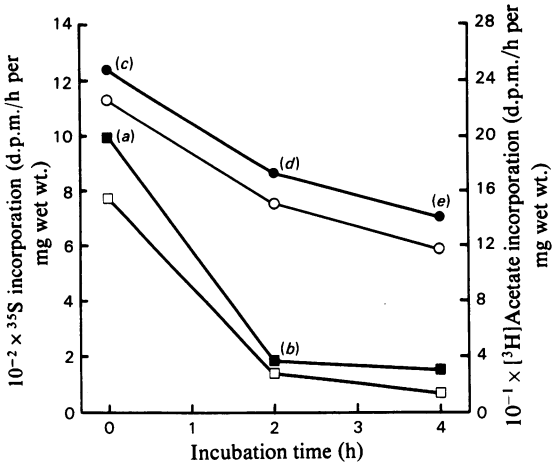

Fig. 5. *Comparison of the effects of cycloheximide on the incorporation of [^{35}S]sulphate and [^3H]acetate into glycosaminoglycans in bovine articular cartilage*

Explant cultures of bovine articular cartilage were set up and maintained for 7 days and then exposed to 0.4mM-cycloheximide for up to 4h. Labelling media containing both [^{35}S]sulphate and [^{3}H]acetate were introduced to individual cultures at 1 and 3h following the addition of cycloheximide. Incorporation of label was stopped after 1h by freezing the cultures at −20°C. Before labelling, some of the cultures were preincubated for 1h with 1mM-benzyl β-D-xyloside. ^{35}S- and ^{3}H-labelled glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography. The rate of incorporation of [^{35}S]sulphate (●, ■) and [^{3}H]acetate (○, □) into glycosaminoglycan was determined with (●, ○) and without (■, □) added benzyl β-D-xyloside. Extracts from points designated (*a*)–(*e*) were further analysed by gel chromatography on Sepharose CL-6B (see Fig. 7).

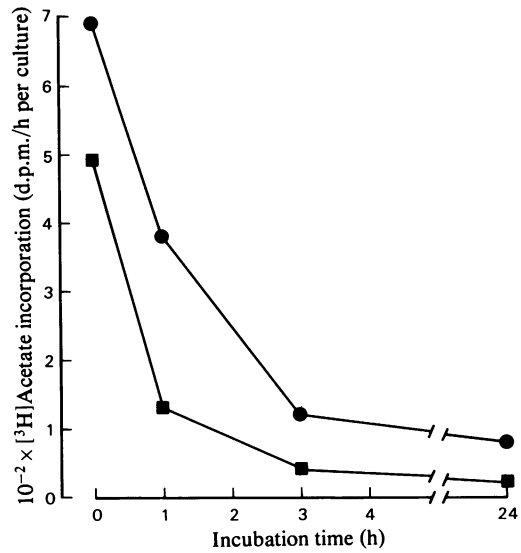

Fig. 6. *Effect of cycloheximide on the incorporation of [^3H]acetate into glycosaminoglycans in chick chondrocyte monolayers*

Chick chondrocyte cultures were set up and treated as in Fig. 4. Cultures were incubated with [^{3}H]-acetate for 1h with (●) or without (■) 1mM-benzyl β-D-xyloside. Incubation was stopped by freezing at −20°C. ^{3}H-Labelled glycosaminoglycans were isolated from the cells plus medium by papain digestion followed by chromatography on DEAE-cellulose. The rate of incorporation of [^{3}H]acetate into glycosaminoglycans was determined.

of cycloheximide on the activity of enzymes involved in glycosaminoglycan synthesis in bovine articular cartilage and isolated chick chondrocytes. This became apparent in xyloside-treated cultures where, on addition of cycloheximide, there was a decrease in glycosaminoglycan synthesis which essentially followed the decrease in the availability of proteoglycan core protein, a result which suggests that as the pool of core protein is depleted there is a concomitant decrease in the activity of the enzymes of glycosaminoglycan synthesis. In experiments using chondrocytes from Swarm rat chondrosarcoma, no such effect of cycloheximide on xyloside-initiated glycosaminoglycan synthesis was observed (Kimura *et al.*, 1981; Mitchell & Hardingham, 1982); however, those authors employed xyloside with a different aglycan group and used different experimental conditions.

The size of the glycosaminoglycan chains synthesized by cartilage cultures, with and without cycloheximide, on to endogenous core protein or exogenous xyloside was examined by gel chromatography on Sepharose CL-6B (Fig. 7). Extracts from zero-time and 2h time points in the presence of cycloheximide (Fig. 5, *a* and *b*) and zero-time, 2h and 4h time points in the presence of cycloheximide with added xyloside (Fig. 5, *c*, *d* and *e*) were used, and $K_{av.}$ values of the peak elution volumes were calculated. Glycosaminoglycans synthesized at zero time (Fig. 7, trace *a*) on to endogenous acceptor were eluted with a peak $K_{av.}$ of 0.62 ($M_r \sim 10.8 \times 10^3$; Wasteson, 1971), whereas glycosaminoglycans synthesized in the presence of excess xyloside were eluted with a peak $K_{av.}$ of 0.69 ($M_r \sim 7.0 \times 10^3$) (Fig. 7, trace *c*). This suggests that, in the presence of excess acceptor (in this case β-D-xyloside), the enzymes of chondroitin sulphate synthesis gave rise to smaller chains.

Glycosaminoglycans synthesized on to core protein after 100min in the presence of cycloheximide (Fig. 7, trace *b*) were eluted with a peak $K_{av.}$ of 0.47 ($M_r \sim 23.0 \times 10^3$), indicating that, as core protein became limiting because of depletion of the pool, the average size of the glycosamino-

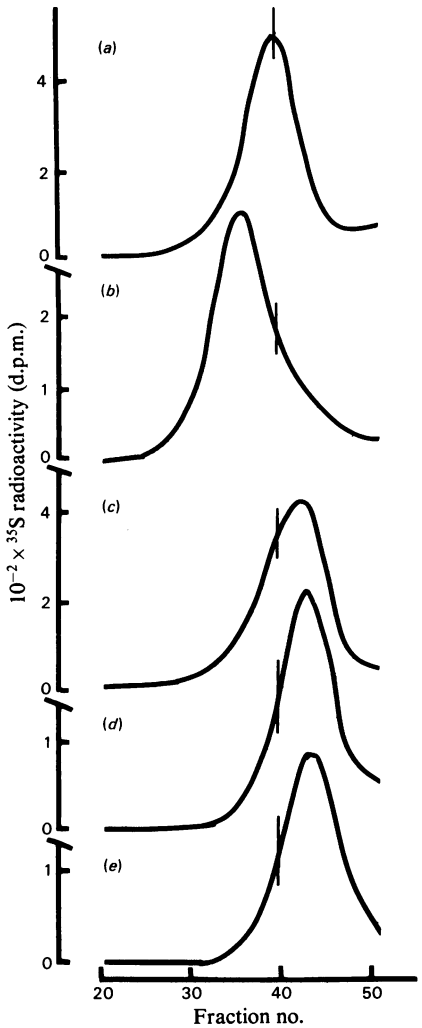

Fig. 7. *Elution from Sepharose CL-6B of glycosaminoglycans synthesized by bovine articular cartilage in the absence and presence of 1 mM-benzyl β-D-xyloside with and without cycloheximide*

Alkali extracts of explant cultures marked (*a*), (*b*), (*c*), (*d*) and (*e*) in Fig. 5 were applied to a column of Sepharose CL-6B eluted with 4M-guanidinium chloride (V_0, fraction 19; V_t, fraction 52). Profiles are shown for labelled glycosaminoglycans from cartilage: (*a*) incubated without additions; (*b*) incubated for 2h with 0.4mM-cycloheximide; (*c*) incubated with 1.0mM-benzyl β-D-xyloside; (*d*) incubated for 2h with 0.4mM-cycloheximide and labelled in the presence of 1.0mM-benzyl β-D-xyloside; and (*e*) incubated for 4h with 0.4mM-cycloheximide and labelled in the presence of 1.0mM-benzyl β-D-xyloside.

glycans increased quite markedly, with a shift in M_r from 10800 to 23000. This is consistent with the model proposed by Mitchell & Hardingham (1982) that glycosaminoglycan chain length is determined by the ratio of elongation activity to xylosyl-acceptor concentration. In the presence of excess xyloside and after exposure to cycloheximide for 2 or 4h, the $K_{av.}$ values on Sepharose CL-6B of the glycosaminoglycan chains were the same as for the zero-time control (Fig. 7, traces *d* and *e*). This indicates that, although the rate of synthesis of glycosaminoglycans was depressed by cycloheximide, the excess concentration of acceptor available to the enzymes of chain elongation determined the chain length of the glycosaminoglycans synthesized.

The supply of energy for chain elongation was shown not to be affected by cycloheximide, by assay of tissue ATP. Cartilage explants and chick chondrocytes were exposed to cycloheximide for 6h and for 24h respectively (Table 2). At the times shown, chondrocyte cultures and batches of articular cartilage were homogenized in 5% trichloroacetic acid and the soluble fractions assayed for ATP. Table 2 shows that at no time during the exposure of the two culture systems to cycloheximide did ATP decrease.

The decrease in glycosaminoglycan synthesis induced by cycloheximide could have been caused by depleted enzyme amounts involved in chain elongation. This was investigated by measuring the activity of some glycosyltransferases involved in chondroitin sulphate synthesis in extracts of both articular cartilage and chick chondrocytes. A linkage enzyme (galactosyltransferase-I), two elongation enzymes of chondroitin sulphate biosynthesis (*N*-acetylgalactosaminyltransferase and glucuronosyltransferase-II), as well as a non-specific glycoprotein-elongation enzyme (UDP-*N*-acetylgalactosamine:chondroitin *N*-acetylgalactosaminyltransferase) were assayed.

Three separate batches of articular cartilage were maintained in culture and exposed to cycloheximide for 0, 2 and 4h respectively. Chick chondrocyte cultures were incubated with cycloheximide for 0, 4 and 24h. After incubation with cycloheximide, the enzymes were extracted from the tissue homogenates and assayed for activity. Table 3 shows the enzyme activity in cartilage explants (nmol of substrate transferred/h per mg dry wt.) and in chick chondrocytes (nmol of substrate transferred/h per culture flask) before and after exposure to cycloheximide. Galactosyltransferase-I catalyses the transfer of galactose to xylosyl residues. This reaction is the second step in the synthesis of the chondroitin sulphate linkage region and may act either on xylosyl residues bound to endogenous core protein or on simple xylosides, such as benzyl β-D-xyloside. The activity of this enzyme in both explants and chondrocytes did not change significantly during the first 4h of

Table 2. *Effect of cycloheximide on ATP content in articular cartilage and isolated chrondrocytes*
Isolated chick chondrocytes and bovine cartilage slices were exposed to cycloheximide (0.4mM for explants, 0.1mM for chondrocytes) for up to 24h. At the appropriate time, cultures were homogenized in 5% trichloroacetic acid and assayed for ATP concentration (see the Experimental section). Each result is the mean±S.D. for three determinations: ND, not determined.

Time in the presence of cycloheximide (h)	ATP content of cartilage explants (pmol/mg dry wt.)	ATP content of isolated chick chondrocytes (nmol/culture)
0	47.3±1.7	15.9±1.7
1	ND	12.8±3.1
2	ND	19.4±2.2
3	59.4±2.2	ND
6	52.2±3.9	21.2±4.4
24	ND	16.6±2.7

Table 3. *Effect of cycloheximide on the activity of glycosyltransferases in articular cartilage and isolated chondrocytes*
Cultures of bovine cartilage were incubated in the presence of 0.4mM-cycloheximide for up to 4h, and chick chondrocytes with 0.1mM-cycloheximide for up to 24h. At the times shown, the cultures were homogenized in enzyme buffer and assayed for glycosyltransferase activity. Enzme activity in cartilage slices is expressed per mg dry wt. of tissue, and in chick chondrocytes as enzyme activity per culture. Each result for the cartilage slices is the mean of two determinations±deviation from the mean. Each result for the chondrocyte cultures is the mean±S.D. for three or more determinations. Abbreviations: Gal-T-I, galactosyltransferase-I; GalNAc-T, *N*-acetylgalactosaminyltransferase; GlcA-T-II, glucuronosyltransferase-II; GlcNAc-Gal-T, UDP-galactose:*N*-acetylglucosaminide galactosyltransferase.

Culture	Time in the presence of cycloheximide (h)	10^{-2} × Activity (nmol of substrate transferred/h)			
		Gal-T-I	GalNAc-T	GlcA-T-II	GlcNAc-Gal-T
Cartilage	0	6.4±0.3	7.6±0.1	4.3±0.2	16.3±1.2
	2	6.6±0.4	7.0±0.4	4.6±0.1	16.4±0.4
	4	5.8±0.5	7.0±0.5	3.9±0.1	14.6±1.4
Chondrocytes	0	1.76±0.15	6.02±0.35	0.81±0.04	4.95±0.04
	4	1.77±0.14	5.46±0.65	0.73±0.11	4.20±0.41
	24	1.27±0.07	3.94±0.17	0.56±0.07	2.64±0.07

exposure to cycloheximide (Table 3, 'Gal-T-I'). The repeating disaccharide structure of chondroitin sulphate is the result of the alternating transfer of *N*-acetylgalactosamine and glucuronic acid to the non-reducing terminus of the growing polysaccharide chain; this requires two specific enzymes, *N*-acetylgalactosaminyltransferase and glucuronosyltransferase-II respectively. The activity of each of these enzymes was measured with artificial substrates under conditions which were probably not optimal; nevertheless, no effect of cycloheximide within the first 4h was detectable (Table 3, 'GalNAc-T, and 'GlcA-T-II') in either articular cartilage or chick chondrocytes. Another enzyme activity was assayed; this was UDP-galactose:*N*-acetylglucosaminide galactosyltransferase, which catalyses the transfer of galactose to *N*-acetylglucosamine residues in glycoproteins. This activity also showed no appreciable decrease within the first 4h of exposure to cycloheximide (Table 3, 'GlcNAc-Gal-T').

After chondrocyte cultures had been exposed to cycloheximide for 24h, each of the glycosyltransferases assayed showed decreased activity compared with control cultures. The inhibition of biosynthesis of protein-initiated and xyloside-initiated chondroitin sulphate caused by cycloheximide cannot, however, be attributed to this slight fall in activity, since that inhibition occurred within 4h of exposure to cycloheximide, before any change in enzyme activity could be detected. It should be stressed, however, that all of these assays were carried out in the presence of the detergent Nonidet P-40, which is known to enhance glycosyltransferase activity (Schwartz & Rodén, 1975; Robinson & Robinson, 1981) and could well mask normal control mechanisms in the chondrocyte. It is also possible that the activities of other glycosyl-

transferases, such as galactosyltransferase-II or glucuronosyltransferase-I, which were not assayed in these experiments, were more sensitive to cycloheximide treatment. Separate experiments with cultured chicken chondrocytes have indicated that galactosyltransferase-II activity is not diminished after cycloheximide treatment, but no information is available about glucuronosyltransferase-I, which could fulfil an unrecognized control function in the chondrocyte. These results established, nevertheless, that glycosyltransferase activity did not change rapidly after exposure of the cultured tissue to cycloheximide. It can be concluded that the turnover of these enzymes in articular cartilage and chick chondrocytes is much slower that that of the proteoglycan core protein.

Except for the xylosyltransferase, it is apparent that the glycosyltransferases of chondroitin sulphate synthesis are tightly associated with membranes and can only be solubilized with the use of detergents (Stoolmiller *et al.*, 1972; Schwartz & Rodén, 1975). Furthermore, it has been suggested that these enzymes may be arranged in membranes into complex structures so that enzymes catalysing subsequent glycosyl transfers may be adjacent to each other (Stoolmiller *et al.*, 1972). Indeed, such organization could indicate that regulation of the glycosyltransferases of chondroitin sulphate synthesis is complex. In the present study cycloheximide produced a decrease in the concerted activity of the glycosyltransferases measured with benzyl β-D-xyloside. This decrease in activity was not caused by an effect of cycloheximide on total enzyme amounts or available cellular ATP. Hence, over the time period discussed, it is unlikely that the turnovers of enzymes are points of control. It appears that these glycosyltransferases are sensitive to some translation product. One possibility is that cycloheximide may affect the synthesis of proteins essential for the synthesis of glycosaminoglycans or nucleotide sugars.

Another possibility is that the activity of the glycosyltransferase enzymes could be sensitive to the presence of core protein, so that maximal activity was expressed only when an optimal amount of core protein was present; perhaps core protein participates in the organization of the glycosyltransferases in the membrane. It is clear that, after cessation of protein synthesis by cycloheximide, a constant rate of glycosaminoglycan synthesis could still be measured when xyloside was included in the incubation medium (Figs. 3–6). This suggests that the glycosyltransferases of chondroitin sulphate synthesis have a basal activity which may be stimulated by translation products. One possible explanation for the difference between data reported here and those described for chondrocytes from the Swarm rat chondrosarcoma (Kimura *et al.*, 1981; Mitchell & Hardingham, 1982) could be that the glycosyltransferases of the latter had a high basal activity which could not be increased.

Irrespective of the underlying mechanism, the results presented here indicate that chondroitin sulphate synthesis in the presence of β-D-xylosides is not necessarily a measure of a cell's total capacity for chondroitin sulphate biosynthesis. Xylosides are useful in measuring the degree to which chondroitin sulphate-proteoglycan biosynthesis is substrate-limited (Schwartz *et al.*, 1974; De Luca *et al.*, 1978) in normally metabolizing cells, but, when perturbations are introduced that could affect protein synthesis, we suggest that the usefulness of xyloside-initiated chondroitin sulphate synthesis is limited and may not be a measure of the cell's maximal synthetic rate.

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