

## Articular cartilage cultured with catabolin (pig interleukin 1) synthesizes a decreased number of normal proteoglycan molecules

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A homogeneous preparation of catabolin from pig leucocytes caused a reversible dose-dependent (0.01–1 nM) decrease in the synthesis of proteoglycan in slices of pig articular cartilage cultured in serum-free medium. The monomers that were synthesized and secreted in the presence of catabolin had the same average hydrodynamic size and ability to aggregate as the controls, and the core protein was substituted with the same number of glycosaminoglycan chains. The chains were the same average length and charge as normal and were sulphated to the same extent as the controls. Newly synthesized extracellular proteoglycan was not preferentially degraded. A 2–3-fold increase in glycosaminoglycan synthesis occurred in control and catabolin-treated cartilage in the presence of  $\beta$ -D-xyloside (1 mM), more than 80% being secreted into the medium as free chains. Decreased incorporation of sulphate was not reversed in the presence of lysosomal-enzyme inhibitors, and there was no evidence in pulse-chase experiments of increased intracellular degradation of glycosaminoglycan chains before secretion. It is concluded that catabolin-treated cartilage synthesizes a smaller number of normal proteoglycan molecules.

In diseases characterized by progressive erosion of articular tissues, the ability of chondrocytes to replace the depleted proteoglycan will determine whether the cartilage can continue to withstand compressive forces and protect the underlying bone (Kempson, 1980).

Articular cartilage grown as explants under suitable culture conditions synthesizes matrix components representative of those found in normal tissue. (Benya & Nimni, 1979; Sandy *et al.*, 1980; Hascall *et al.*, 1983). Such a system provides an excellent means of studying the factors that contribute to the maintenance of a functional extracellular matrix.

Many of the agents that can stimulate chondrocytes to induce net depletion of cartilage proteoglycan also decrease the rate at which [ $^{35}$ S]sulphate is incorporated into glycosaminoglycans. Thus retinol (Jubb, 1984), medium conditioned by mononuclear cells (Taplits *et al.*, 1979; Herman *et al.*, 1981) or synovium (Jubb & Fell, 1980), and lipopolysaccharides (Morales *et al.*, 1984), all decrease the production of  $^{35}$ S-proteoglycan. Catabolin was originally defined by its ability to in-

duce proteoglycan resorption in cultured cartilage (Dingle *et al.*, 1979), and has now been purified to homogeneity from the medium of mononuclear leucocytes incubated with concanavalin A (Saklatvala *et al.*, 1983). It is a protein with an  $M_r$  of 21 000 and an isoelectric point of 4.9, and it is probably a form of interleukin 1 (Saklatvala *et al.*, 1984). The same protein can be produced by cultured synovium and fibroblasts (Pilsworth & Saklatvala, 1983), and it is thought to be responsible for most of the non-enzymic cartilage-degrading activity found in medium conditioned by these cells. Partially purified catabolin from synovium (Dingle, 1984) also decreases the incorporation of [ $^{35}$ S]sulphate into cartilage.

Newly synthesized proteoglycan released by the action of catabolin has a non-functional binding region and therefore diffuses out of the matrix. These molecules have an average hydrodynamic size only slightly smaller than that of normal monomers. There was no significant degradation of the glycosaminoglycan chains, suggesting that diminished incorporation of [ $^{35}$ S]sulphate was a separate event (Tyler, 1985).

The reason why smaller amounts of [ $^{35}\text{S}$ ]sulphate are incorporated into cartilage proteoglycan in the presence of catabolin is the subject of the present paper.

## Materials

Iscove's modified Eagle's medium, supplements and serum were from Gibco, Grand Island, NY, U.S.A. Chondroitin sulphate, chondroitinase ABC and standard disaccharides were from Miles Laboratories, Stoke Poges, Slough, Bucks., U.K. 1,9-DimethylMethylene Blue was from Serva Feinbiochemica, Heidelberg, Germany. Sepharose CL-2B and Sephacryl S-200 were from Pharmacia, Uppsala, Sweden.

Pepstatin was from the Peptide Institute, Osaka, Japan. [ $^{35}\text{S}$ ]Sulphate and [ $^3\text{H}$ ]acetate were from Amersham International, Amersham, Bucks., U.K. All other chemicals were analytical reagent or best available grade.

## Methods

### Tissue culture

Cartilage slices (12 mm  $\times$  2 mm  $\times$  0.3 mm) were cut from the condylar ridge of the forefeet from pigs aged about 25 weeks. The strips were cultured at 37°C in an atmosphere of  $\text{CO}_2/\text{air}$  (1:19) for 48 h in control medium to stabilize them before the experiment was set up (day 1). The slices were cut transversely into two equivalent pieces (7–11 mg wet wt.). Six to eight halves were cultured as a control in Falcon multiwell (2 cm<sup>2</sup>) plates containing 1 ml of Iscove's medium. The other halves received various experimental treatments as indicated in the text. The medium was renewed once or twice daily and contained the following supplements per ml: bovine serum albumin (60  $\mu\text{g}$ ), soya-bean lipid (15  $\mu\text{g}$ ), human transferrin (5  $\mu\text{g}$ ), ascorbic acid (100  $\mu\text{g}$ ), streptomycin (100  $\mu\text{g}$ ) and penicillin (150  $\mu\text{units}$ ).

### Purification of catabolin

The homogeneous preparation of catabolin used for these experiments was generously given by Dr. J. Saklatavala, Strangeways Research Laboratory, Cambridge, U.K. The protein was isolated from the conditioned medium of cultured pig mononuclear cells stimulated with lectins. Activity was concentrated by ultrafiltration, chromatographed on Ultrogel AcA-54, chromatofocused on a pre-packed Mono P HRS/20 column, and then eluted from hydroxyapatite, a Mono Q anion-exchanger, and Zorbax ODS (h.p.l.c.) (Saklatavala *et al.*, 1983). The activity ran as a single protein band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis ( $M_r$  21 000, pI 4.9).

### Glycosaminoglycan synthesis

The explants were incubated for up to 3 h in medium containing 5% of the sulphate concentration of normal medium. [ $^{35}\text{S}$ ]Sulphate or [ $^3\text{H}$ ]acetate at 20  $\mu\text{Ci/ml}$  was used for the 3 h pulse, and 100  $\mu\text{Ci/ml}$  for periods of less than 1 h. The radiolabelled cartilage was washed twice in ice-cold normal medium and frozen on a glass plate over solid  $\text{CO}_2$ . For pulse-chase experiments the cartilage was washed four times with warm medium, then incubated for various chase times. The tissue was digested with papain (300  $\mu\text{g/ml}$ ) in 50 mM-sodium phosphate buffer, pH 6.5, containing 2 mM-*N*-acetylcysteine and 2 mM-EDTA at 65°C for 1 h. Glycosaminoglycans from the  $^3\text{H}$ -labelled samples were eluted as a  $V_0$  peak from a 10 ml Sephadex G-50 column equilibrated with 50 mM-sodium acetate buffer, pH 6.0, then isolated by anion-exchange chromatography on Whatman DE-52 DEAE-cellulose (acetate form) as described below. Samples of the  $^{35}\text{S}$ -labelled digest and pulse medium were either eluted as a  $V_0$  peak from a Sephadex G-50 column or dialysed against water and spotted on to squares of Whatman 1 MM filter paper. Radiolabelled glycosaminoglycans were precipitated directly on to the paper by soaking the squares in aq. 1% (w/v) cetylpyridinium chloride for 30 min at room temperature. The papers were washed twice in 0.3 M-NaCl/0.1% cetylpyridinium chloride containing 20 mM- $\text{Na}_2\text{SO}_4$  and dried. Radiolabelled samples (up to 250  $\mu\text{l}$ ) or paper squares were placed in scintillation Minivials with 2.5 ml of Picafluor/toluene (1:1, v/v) and their radioactivities counted on a Packard Tri-Carb 300 scintillation counter. The c.p.m. values were corrected to d.p.m. values with the aid of the use of an external standard.

### Extraction of $^{35}\text{S}$ -labelled proteoglycans

Radiolabelled explants were sliced into 30  $\mu\text{m}$  cryostat sections and sequentially extracted at 4°C twice with low-salt buffer (0.5 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8) and twice with high-salt buffer (4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8). Residual radioactivity was solubilized with papain. The following inhibitors were present in all the extraction/dialysis procedures: pepstatin (1  $\mu\text{g/ml}$ ), 1,10-phenanthroline (1 mM), iodoacetic acid (1 mM), phenylmethanesulphonyl fluoride (1 mM) and toluene (0.03%, v/v).

### Gel chromatography

The average hydrodynamic size of proteoglycans and glycosaminoglycans was determined by gel chromatography on columns (100 cm  $\times$  6.6 cm) of Sepharose CL-2B and Sephacryl S-200, as previously described (Tyler, 1985).

Samples were eluted with 0.5M-sodium acetate buffer, pH 7.0, at 4 ml/h at room temperature in the presence and in the absence of 4M-guanidinium chloride. Samples to be tested for the ability to aggregate were first mixed with hyaluronic acid (2% of the sample uronic acid) at 4°C for 4h.

#### *Preparation of $^{35}\text{S}$ -glycosaminoglycan chains*

Extracted  $^{35}\text{S}$ -proteoglycan was digested with papain or hydrolysed with alkali (1M- $\text{NaBH}_4$  in 50mM- $\text{NaOH}$  at 45°C for 48h; Carlson, 1968) and chromatographed on Sephacryl S-200. The peak of  $^{35}\text{S}$ -glycosaminoglycans was dialysed against water and freeze-dried. Samples were applied to a Whatman DE-52 DEAE-cellulose (acetate form) anion-exchange column (0.66cm  $\times$  15cm) equilibrated with 10mM-Tris/HCl buffer, pH 7.2, and eluted with a 100ml linear 0–0.8M- $\text{NaCl}$  gradient in the same buffer at room temperature. The chondroitin sulphate chains were usually eluted at about 0.6M- $\text{NaCl}$ , and they were dialysed against water and freeze-dried.

#### *Standard proteoglycan*

Radiolabelled proteoglycan monomers (A1–D1) were extracted from bovine nasal cartilage and isolated by associative and then dissociative centrifugation in  $\text{CsCl}$  gradients (Hascall & Sajdera, 1969).

#### *Chondroitinase ABC assay*

The conditions for optimum digestion were worked out with the use of chondroitin sulphate prepared from radiolabelled standard (A1–D1) proteoglycan. Samples (50  $\mu\text{l}$ ) of the assay mixture were mixed with 1 ml of 0.1M-HCl and read at 232nm to monitor increased production of disaccharides (Saito *et al.*, 1968). A mixture that gave a maximum increase in absorbance after 1h resulted in 85% digestion of the glycosaminoglycan. Samples were usually incubated with 0.2 unit of enzyme/mg of substrate in 300  $\mu\text{l}$  of 0.1M-sodium acetate/0.1M-Tris/HCl buffer, pH 7.4, at 40°C for 5h. Samples were analysed by gel chromatography and t.l.c.

#### *T.l.c.*

Samples up to 50  $\mu\text{l}$  were applied to plastic-backed pre-coated cellulose t.l.c. plates (Kodak) in 5  $\mu\text{l}$  spots and dried in a stream of hot air. A standard mixture containing 20  $\mu\text{g}$  each of the following disaccharides was run as a reference standard:  $\Delta\text{di-6-S}$ ,  $\Delta\text{di-4-S}$  and  $\Delta\text{di-0-S}$  (Suzuki *et al.*, 1968). The disaccharides were de-salted with butan-1-ol/ethanol/water (13:8:4, by

vol.), then separated with butan-1-ol/acetic acid/2M- $\text{NH}_3$  (2:3:1, by vol.) essentially as described by Mason *et al.* (1982). The plate was dried and examined under u.v. light to mark the position of the standards. Each sample lane was then cut into 1.4cm  $\times$  0.5cm strips, each of which was incubated with 1 ml of 0.5M-HCl at 60°C for 16h. Samples were then mixed with Picafluor and their radioactivities counted.

#### *Analytical procedures*

Glycosaminoglycan was measured from the amount of polyanionic material reacting with 1,9-dimethylMethylene Blue (Farndale *et al.*, 1982) and as uronic acid by the method of Bitter & Muir (1962). Shark chondroitin sulphate and glucuronolactone were used as a standard. Glucose was determined from the formation of NADPH after conversion into glucose 6-phosphate by ATP in the presence of hexokinase (Sigma kit no. 15-uv).

Collagen was measured as hydroxyproline in neutralized acid hydrolysates of the medium or tissue (6M-HCl, 105°C, 20h) as described by Tougaard (1973). DNA was measured in papain digests of the tissue by the method of Royce & Lowther (1979).

## **Results**

#### *Inhibition of glycosaminoglycan synthesis*

Equivalent halves of articular cartilage slices were cultured for 3 days in serum-free medium in the presence of increasing concentrations of homogeneous catabolin. Incorporation of [ $^{35}\text{S}$ ]sulphate (3h pulse) into glycosaminoglycans was diminished in a dose-dependent manner (Fig. 1). Catabolin induces chondrocytes to release increased amounts of matrix proteoglycan into the medium. The values given in parentheses in Fig. 1 represent the proportion of proteoglycan as percentages of the total released from the cartilage during the 3 days before the labelling. The newly synthesized extracellular material released during the 3h labelling period was all recovered and is shown as  $^{35}\text{S}$ -glycosaminoglycan in the medium (Fig. 1, hatched area).

The rate of incorporation of [ $^{35}\text{S}$ ]sulphate into total glycosaminoglycans of both the control and the catabolin-treated cartilage (Fig. 2a) was linear between 5min and 16h (up to 3h only shown), indicating a very rapid equilibration of the radio-label. At this dose (0.14nM only shown) synthesis was decreased in the presence of catabolin by about 75% during the first hour of linear labelling. Incorporation of [ $^3\text{H}$ ]acetate was also diminished in the presence of catabolin, but not to the same extent as that of [ $^{35}\text{S}$ ]sulphate (Fig. 2b).

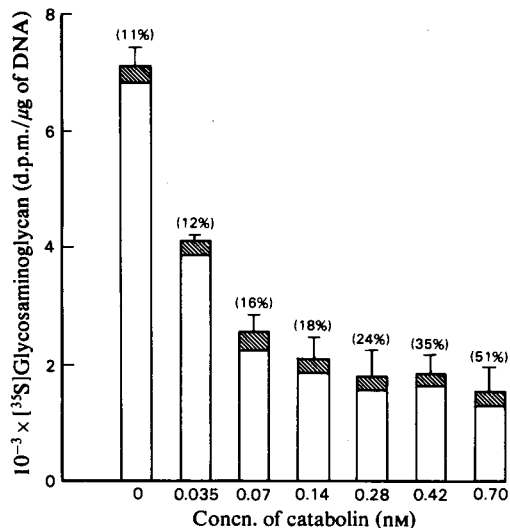


Fig. 1. Glycosaminoglycan synthesis in cartilage cultured with increasing concentrations of catabolin

Cartilage was cultured for 3 days in the absence and in the presence of catabolin as indicated. Incorporation of [<sup>35</sup>S]sulphate (3 h pulse) into a glycosaminoglycan in the cartilage (□) and medium (▨) is shown as the means for four experiments. Bars indicate S.E.M. Values in parentheses represent the amounts of unlabelled proteoglycan released into the medium (as percentages of total) before the labelling.

#### Distribution and average molecular size of newly synthesized proteoglycan

Cartilage halves were cultured for 1, 2 or 3 days in the presence or in the absence of catabolin (0.14 nM), then labelled for 3 h with [<sup>35</sup>S]sulphate. The explants were washed well, sliced into 30 μm sections and sequentially extracted with 0.5 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, for 24 h (twice) and 4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 5.8, for 24 h (twice). The residue was solubilized with papain. The extracts and the medium were dialysed against water and freeze-dried. The distribution of <sup>35</sup>S-labelled glycosaminoglycans is shown in Fig. 3(b). Total glycosaminoglycan synthesis ([<sup>35</sup>S]sulphate, 3 h pulse) was progressively decreased to 66%, 48% and 21% of that in the controls after 1, 2 or 3 days' treatment. At this dose of catabolin 16% of the unlabelled proteoglycan is released into the medium after 3 days compared with 7% of the controls. The overall distribution therefore remains very similar (Fig. 3a).

The average molecular size and ability to aggregate of molecules synthesized in the presence

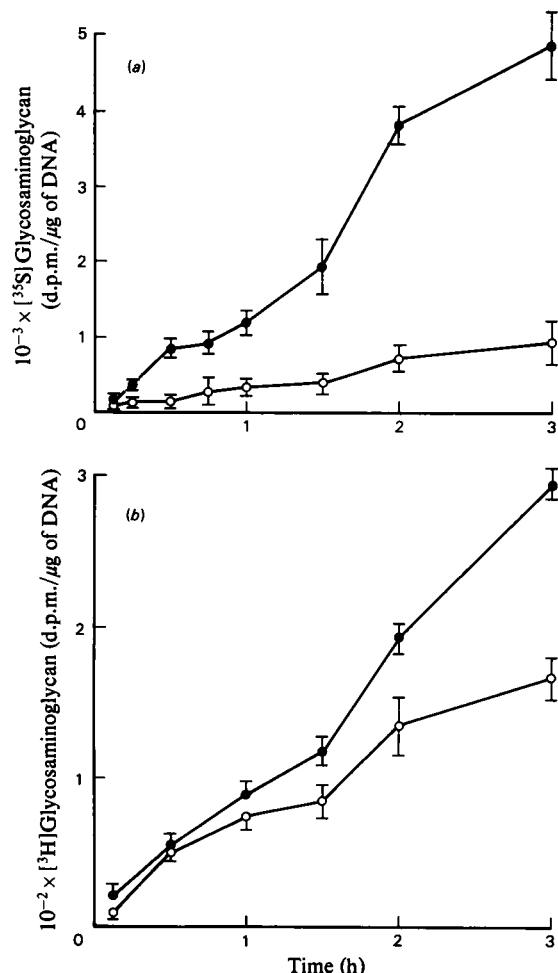


Fig. 2. Incorporation of [<sup>35</sup>S]sulphate and [<sup>3</sup>H]acetate with time into total glycosaminoglycans

Equivalent halves of cartilage were cultured in the absence and in the presence of catabolin (0.14 nM) for 3 days. The rate of incorporation of (a) [<sup>35</sup>S]sulphate and (b) [<sup>3</sup>H]acetate into total glycosaminoglycans is shown as d.p.m./μg of DNA for various periods of labelling. Values are the means for three experiments. Bars indicate S.E.M. ●, Control; ○, catabolin.

of catabolin was measured by elution from columns of Sepharose 2B (Fig. 4b). These data represent material extracted with guanidinium chloride from the day-3 cultures, but the same results were obtained for days 1 and 2. The average size of monomers extracted from the cartilage with high-salt buffer was the same as that of the controls (Fig. 4a) and the same as the unlabelled proteoglycan when eluted in the presence of 4 M-guanidinium chloride (Figs. 4c and 4d). Most of this material was able to aggregate when mixed

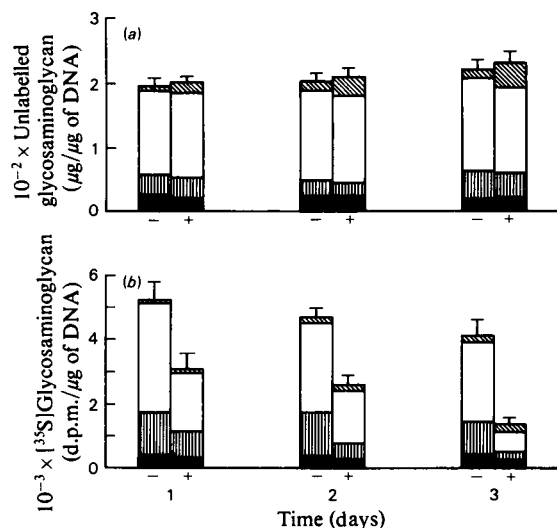


Fig. 3. Distribution of newly synthesized proteoglycan. Equivalent halves of cartilage were cultured in the absence (–) and in the presence (+) of catabolin (0.14 nM) for 1, 2 or 3 days, then labelled with [ $^{35}\text{S}$ ]sulphate (3 h pulse). Sections (30  $\mu\text{m}$ ) of the tissue were sequentially extracted with low-salt buffer (24 h;  $\times 2$ ) (■) and with 4M-guanidinium chloride (24 h;  $\times 2$ ) (□) and solubilized by papain treatment (■). Unlabelled glycosaminoglycan (a) and  $^{35}\text{S}$ -glycosaminoglycans (b) in each extract and in the medium (▨) are shown as the means for three experiments. Bars indicate S.E.M.

with hyaluronate and was eluted in the void volume under associative conditions.

#### Average size and charge of newly synthesized glycosaminoglycan chains

Materials eluted in the peaks shown in Figs. 4(a) and 4(b) were pooled as indicated by the bars, dialysed against water and freeze-dried. Glycosaminoglycans were prepared from the samples as described in the Methods section and eluted from a column of Sephacryl S-200 before and after digestion with chondroitinase ABC. The average size of the glycosaminoglycan chains from proteoglycan synthesized in the presence of catabolin (Fig. 5b) was the same as that of the controls (Fig. 5a), and a similar proportion (80–90%) was susceptible to digestion with chondroitinase ABC (Figs. 5c and 5d). The  $^{35}\text{S}$ -glycosaminoglycan chains synthesized in the presence of catabolin migrated with the same mobility (Fig. 5f) after electrophoresis in HCl as that of those from the controls (Fig. 5e) and as that of the unlabelled chondroitin sulphate stained with Alcian Blue.

Disaccharides in the digest were separated by

Table 1. Composition of the  $^{35}\text{S}$ -glycosaminoglycans. The data were calculated from the values shown in Fig. 6. The proportions of  $^{35}\text{S}$ -disaccharides identified as 4-sulphate or 6-sulphate are shown as percentages of total chondroitin sulphate recovered.

Sample	Composition (% of total recovered)	
	Control	Catabolin-treated
Undigested	9	11
Chondroitin 6-sulphate	36	33
Chondroitin 4-sulphate	50	48
Chondroitin	6	8
Radioactivity recovered	86%	93%
6-Sulphate/4-sulphate ratio	0.70	0.68

t.l.c. after a desalting step as described by Mason *et al.* (1982) (Fig. 6). The proportion of different isomers is shown in Table 1 as percentages of the total, together with the ratio of chondroitin 4-sulphate to chondroitin 6-sulphate. Catabolin did not alter the pattern of sulphation.

#### Decreased incorporation of [ $^{35}\text{S}$ ]sulphate is reversed in the presence of $\beta$ -D-xyloside

Cartilage was cultured for 3 days in the presence and in the absence of catabolin, methyl  $\beta$ -D-xyloside (1 mM) being added for 2 h before and during a 3 h pulse with [ $^{35}\text{S}$ ]sulphate. Incorporation of the radiolabel into glycosaminoglycans was stimulated 2–3-fold in both the control and the catabolin-treated cartilage (Fig. 7b). More than 80% of the chains in the xyloside cultures were recovered from the medium, compared with the 10–15% normally found. Incorporation of [ $^{35}\text{S}$ ]sulphate in parallel cultures in the presence of  $\text{NH}_4\text{Cl}$  (Fig. 7c) or of 7-amino-1-chloro-3-tosylamidoheptan-2-one (Fig. 7d) was not significantly different from that by the control or catabolin-treated cartilage incubated with buffer (Fig. 7a).

#### Intracellular degradation of newly synthesized proteoglycan

It seemed that decreased incorporation of [ $^{35}\text{S}$ ]sulphate in the presence of catabolin was not due to defective synthesis of undersulphated or under-substituted proteoglycan monomers, but to synthesis of a decreased number of normal molecules. The fact that glycosaminoglycan synthesis was stimulated 2–3-fold in the presence of xyloside acceptors indicated that the rate-limiting step was not delayed elongation or sulphation. The amount of radiolabel remaining in newly synthesized proteoglycan was therefore measured in a series of pulse-chase experiments.

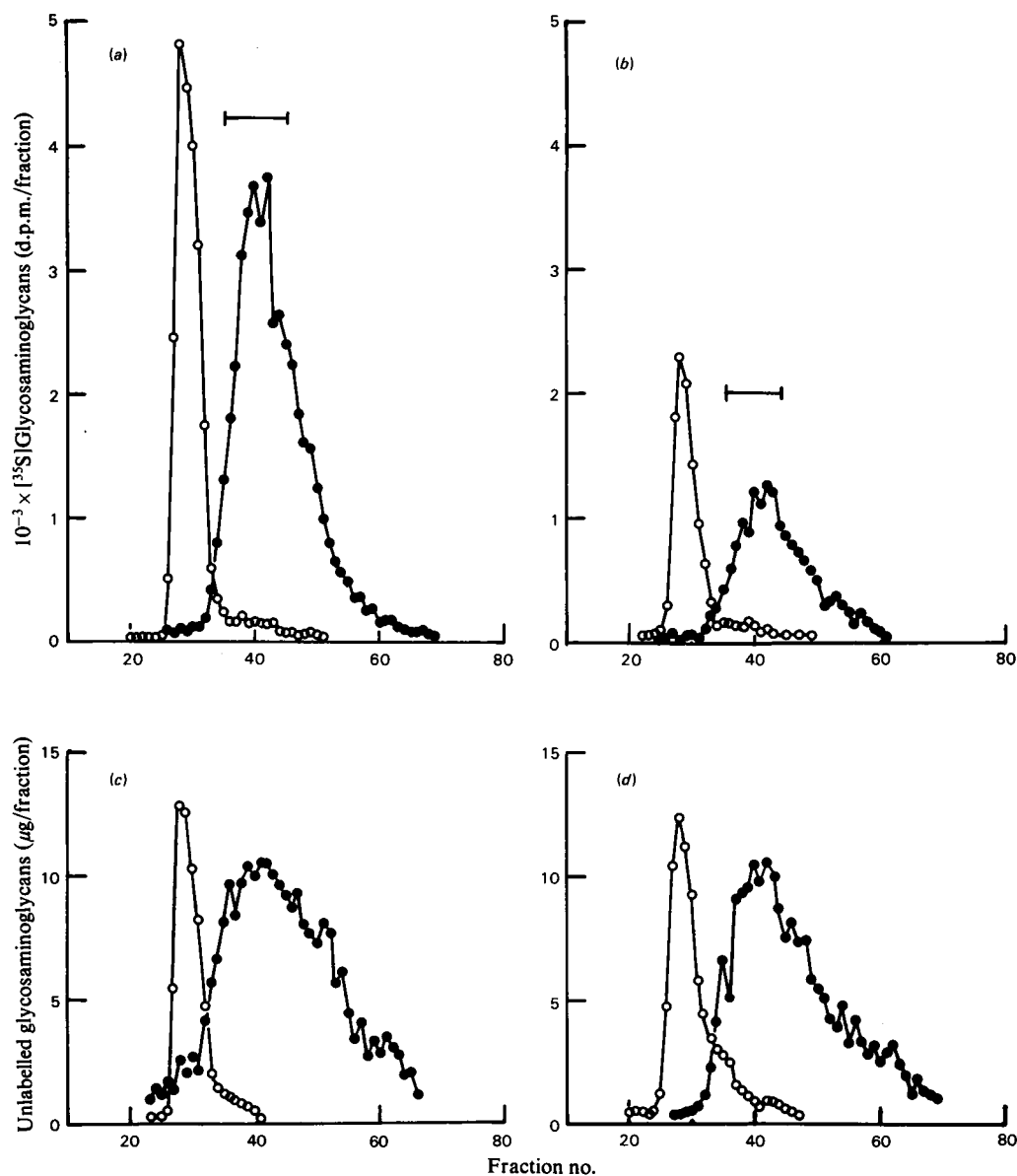


Fig. 4. Gel chromatography on Sepharose CL-2B of newly synthesized proteoglycans extracted with 4M-guanidinium chloride. Samples of proteoglycan from the 4M-guanidinium chloride extracts of control (a and c) and catabolin-treated (b and d) cultures shown in Fig. 3 were eluted from a column of Sepharose CL-2B in the presence of 4M-guanidinium chloride (●) and under associative conditions after mixing with hyaluronate (○). Fractions were analysed for unlabelled (c and d) and radiolabelled (a and b) glycosaminoglycans.

Cartilage halves were cultured for 3 days in the presence or in the absence of catabolin. The cultures were pulse-labelled for 20 min, washed well and then chased for 0, 20, 40, 60 or 180 min, in the presence and in the absence of 7-amino-1-chloro-3-tosylamidoheptan-2-one, as described in the Methods section. Explants were frozen on glass

Petri dishes over solid CO<sub>2</sub> at the times indicated, sliced into 30 μm sections and extracted with 4M-guanidinium chloride/sodium acetate buffer as described above. The residue was solubilized with papain. The changes in total radioactivity were measured as a function of chase time (Fig. 8). The <sup>35</sup>S radiolabel recovered in the proteoglycan of

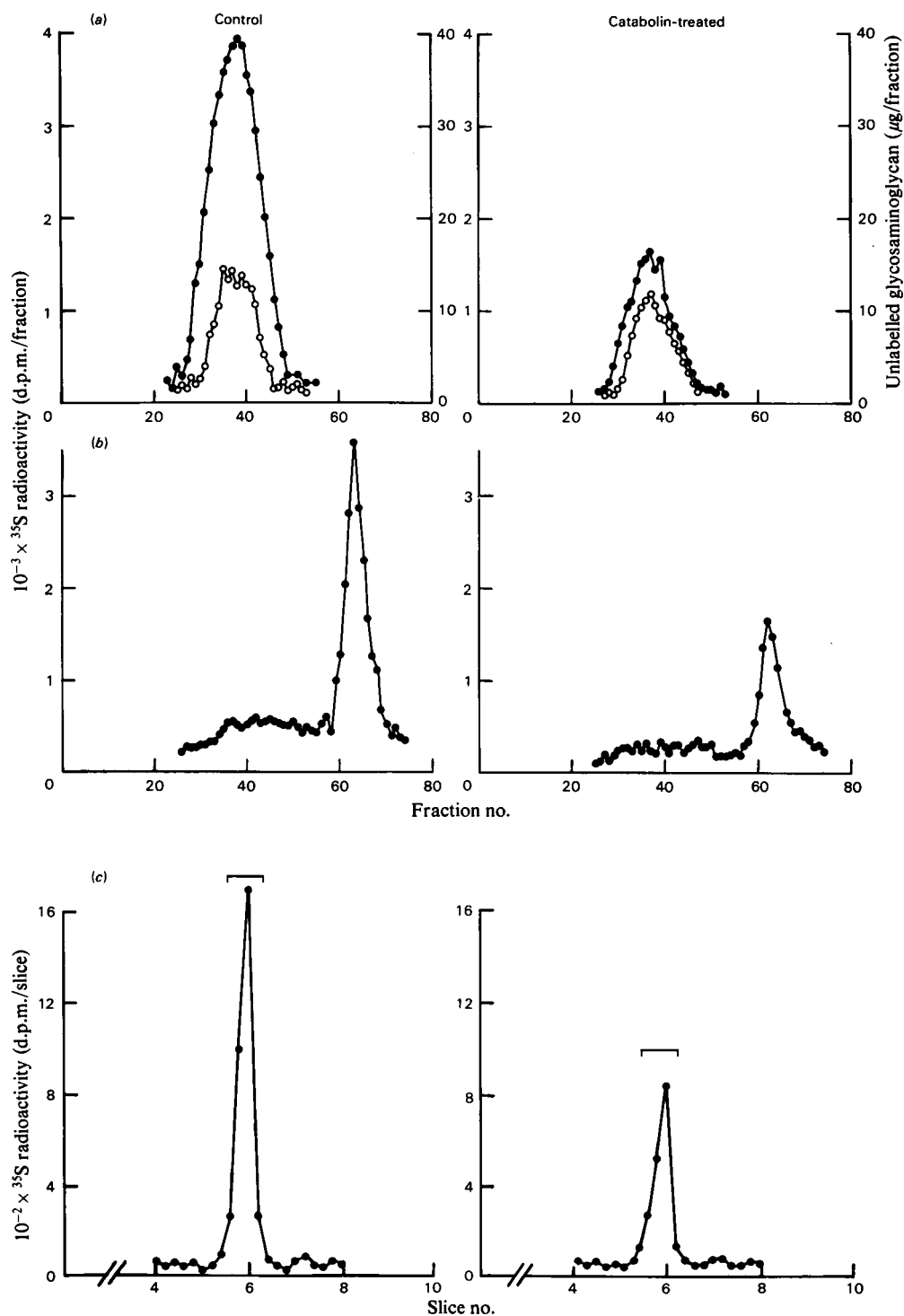


Fig. 5. Comparison of the average molecular size and charge and composition of glycosaminoglycan chains. Glycosaminoglycans extracted from control and catabolin-treated cartilage were eluted from a column of Sephadex S-200 before (a) and after (b) digestion with chondroitinase ABC as described in the Methods section. Samples were also analysed by electrophoresis in HCl on strips of cellulose acetate (c). Duplicate samples were stained with Alcian Blue or cut into 0.2 cm slices and their radioactivities counted. The position of the Alcian Blue stain is denoted by  $\square$ .

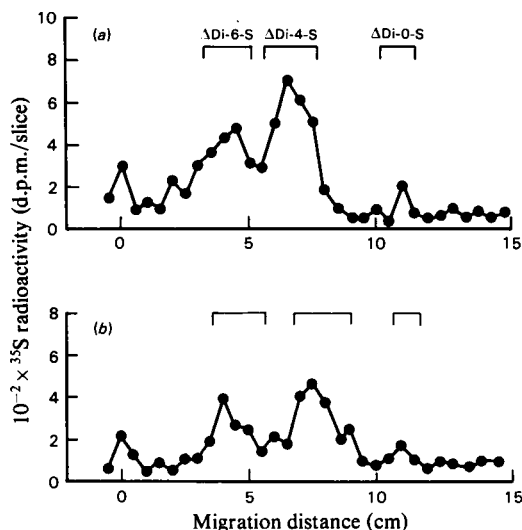


Fig. 6. T.l.c. analysis of disaccharides in the chondroitinase ABC digests

$^{35}\text{S}$ -glycosaminoglycans were digested with chondroitinase ABC and applied to cellulose t.l.c. plates. The  $^{35}\text{S}$ -labelled unsaturated disaccharides were desalted and then separated in butan-1-ol/acetic acid/ $2\text{M-NH}_3$  (2:3:1, by vol). The sheets were cut into 0.5 cm strips and their radioactivities counted. (a) Controls; (b) catabolin-treated.

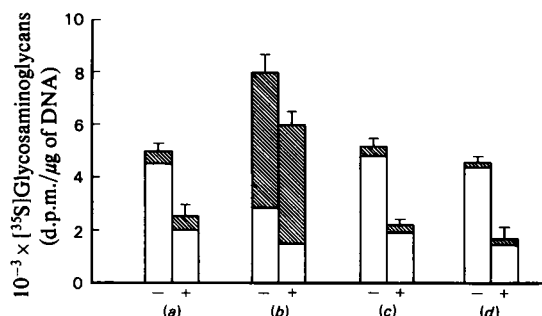


Fig. 7. Synthesis of glycosaminoglycans in the presence of  $\beta$ -D-xyloside and lysosomal-enzyme inhibitors

Cartilage was cultured for 3 days in the absence (—) and in the presence (+) of catabolin ( $0.14 \text{ nM}$ ) 2 h before the labelling. Parallel cultures received (a) buffer, (b) methyl  $\beta$ -D-xyloside (1 mM), (c)  $\text{NH}_4\text{Cl}$  (25 mM) and (d) 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ( $10 \mu\text{M}$ ). The amount of  $^{35}\text{S}$  sulphate incorporated in the presence of these agents (3 h pulse) was measured and is shown as  $^{35}\text{S}$ -glycosaminoglycans in the cartilage ( $\square$ ) and in the medium ( $\blacksquare$ ). The values are the means for three experiments. Bars indicate S.E.M.

control and of catabolin-treated cartilage decreased at similar rates during the chase period.

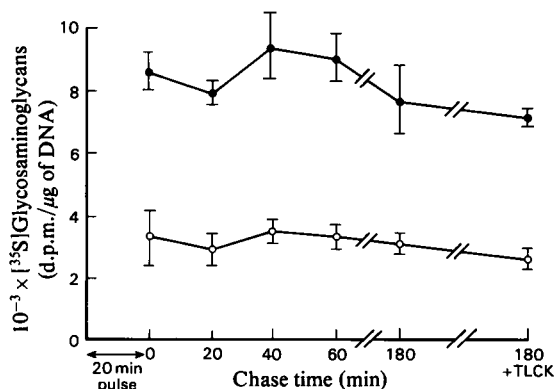


Fig. 8. Degradation of newly synthesized proteoglycan

Cartilage was cultured for 3 days in the absence and in the presence of catabolin ( $0.14 \text{ nM}$ ). Parallel cultures were labelled with  $^{35}\text{S}$  sulphate for 20 min, washed well and incubated in chase medium for 0, 20, 40, 60 or 180 min in the presence or absence of 1-amino-7-chloro-3-L-tosylamidoheptan-2-one (TLCK). The explants were sliced into  $30 \mu\text{m}$  sections and  $^{35}\text{S}$ -proteoglycans were extracted. The residue was solubilized by papain treatment. The amount of total  $^{35}\text{S}$ -proteoglycan recovered is shown as a function of chase time.  $\bullet$ , Control;  $\circ$ , catabolin-treated. Values are the means for four experiments. Bars indicate S.E.M.

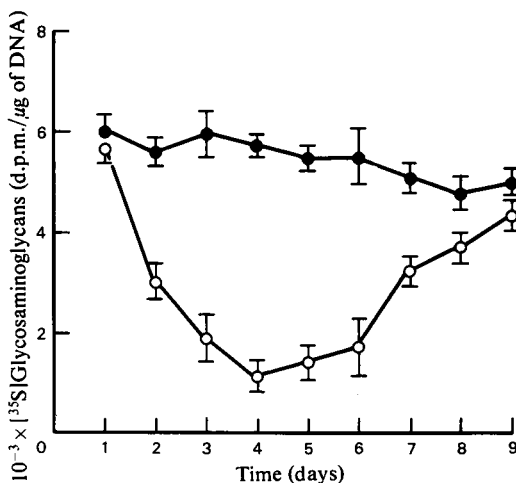


Fig. 9. Synthesis of proteoglycan during recovery

Equivalent halves of cartilage were cultured for 9 days in control medium ( $\bullet$ ) or for 4 days with catabolin and then 5 days in control medium ( $\circ$ ). Parallel cultures were labelled with  $^{35}\text{S}$  sulphate (3 h) every 24 h, and the amount of  $^{35}\text{S}$ -glycosaminoglycan in papain digests of the cartilage was measured. Values are the means for three experiments. Bars indicate S.E.M.

### *Synthesis of proteoglycan during recovery*

The rate of proteoglycan synthesis was measured every 24 h ( $[^{35}\text{S}]$ sulphate, 3 h pulse) during a 4-day period with catabolin, then for 5 days during recovery in control medium (Fig. 9). Incorporation of the radiolabel increased during recovery and was approaching that of the controls after 5 days.

### **Discussion**

Pig leucocyte catabolin (0.01–1.0 nM) causes a dose-dependent reversible inhibition of proteoglycan synthesis in pig cartilage cultured in serum-free medium. After 3 days' exposure at 0.14 nM, incorporation of  $[^{35}\text{S}]$ sulphate was diminished to 30% of that in the controls. Serum contains undefined amounts of various factors that enhance proteoglycan synthesis (Castor & Whitney, 1978; Stevans & Hascall, 1981; Prins *et al.*, 1982), and was therefore omitted in these experiments. In the presence of heat-inactivated foetal-calf serum (5%, v/v) both absolute rates of proteoglycan synthesis were higher (results not shown) and the relative inhibition with catabolin was maintained. This was also observed in lipopolysaccharide-treated cartilage grown with or without serum (Morales *et al.*, 1984).

Cartilage proteoglycan synthesis is initiated by the transfer of xylose to serine residues on a pre-formed core protein (Robinson *et al.*, 1966). The sequential addition of two galactose residues and the first glucuronic acid residue of the chondroitin sulphate chain is then catalysed by distinct and specific enzymes (Helting & Rodén, 1969). Elongation of the chain occurs by the addition of alternate residues of *N*-acetylgalactosamine and glucuronic acid (Tesler *et al.*, 1966). Sulphation of the C-4 or C-6 position of almost every galactosamine residue occurs concurrently with elongation (DeLuca *et al.*, 1973). The rate of incorporation of  $[^{35}\text{S}]$ sulphate in a normal system is therefore equivalent to the rate of glycosaminoglycan synthesis.  $\beta$ -D-Xyloside acts as an acceptor for the galactosyltransferase (Okayama *et al.*, 1973; Schwartz *et al.*, 1974; Robinson *et al.*, 1975) and provides a substrate to which chains can be attached and synthesized independently of the core protein.

Defective synthesis resulting in undersulphated residues, chains of a shorter length or diminished substitution of serine residues leading to fewer chains per monomer, would all decrease the amount of  $[^{35}\text{S}]$ sulphate in newly synthesized proteoglycan. However, the monomers and glycosaminoglycan chains synthesized in the presence of catabolin had the same average hydrodynamic size and charge as those of the controls and were sulphated to the same extent. A 3-fold increase in the rate of chain synthesis occurred in catabolin-

treated cartilage as well as the controls in the presence of xyloside, confirming that elongation and sulphation were not rate-limiting.

Incorporation of  $[^3\text{H}]$ acetate into glycosaminoglycans was not diminished to the same extent as that of  $[^{35}\text{S}]$ sulphate in cartilage cultured with catabolin for 3 days. As the newly synthesized chains were not undersulphated, this presumably is due to an increased specific radioactivity of  $^3\text{H}$  in the proteoglycan from the catabolin-treated cartilage. The metabolic precursors of glycosaminoglycans are common to several carbohydrate pathways. An increased specific radioactivity of the  $[^3\text{H}]$ acetylhexosamine pool in cartilage exposed to catabolin would imply an altered demand in some other area of carbohydrate metabolism. A low  $^3\text{H}/^{35}\text{S}$  ratio was also observed in chondroitin sulphate isolated from lipopolysaccharide-treated cartilage labelled with  $[^3\text{H}]$ glucosamine and  $[^{35}\text{S}]$ sulphate (Morales *et al.*, 1974). These authors suggested that less glucose may be metabolized by cells synthesizing decreased amounts of proteoglycan, allowing increased amounts of  $[^3\text{H}]$ glucosamine to enter the precursor pool.

Catabolin does not cause degradation of extracellular glycosaminoglycan chains (Tyler, 1985). However, it was not known whether an increased number of molecules, especially if defective, were being shunted to the lysosomes to be degraded before secretion (Bienkowski, 1984). Increased intracellular degradation of underhydroxylated collagen can be significantly diminished by the addition of  $\text{NH}_4\text{Cl}$ , leupeptin, chloroquine or 7-amino-1-chloro-3-tosylamidoheptan-2-one, which inhibit lysosome function (Berg *et al.*, 1980). The decrease in the uptake of  $[^{35}\text{S}]$ sulphate was not reversed in the presence of these inhibitors in the catabolin-treated cultures. It was concluded that the decreased synthesis of proteoglycan in cartilage exposed to catabolin was probably caused by a lack of xylose-substituted core protein. This could be due either to decreased activity of the xylosyltransferase or to decreased amounts of core protein.

It is not known whether this is a direct effect of catabolin or is due to a 'feedback inhibition' caused by increased concentrations of prostaglandin  $\text{E}_2$ , proteoglycan subunit or hyaluronic acid, all of which are said to inhibit proteoglycan synthesis (Lipiello *et al.*, 1978; Handley *et al.*, 1980; Wiebkin *et al.*, 1975; Handley & Lowther, 1976). Catabolin-induced inhibition of proteoglycan synthesis is independent of the stimulation of prostaglandin  $\text{E}_2$ , as the pig cartilage cultured in the absence of serum secreted undetectable ( $<5\text{ pmol/ml}$ ; S. J. Sarsfield, unpublished work) amounts.

Decreased synthesis of normal proteoglycan occurs also in cartilage stimulated with lipopoly-

saccharide (Morales *et al.*, 1984), bromodeoxyuridine (Schwartz, 1976), colchicine (Lohmander *et al.*, 1976) and retinol (Jubb, 1984). It is possible that these agents might stimulate endogenous catabolin production. Alternatively, chondrocytes may simply respond in a similar manner to a variety of traumatic events. The fact that new proteoglycan synthesis is drastically diminished during chondrocyte-mediated degradation of the extracellular matrix precludes any attempt at repair while the stimulus persists.

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