Biosynthesis of Proteoglycans in Cartilage Slices

FRACTIONATION BY GEL CHROMATOGRAPHY AND EQUILIBRIUM DENSITY-GRADIENT CENTRIFUGATION

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The kinetics of incorporation of [35 S]sulphate into slices of pig laryngeal cartilage *in vitro* was linear with time up to 6 h. The specific radioactivities of the extracted proteoglycans (containing about 80% of the uronic acid of the cartilage) and the glycosaminoglycans remaining in the tissue after extraction were measured after various times of continuous and 'pulse-chase' radioactivity incorporation. Radioactivity was present in the isolated chondroitin sulphate after 2 min, but there was a 35 min delay in its appearance in the extractable proteoglycan fraction. Fractionation of the proteoglycans by gel chromatography showed that the smallest molecules had the highest specific radioactivity, but 'pulse-chase' experiments over 5 h did not demonstrate any precursor-product relationships between fractions of different size. Equilibrium density-gradient centrifugation in 4 M-guanidine hydrochloride showed that among the proteoglycan fractions the specific radioactivity increased as the chondroitin sulphate content decreased, but with preparations from 'pulse-chase' experiments there was again no evidence for precursor-product relationships between the different fractions. Differences in radioactive incorporation would seem to reflect metabolic heterogeneity within the proteoglycans extracted from cartilage. This may be due either to a partial separation of different types of proteoglycans or to differences in the rates of degradation of the molecules of different size and composition as a result of the nature and specificity of the normal degrading enzymes. The results suggest that molecules of all sizes were formed at the same time.

Proteoglycans account for 6% of the wet weight of pig laryngeal cartilage and consist of molecules that vary in size and in relative content of protein, chondroitin 4-sulphate and keratan sulphate (Tsiganos & Muir, 1969*a,b*; Tsiganos *et al.*, 1971). Chondroitin 4-sulphate accounts for about 90% and keratan sulphate for about 10% of the total hexosamine and they appear to be part of the same molecule (Tsiganos & Muir, 1967).

Previous studies of the biosynthesis of proteoglycans of cartilage from different species and anatomical sites (Gross *et al.*, 1960; Davidson & Small, 1963; Kresse & Buddecke, 1968; Kleine *et al.*, 1971) showed that there was considerable heterogeneity among proteoglycan fractions separated by various methods, which appeared incompatible with the turnover of a single proteoglycan species.

Hascall & Sajdera (1969) showed that in extracts from the cartilage of bovine nasal septa, the largest proteoglycans were aggregates of smaller molecules held together by a 'link' glycoprotein, a fact that raises further problems in examining the biosynthesis of proteoglycans. Moreover, the large degree of heterogeneity of proteoglycans that exists even

under disaggregating conditions (Tsiganos *et al.*, 1971) indicated that a further assessment of the metabolic heterogeneity of the proteoglycans of cartilage was required. By applying more effective methods to fractionate proteoglycans, their metabolic inter-relationship has been examined by using the incorporation *in vitro* of [35 S]sulphate into proteoglycans, in slices of pig laryngeal cartilage.

Experimental

Materials

All reagents were of analytical grade, with the exception of 9-aminoacridine hydrochloride, galactosamine hydrochloride, glucosamine hydrochloride, glucuronolactone, carbazole, naphthalene, 2-methoxyethanol and guanidine hydrochloride, which was purified with activated charcoal (Norit NK; Hopkin and Williams, Chadwell Heath, Essex, U.K.) and acetylacetone, which was redistilled (b.p. 133–134°C).

Cetylpyridinium chloride (special grade, lot 30601) was obtained from A. B. Kabi, Stockholm, Sweden.

Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., supplied scintillation-grade 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen and crude papain (EC 3.4.4.10). Twice-crystallized papain (4 units/mg of protein) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; gelatin (Bacto) from Difco Laboratories, Detroit, Mich., U.S.A.; and naphthalene flakes from Boots Pure Drug Co. Ltd., Nottingham, U.K. Visking tubing (width 24/32 in and 8/32 in) for dialysis was obtained from Scientific Instruments Centre, London W.C.1, U.K.

Methods

Analytical methods. Uronic acid was measured by an automated modification (D. Heinegård, personal communication) of the method of Bitter & Muir (1962) with glucuronolactone as standard. Hexosamine was measured by the procedure of Kraan & Muir (1957) with galactosamine hydrochloride as standard. Samples were hydrolysed in sealed glass tubes under N_2 in 8M-HCl for 3 h at 95°C (Swann & Balazs, 1966). Excess of acid was removed by rotary evaporation at 40°C and the hydrolysate made up to a known volume with water. Glucosamine/galactosamine molar ratios were determined in the same hydrolysates by using a Locarte amino acid analyser (Tsiganos & Muir, 1969b). Sulphate was determined after hydrolysis in 60% (v/v) formic acid for 8 h at 100°C or in 8M-HCl for 3 h at 95°C. In either case excess of acid was removed from the hydrolysate by rotary evaporation below 40°C and sulphate was determined after the addition of a known volume of water by the method of Dodgson (1961). Protein was measured by an automated modification (D. Heinegård, personal communication) of the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as standard.

Measurement of radioactivity. Water-based samples of up to 500 μ l were placed in low-background glass vials containing 10 ml of toluene-2-methoxyethanol (3:2, v/v) scintillation fluid, containing (per litre) 80 g of naphthalene and 4 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen, and the radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. Counting rates were corrected for quenching by using a channels-ratio method with the external standard.

Incubation experiments: continuous incorporation. Larynges from pigs (6-9 months old) were obtained fresh from the slaughterhouse. The thyroid cartilage was dissected free of adhering tissue and perichondrium in the cold-room, and was then sliced into sections 50-100 μ m thick while being kept moist with 0.15M-NaCl. Excess of liquid was rapidly removed from the slices on a coarse sintered-glass funnel and weighed samples were added to incubation media, about 4 ml/g of wet cartilage being used.

All samples were equilibrated in a shaking water-bath at 37°C for 30 min before addition of radioactive isotope, to avoid any abnormal effect on the subsequent radioactivity incorporation (Hardingham & Muir, 1970). The medium was Krebs-Ringer bicarbonate, pH 7.4, (Umbreit *et al.*, 1964), in which $MgCl_2$ replaced $MgSO_4$ and which contained 20 mM-D-glucose and 10 mM-L-glutamine. Carrier-free [^{35}S]-sulphate (The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the incubation mixtures, 100 μ Ci/ml being used for incubations of less than 30 min and 20 μ Ci/ml for longer times of incorporation.

At the end of the incubation, when proteoglycans were to be isolated, the cartilage slices were filtered on a sintered-glass funnel and transferred to cold extracting medium (2M- $CaCl_2$ -50 mM-sodium acetate, pH 5.8, or 4M-guanidine hydrochloride-50 mM-sodium acetate, pH 5.8, at 4°C). When glycosaminoglycans alone were to be isolated the incubation tubes were immersed in a boiling-water bath for 3 min and then cooled in ice before proteolysis.

When incubation was continued for 24 h, streptomycin (5 μ g/ml) and penicillin (50 units/ml) were included in the incubation medium; [^{35}S]-sulphate was added to the incubation mixture after 30 min at 37°C and to a duplicate incubation mixture after 23 h at 37°C. In both, incorporation was stopped 1 h later and the cartilage from each was divided in two. The glycosaminoglycans were isolated from one half and their specific radioactivities based on uronic acid determined. The proteoglycans from the other half were extracted with 4M-guanidine hydrochloride at pH 5.8, and isolated by equilibrium density-gradient centrifugation (Hascall & Sajdera, 1969). The samples were subjected to chromatography on Sepharose 2B. The medium from 24 h experiments was dialysed against several changes of water, concentrated to a small volume by pressure dialysis and any proteoglycans present were isolated by precipitation with 9-aminoacridine. Samples were fractionated by gel chromatography on Sepharose 2B. The clear medium and normal appearance of proteoglycans isolated from cartilage after incubation for 24 h suggested that bacterial contamination was insignificant.

'Pulse-chase' experiments. In 'pulse-chase' experiments cartilage was incubated with [^{35}S]-sulphate in the normal way for up to 15 min. It was then filtered on a sintered glass funnel, washed twice with about 5 ml of warm incubation medium containing 1 mM- Na_2SO_4 , and then replaced in fresh medium containing 1 mM- Na_2SO_4 at 37°C for various times of non-radioactive 'chase'.

Isolation of glycosaminoglycans. Glycosaminoglycans were isolated, after proteolytic digestion of the cartilage with papain, by precipitation with cetylpyridinium chloride. The cartilage slices were suspended in 0.1M-sodium acetate, pH 5.5, containing

50mM-EDTA (disodium salt) and 10mM-L-cysteine. Activated (Kimmel & Smith, 1954) crude papain (20mg/g wet wt. of cartilage) was added to the suspension, which was incubated at 60°C for 4h. The small amount of insoluble residue remaining after digestion contained no hexosamine and was removed by centrifugation and discarded. After dilution of the digest with an equal volume of water and a few drops of saturated Na₂SO₄, the glycosaminoglycans were precipitated by the dropwise addition of 10% (w/v) cetylpyridinium chloride. After 1h at room temperature the precipitate was centrifuged and washed twice with 0.05% (w/v) cetylpyridinium chloride containing 5mM-Na₂SO₄ and once with 0.05% (w/v) cetylpyridinium chloride alone. The cetylpyridinium chloride complex was dissolved in a minimum volume of propan-1-ol and isolated and dried as described above. Uronic acid and radioactivity were determined in samples redissolved in known volumes of water.

Isolation of proteoglycans. Cartilage slices from incubations were suspended in 10vol. of 2M-CaCl₂, pH5.8, and agitated on a mechanical roller for 24h at 4°C. The extracted proteoglycans were purified by precipitation with 9-aminoacridine hydrochloride (Tsiganos & Muir, 1969a) or by equilibrium density-gradient centrifugation (Hascall & Sajdera, 1969). The proteoglycans extracted with either 2M-CaCl₂ or 4M-guanidine hydrochloride accounted for 77–80% of the total uronic acid. Of the remaining uronic acid, 14–16% was in the cartilage residue and 5–8% appeared in the incubation medium. Even after 6h incubation, however, the latter contained only a small amount of radioactivity and was discarded.

(a) Precipitation with 9-aminoacridine. At the end of the extraction with 2M-CaCl₂ the cartilage slices were filtered through a double layer of lint and rinsed with a small volume of 2M-CaCl₂. The combined extract and washings were dialysed against several changes of water overnight. The proteoglycans were isolated as their sodium salts after purification by precipitation with 9-aminoacridine hydrochloride (Tsiganos & Muir, 1969a). Dried samples were redissolved overnight with agitation in known volumes of 0.5M-sodium acetate, pH6.8, for measurement of uronic acid and radioactivity or for gel chromatography.

(b) Equilibrium density-gradient centrifugation. The extract from cartilage slices with 2M-CaCl₂ or 4M-guanidine hydrochloride was dialysed overnight against 9vol. of 50mM-sodium acetate, pH5.8. Solid CsCl was added to bring the density to 1.50g/ml and the solution was centrifuged in an M.S.E. 65 centrifuge in an 8×25ml angle head at 95000g_{av.} for 48h at 20°C. The proteoglycans, which accounted for 95% of the total uronic acid, were recovered from the bottom two-fifths of the gradient. They were free of collagen and other unbound protein contaminants (Tsiganos *et al.*, 1971).

After extraction of the proteoglycans the residual glycosaminoglycans were isolated from the cartilage slices by digestion with crude papain as described above after the slices had been soaked in water overnight to remove excess of CaCl₂ or guanidine hydrochloride.

Chromatographic methods

Micro-column fractionation of glycosaminoglycans as cetylpyridinium chloride complexes. Chondroitin sulphate and keratan sulphate obtained from proteoglycans after proteolysis were separated by cetylpyridinium chloride–cellulose chromatography on micro-columns essentially as described by Antonopoulos *et al.* (1964).

The proteoglycan sample in 0.1M-sodium acetate, pH5.5, containing 5mM-EDTA (disodium salt) and 5mM-cysteine was digested with activated (Kimmel & Smith, 1954) twice-crystallized papain for 4h at 60°C. A sample of the digest containing 100–200μg of hexosamine was applied to micro-columns (6.0cm×0.3cm) of cellulose (Whatman CF 11; H. Reeve Angel and Co. Ltd., London E.C.4, U.K.) that was saturated with 1% (w/v) cetylpyridinium chloride containing 5mM-Na₂SO₄. Keratan sulphate was eluted with 2.0ml of 1% (w/v) cetylpyridinium chloride containing 5mM-Na₂SO₄ and 1.0ml of 0.05% (w/v) cetylpyridinium chloride, and chondroitin sulphate was eluted with 2.0ml of 0.75M-MgCl₂ in 0.05% (w/v) cetylpyridinium chloride and 1ml of 0.05% (w/v) cetylpyridinium chloride (Antonopoulos *et al.*, 1964). The column was finally washed with 2.0ml of 6M-HCl. The radioactivity was measured of samples of both 1% cetylpyridinium chloride fractions and of 6M-HCl fractions, and their hexosamine contents were determined after hydrolysis in 6M-HCl for 8h at 100°C. The 0.75M-MgCl₂ fraction was diluted with 3vol. of water and the precipitated cetylpyridinium chloride complex was collected by centrifugation after 1h at room temperature. The precipitate was washed in 0.05% (w/v) cetylpyridinium chloride, then was dissolved in a minimum volume of propan-1-ol and reprecipitated as the sodium salt by the addition of a few drops of saturated sodium acetate and 5vol. of ethanol. After being left overnight at 4°C the precipitate was collected by centrifugation, then washed with ethanol and dried *in vacuo* over silica gel. It was then dissolved in a known volume of water and portions were taken for measurement of radioactivity and for hexosamine content. The 6M-HCl fractions contained too little hexosamine and radioactivity for the specific radioactivity to be accurately measured.

Gel chromatography. Samples of proteoglycan containing about 1mg of uronic acid in 1.0ml of 0.5M-sodium acetate, pH6.8, were applied to a column (180cm×1.1cm) of Sepharose 2B (Pharmacia,

Uppsala, Sweden) that was eluted with 0.5M-sodium acetate, pH 6.8, at the rate of 5 ml/h at 4°C by using a peristaltic pump. Fractions of about 2.5 ml (40 drops) were collected and their uronic acid content and radioactivity determined.

Recombination of gel-chromatographic fractions under disaggregating conditions. A sample of radioactive proteoglycan was fractionated by gel chromatography on Sepharose 2B into material excluded and retarded by the gel. These pooled fractions were concentrated to a small volume by pressure dialysis (Diaflo UM-2 membrane; Amicon Co., Lexington, Mass., U.S.A.) and similar fractions from a non-radioactive sample of proteoglycan were also prepared. The radioactive retarded fraction was then mixed with the non-radioactive excluded fraction under disaggregating and non-disaggregating conditions (see Fig. 4). When disaggregating conditions were required the solutions were adjusted to 4M-guanidine hydrochloride by adding 2 vol. of 8.0M-guanidine hydrochloride containing 50 mM-sodium acetate, pH 5.8, before mixing. After mixing, fractions were left overnight at 5°C and then dialysed against at least 100 vol. of 0.5M-sodium acetate, pH 6.8. Solutions were then concentrated by vacuum dialysis until they contained about 1 mg of uronic acid/ml and were then chromatographed again on a column of Sepharose 2B.

Other methods

Equilibrium density-gradient centrifugation under disaggregating conditions. Proteoglycans prepared by equilibrium density-gradient centrifugation in the absence of high concentrations of guanidine hydrochloride were centrifuged again in a CsCl density gradient in the presence of 4M-guanidine hydrochloride essentially as described by Tsiganos *et al.* (1971) except that the number and volume of the fractions taken were varied.

Radioautography. Slices of cartilage from incubations were fixed for 1 h in acetic acid-ethanol (1:3, v/v) then for 24 h in 0.15M-NaCl containing formaldehyde (4%, w/v). The slices were embedded in paraffin wax, sectioned at 5–6 μ m and radioautographs were prepared by the stripping-film technique by using Kodak AR.10 film (Pelc, 1956). The preparations were exposed for 44 days at –25°C and, after photographic processing, were stained with Haematoxylin and Eosin. Slices of cartilage that had been extracted with 4M-guanidine hydrochloride were soaked in water for several hours before fixing, to remove excess of the extracting salts.

Results

Although the experiments reported here were limited to the period of linear incorporation of

radioactivity, the cartilage slices used as a routine in this study appeared viable in the medium for a considerably longer time. Thus [³⁵S]sulphate was incorporated into apparently normal proteoglycans (as assessed by gel chromatography on Sepharose 2B) at 60% of the initial rate after 24 h in the medium at 37°C. During this period proteoglycans accounting for about 12% of the uronic acid of the cartilage appeared in the medium, but chromatography on Sepharose 2B showed this to be relatively undegraded. As 5–8% of the uronic acid appeared in the medium after only 30 min of incubation (Hardingham & Muir, 1970) most of the proteoglycan in the medium appeared to have diffused out from the newly exposed surfaces of the cartilage slices rather than to have resulted from autolysis.

It has been noted (Hardingham & Muir, 1970) that there was a transient abnormal incorporation of [³⁵S]sulphate into a chondroitin sulphate fraction of long chain length when cartilage at 4°C was added directly to radioactive medium at 37°C. As this effect was abolished by preincubating the cartilage at 37°C for 30 min before the radioactive isotope was added to the incubation mixture, this procedure was used in all the experiments described here.

No extra inorganic sulphate was added to the medium. The incorporation of [³⁵S]sulphate was linear with time up to 6 h (Hardingham & Muir, 1970), and higher sulphate concentrations (1 mM) did not extend the time of linear incorporation. There was

Fig. 1. Incorporation of [³⁵S]sulphate with time into slices of pig laryngeal cartilage incubated *in vitro* at 37°C

Incorporation into the total glycosaminoglycan fraction of the cartilage (●) and into the extractable proteoglycan fraction (■) from a parallel series of incubations is shown.

a short initial delay of about 5 min in the incorporation of radioactivity into glycosaminoglycans (Fig. 1). The incorporation into the proteoglycans extracted with 2M-CaCl₂ or 4M-guanidine hydrochloride was also linear with time, but only after a much longer delay of about 40 min (Fig. 1). The difference between these times was the average time, i.e. 35 min, that it took proteoglycans to become extractable with either 2M-CaCl₂ or 4M-guanidine hydrochloride. The earliest time at which any radioactivity could be detected in the extracted proteoglycans was about 15 min after the addition of label (Fig. 1).

Residual glycosaminoglycans

The specific radioactivity of the residual glycosaminoglycans obtained by papain digestion was higher than that of the extracted proteoglycan, but during 'pulse-chase' experiments the specific radioactivity fell rapidly over the first hour of radioactive 'chase', whereas that of the extracted proteoglycans correspondingly increased (Fig. 2). However, the specific radioactivity of the extract never exceeded that of the residue, and after 1 h the specific radioactivities showed little further change, that of the residual glycosaminoglycans remaining somewhat higher than that of the extracted proteoglycans.

Glycosaminoglycans isolated by precipitation as cetylpyridinium chloride complexes contain very little keratan sulphate (Hardingham & Muir, 1970), but,

as proteoglycans contain both chondroitin sulphate and keratan sulphate, some of the radioactive sulphate would also be incorporated into keratan sulphate. This, however, was found to be only about 5% of the total radioactivity, because keratan sulphate accounts for only 10% of the glycosaminoglycans in pig laryngeal cartilage and it had about half the specific radioactivity of chondroitin sulphate in a preparation of ³⁵S-labelled proteoglycan (Table 3; see below).

Fractionation by gel chromatography

Proteoglycans extracted from cartilage slices after various times of incubation were fractionated by gel chromatography on Sepharose 2B (Fig. 3). Radioactivity was distributed throughout the uronic acid profile, but the specific radioactivity was higher in smaller than in larger molecules (Table 1). Nevertheless, the distribution of radioactivity did not change with longer times of incubation and was similar in preparations isolated either by precipitation with 9-aminoacridine or by equilibrium density-gradient centrifugation. Day-to-day variation in the relative incorporation into gel-chromatographic fractions was quite large, but in all instances the smaller components were more highly labelled than larger ones (Table 1).

Possible metabolic relationships between molecules of different size were therefore sought in 'pulse-chase'

Fig. 2. Changes in the specific radioactivities of proteoglycans and corresponding residual glycosaminoglycans

The materials were isolated from slices of pig laryngeal cartilage incubated with [³⁵S]sulphate for 15 min and for various times of non-radioactive 'chase'. Proteoglycans were extracted with 2M-CaCl₂, pH 5.8 (●), or with 4M-guanidine hydrochloride, pH 5.8 (○), and the corresponding glycosaminoglycans remaining in the cartilage after extraction, (■) and (□) respectively, were isolated after proteolytic digestion.

Fig. 3. Gel chromatography of proteoglycans

The proteoglycans were extracted with 2M-CaCl₂, pH 5.8, from slices of pig laryngeal cartilage incubated *in vitro* for 60 min with [³⁵S]sulphate. Samples were applied to a column (180 cm × 1.1 cm) of Sepharose 2B eluted with 0.5M-sodium acetate, pH 6.8, at the rate of 5 ml/h at 4°C. Fractions (2.5 ml) were collected and the uronic acid content (—) and radioactivity (---) were measured. Fractions containing the largest (E), medium (R₁) and smallest (R₂) size proteoglycans were pooled separately for analysis. V₀ and V_t mark the void volume and total volume of the column respectively.

Table 1. Incorporation of [³⁵S]sulphate into proteoglycans fractionated by gel chromatography (Fig. 3) after various times of continuous and 'pulse-chase' incorporation

The proteoglycans were extracted either with 2M-CaCl₂ or 4M-guanidine hydrochloride and then purified by selective precipitation with 9-aminoacridine hydrochloride (SP) or by equilibrium density-gradient centrifugation (DG). Some relative specific radioactivities are means ± S.D. for five experiments where given.

Time of radioactive incubation (min)	Time of non-radioactive 'chase' (min)	Extracting solvent	Method of purification	Relative specific radioactivities of Sepharose 2B fractions shown in Fig. 3 (d.p.m./mg of uronic acid)		
				E	R ₁	R ₂
60	0	2M-CaCl ₂	SP	1.00	1.26 ± 0.16	1.74 ± 0.37
120	0	2M-CaCl ₂	SP	1.00	1.07	1.52
120	0	2M-CaCl ₂	DG	1.00	1.10	1.57
120	0	4M-Guanidine hydrochloride	DG	1.00	1.36 ± 0.15	1.68 ± 0.29
15	0	2M-CaCl ₂	SP	1.00	1.14	1.38
15	60	2M-CaCl ₂	SP	1.00	1.15	1.44
15	120	2M-CaCl ₂	SP	1.00	1.07	1.44
15	180	2M-CaCl ₂	SP	1.00	1.08	1.35
15	300	2M-CaCl ₂	SP	1.00	1.13	1.46
15	0	4M-Guanidine hydrochloride	DG	1.00	1.25	1.75
15	60	4M-Guanidine hydrochloride	DG	1.00	1.18	1.66
15	180	4M-Guanidine hydrochloride	DG	1.00	1.31	1.81

experiments in which cartilage slices were labelled for 15 min with [³⁵S]sulphate and then incubated for up to 5 h in non-radioactive medium that contained 1 mM-sodium sulphate. When the extracted proteo-

glycans were fractionated by gel chromatography on Sepharose 2B there was no change in the distribution of radioactivity during the non-radioactive 'chase' (Table 1). Thus no precursor-product relationships

were evident between molecules of different size over the time-scale of these experiments.

Hascall & Sajdera (1969) showed that some of the proteoglycans extracted from bovine nasal septal cartilage consisted of aggregates of smaller molecules. The results of Tsiganos & Muir (1970) with pig laryngeal cartilage suggested that such aggregates would be excluded from Sepharose 2B. Under the

conditions used here these would be disaggregated during the extraction but would form again during dialysis of the extracting salts. If the material retarded by Sepharose 2B represented subunits from which aggregates may be formed, the process of extraction and isolation should have randomized the radioactivity between the excluded and retarded fractions. The results given in Fig. 4 show that this did not

Fig. 4. Gel chromatography of proteoglycan fractions combined under disaggregating and non-disaggregating conditions

(a) Labelled starting material; (b) labelled fraction retarded by Sepharose 2B; (c) labelled fraction retarded by Sepharose 2B mixed in 0.5M-sodium acetate, pH 6.8, with unlabelled fraction excluded from Sepharose 2B; (d), as (c) but mixed under disaggregating conditions in 4M-guanidine hydrochloride, pH 5.8, then dialysed (see the text). Uronic acid (—) and radioactivity (---) were measured in the fractions. The conditions of gel chromatography were as described in Fig. 3.

occur, because when labelled retarded fractions were mixed with unlabelled excluded fractions under disaggregating conditions and then dialysed, the distribution of radioactivity was almost the same as when these fractions were mixed under non-disaggregating conditions. Therefore the material retarded by gel chromatography on Sepharose 2B was different from the material in the excluded fractions, which appeared to be capable of reversible disaggregation. Hence the differences in the distribution

of radioactivity between the fractions retarded and excluded by Sepharose 2B were not artifacts of preparation.

Fractionation by equilibrium density-gradient centrifugation in 4M-guanidine hydrochloride

Proteoglycans extracted with 2M-CaCl₂ or 4M-guanidine hydrochloride from cartilage incubated with [³⁵S]sulphate were fractionated by equilibrium

Fig. 5. Equilibrium density-gradient centrifugation of proteoglycans

The proteoglycans were extracted with 4M-guanidine hydrochloride, pH 5.8, from pig laryngeal cartilage that was incubated *in vitro* with [³⁵S]sulphate for 90 min at 37°C. (a) Proteoglycans centrifuged in 4M-guanidine hydrochloride-CsCl, starting density 1.50 g/ml, in the 8×25 ml angle rotor of the M.S.E. 65 centrifuge run at 95000*g*_{av} for 48 h at 20°C. (b) Proteoglycans from fractions A1-4 re-fractionated under similar conditions except that the starting density was 1.65 g/ml.

Table 2. Analyses of proteoglycan fractions from equilibrium density-gradient centrifugation

Fraction	Fractions are defined in Fig. 5.		Specific radioactivity (d.p.m./mg of uronic acid)
	Uronic acid protein weight ratio	Galactosamine glucosamine molar ratio	
A5	0.05	2.4	150
A4	0.35	3.4	220
A3	0.80	6.1	170
A2	1.66	11.9	66
A1	2.00	16.8	40
B6	0.35	3.2	943
B5	0.50	—	505
B4	0.75	4.9	243
B3	1.37	11.0	52.1
B2	1.95	17.1	17.3
B1	2.04	18.6	15.4

density-gradient centrifugation in 4M-guanidine hydrochloride, pH 5.8, as described by Tsiganos *et al.* (1971) (Fig. 5a). The distribution of radioactive sulphate was qualitatively similar to that of uronic acid, but the fractions of intermediate density had higher specific radioactivity (based on uronic acid) than the fraction at the bottom of the gradient, which contained most of the uronic acid (Fig. 5a). The fraction at the top of the gradient contained very little uronic acid but a large amount of protein, distinguishing it from all other fractions. Its specific

radioactivity was somewhat lower than that of the middle fraction. Further fractionation of the middle and bottom fractions at a higher starting density (Fig. 5b) showed more striking differences in specific radioactivity in proteoglycans of different composition (Table 2). The top fraction (B6, Fig. 5), which contained the least amount of chondroitin sulphate, had a specific radioactivity 60 times higher than the bottom fraction (B1, Fig. 5), which contained the most chondroitin sulphate.

Gel chromatography of each density-gradient

Fig. 6. Gel chromatography on Sepharose 2B of proteoglycan fractions A1, A2, A3, A4 and A5 after equilibrium density-gradient centrifugation in 4M-guanidine hydrochloride (Fig. 5)

The conditions of gel chromatography were as described in Fig. 3. Uronic acid (—) and radioactivity (---) were measured in the fractions collected.

fraction on Sepharose 2B (Fig. 6) showed that there was a broad distribution of size in all density-gradient fractions, but the uppermost fractions (Fig. 6, fractions A4 and A5) were much more polydisperse than the fractions of higher density (Fig. 6, fractions A1 and A2), which contained most of the uronic acid. The specific radioactivities of different regions of the elution profiles of each density gradient fraction, however, showed relatively small variation with size.

Proteoglycans from 'pulse-chase' experiments were also fractionated by equilibrium density-gradient centrifugation in 4M-guanidine hydrochloride (Fig. 7), but there was no apparent exchange of radioactivity with time between fractions of different buoyant densities and again no precursor-product relationships were evident during these experiments of up to 5 h.

Separation of keratan sulphate and chondroitin sulphate from proteoglycans

Keratan sulphate contains equimolar amounts of glucosamine and galactose, whereas chondroitin sulphate contains equimolar amounts of galactosamine and glucuronic acid. The molar ratios of the hexosamines were thus measured in proteoglycan fractions to determine their keratan sulphate content.

To assess how much of the radioactivity was attributable to keratan sulphate, selected fractions were digested with papain. Keratan sulphate and chondroitin sulphate were then separated by cetylpyridinium chloride-cellulose chromatography (Antonopoulos *et al.*, 1964) and their specific radioactivities based on hexosamine were determined. From the specific radioactivity of keratan sulphate and the amount in each fraction the relative proportion of the radioactivity contributed by keratan sulphate was calculated (Table 3). Even in fractions of high keratan sulphate content the radioactivity in keratan sulphate could not account for the large differences in labelling observed between different

Fig. 7. Equilibrium density-gradient centrifugation in 4M-guanidine hydrochloride of 'pulse-chase'-labelled proteoglycans

The conditions of centrifugations were as described in Fig. 5(a). Proteoglycans were extracted with 4M-guanidine hydrochloride, pH 5.8, from slices of pig laryngeal cartilage after 15 min incorporation of [35 S]sulphate and non-radioactive 'chase' for (a) 0 min; (b) 20 min; (c) 60 min; (d) 180 min; (e) 300 min. Uronic acid (—) and radioactivity (---) were measured in the fractions after dialysis against 0.5M-sodium acetate, pH 6.8.

EXPLANATION OF PLATE I

Radioautography of sections of cartilage slices incubated with [^{35}S]sulphate at 37°C. (a) Incubated with [^{35}S]sulphate for 30 min; (b) incubated with [^{35}S]sulphate for 30 min followed by a non-radioactive 'chase' for 180 min; (c) incubated as (a) but subsequently extracted with 4M-guanidine hydrochloride, pH 5.8, for 24 h at 4°C; (d) incubated and 'chased' as in (b), but subsequently extracted with 4M-guanidine hydrochloride, pH 5.8, for 24 h at 4°C.

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Table 3. *Analysis of proteoglycan fractions from gel chromatography and from equilibrium density-gradient centrifugation*

Radioactive proteoglycans were extracted from cartilage slices incubated with [^{35}S]sulphate for 90 min at 37°C. Keratan sulphate and chondroitin sulphate were separated after proteolysis by cetylpyridinium chloride-cellulose chromatography (Antonopoulos *et al.*, 1964).

Fraction	Molar ratios		Specific radioactivity of keratan sulphate (% of specific radioactivity of chondroitin sulphate)	Radioactivity in keratan sulphate (% of total)
	Galactosamine glucosamine	Sulphate hexosamine		
Starting material	11.9	0.933	60	4.6
Sephacrose 2B excluded fraction	8.4	0.938	65	6.9
Sephacrose 2B retarded fraction	14.7	0.928	57	3.6
Fractions as shown in Fig. 5				
A1	} 13.4	0.932	} 65	4.5
A2		0.935		
A3		0.933		
A4	3.6	0.903	55	12.0
A5	2.4	0.912	78	22.9

fractions on gel chromatography (Table 1), or equilibrium density-gradient centrifugation (Table 2).

Thus, although the differential labelling of keratan sulphate was not examined in detail in this study, the results showed that the differences in specific radioactivity observed between fractions, based on uronic acid analyses, were modified only slightly if the contribution of keratan sulphate was taken into account, but the large qualitative differences remained.

Discussion

In assessing the results it was assumed that the incorporation of [^{35}S]sulphate was proportional to the rate of sulphation of polymers and was also a measure of glycosaminoglycan synthesis, since differences in the degree of sulphation of different fractions were small (Table 3).

When cartilage slices were incubated with [^{35}S]sulphate, radioactivity was incorporated rapidly into glycosaminoglycans isolated after proteolytic digestion, but there was a delay of about 35 min before the label appeared in extractable proteoglycans. Results of radioautography were comparable with previous reports on the incorporation of radioactivity into polysaccharide material in chondrocytes both *in vivo* (Pelc & Glücksmann, 1955) and *in vitro* (Revel & Hay, 1964; Peterson & Leblond, 1964; Godman & Lane, 1964; Neutra & Leblond, 1966; Revel, 1970), but it was also noticed that the incorporation of radioactivity into cells was very uneven, and some remained unlabelled (e.g. Plate 1a). This was not

observed with the chondrocytes of adult mouse tracheal cartilage labelled with [^{35}S]sulphate *in vivo* (Pelc & Glücksmann, 1955), and its significance is not yet known. After incorporation for 30 min, radioactivity in labelled cells was most intense in the perinuclear region (Plate 1a) and most of the radioactivity remained within the cells of tissue that had been extracted with 4M-guanidine hydrochloride (Plate 1c). In contrast, after 30 min incorporation followed by 3 h of non-radioactive 'chase', most of the radioactivity had migrated from the centre of the cells towards the periphery and a large proportion appeared to be in the matrix outside the cell (Plate 1b). By this time the radioactivity could be largely removed from cartilage slices by extraction with 4M-guanidine hydrochloride (Plate 1d). There was thus a correlation between the movement of newly synthesized material towards the periphery and out of the cell, and the appearance of radioactivity in proteoglycans extracted by 2M-CaCl₂ or 4M-guanidine hydrochloride. Newly synthesized proteoglycans were not extracted by these solvents, probably because they were still within membranous vesicles (Revel, 1970).

The inextractable proteoglycans of cartilage would thus seem to be of two kinds, those newly synthesized that are not yet secreted from the cells and those firmly associated with collagen. This is demonstrated in Fig. 2. After incorporation for 15 min most of the radioactivity was present in intracellular proteoglycans, but during the first hour of non-radioactive 'chase' incubation a large proportion of the

radioactivity appeared in the extractable fraction, although the amount did not increase with time. The radioactivity that remained in the residue after the non-radioactive 'chase' was probably due to that in the proteoglycans more firmly associated with collagen. These were apparently labelled more rapidly than the extractable proteoglycans, although the homogeneity of this fraction could not be assessed.

Fractionation of the extracted proteoglycans according to size under non-disaggregating conditions by gel chromatography on Sepharose 2B showed that the smallest molecules had a specific radioactivity that was about 70% higher than that of the largest molecules, but 'pulse-chase' experiments revealed no precursor-product relationships between the different fractions. There was thus no evidence of larger molecules being formed from smaller ones, at least among those extracted from the cartilage. Moreover, after 15 min of radioactive incubation, molecules large enough to be excluded from Sepharose 2B were labelled, which was as soon as radioactivity was detectable in the whole proteoglycan fraction. If the Sepharose 2B-excluded fraction contained aggregates, the method of extraction of proteoglycans would have disaggregated any that were present initially in the cartilage matrix. The results showed, however, that all proteoglycans were synthesized at about the same time, but suggested that the smaller proteoglycans had a faster turnover than those of larger size.

The biosynthesis of proteoglycans requires the formation of the polypeptide before the carbohydrate chains are attached (Telser *et al.*, 1965). However, the number of polypeptides involved and whether they have a fixed or variable number of carbohydrate chains attached is not yet known. Equilibrium density-gradient centrifugation under disaggregating conditions in 4M-guanidine hydrochloride fractionated the proteoglycans mainly according to relative carbohydrate and protein contents. Differences in the amino acid analyses (Tsiganos *et al.*, 1971) and in the antigenic determinants (Tsiganos, 1971) of different fractions suggested that two or more polypeptide types were present. Omitting the protein-rich fraction at the top of the gradient there was a 10-fold decrease in the proportion of chondroitin sulphate, compared with protein in fractions of decreasing buoyant density (Tsiganos *et al.*, 1971), which was not accompanied by a corresponding decrease in serine content (C. P. Tsiganos, unpublished work). Since there was little difference in the average length of the chondroitin sulphate chains in different fractions (Tsiganos *et al.*, 1971), the degree of glycosylation of serine residues varied, and hence the number of chondroitin sulphate chains attached to each type of polypeptide, so that each would be spread through the gradient.

After equilibrium density-gradient fractionation in

4M-guanidine hydrochloride most of the radioactivity was in the fraction at the bottom of the gradient that contained most of the chondroitin sulphate. However, when specific radioactivities based on uronic acid were compared it appeared that proteoglycans of low chondroitin sulphate content were labelled faster than the fractions with a high content of chondroitin sulphate. The differences in labelling may be due to the partial separation in the gradient of proteoglycans with different polypeptide cores, or it may reflect different degrees of glycosylation of proteoglycans of each polypeptide type. All types, although possibly synthesized independently of each other, are presumably subject to the same processes of degradation in the matrix, but it is unlikely that with such large differences in size and composition all proteoglycans are degraded at equal rates. If one particular type were degraded more rapidly than others it would be labelled more intensely in experiments of the type described here. Cathepsin D has been implicated in the degradation process (Dingle *et al.*, 1971) and it is possible that molecules with fewer chondroitin sulphate chains, such as in fraction A4 (Table 2), may be degraded more rapidly by a proteolytic enzyme than those protected by a higher density of attached chondroitin sulphate chains, such as those in fraction A1 (Table 2). The differences in labelling could thus reflect either the biosynthesis of different proteoglycan types at different rates or the differential degradation of proteoglycans, depending on their composition, or a combination of these two effects. However, it is not possible to distinguish between these alternatives by experiments *in vitro* of only a few hours duration, because Mankin & Lippiello (1969) showed that the half-life of proteoglycans in adult rabbit articular cartilage is about 8 days *in vivo*, and it is somewhat longer in other types of cartilage (Gross *et al.*, 1960; Davidson & Small, 1963).

By using calf rib cartilage incubated with [³⁵S]-sulphate and [³H]leucine, Kleine *et al.* (1971) also observed metabolic heterogeneity among proteoglycans fractionated by equilibrium density-gradient centrifugation and cetylpyridinium chloride-cellulose chromatography, in which fractions with the highest specific radioactivity also had the highest protein content. However, they found that the specific radioactivity of the residual glycosaminoglycans was about three times greater than that of the purified extracted proteoglycans. This difference from the present results might be caused by the experimental design, as no non-radioactive 'chase' was done and hence a considerable proportion of labelled proteoglycan would still be inside the cells and so included in the residue fraction.

Radioautography indicated that the extractable proteoglycans came mainly from the extracellular matrix, and so the failure to show metabolic relation-

ships between different proteoglycan fractions does not exclude the possibility that there were such relationships while proteoglycans were still inside cells within a few minutes of synthesis. The results do show, however, that a slow aggregation or cross-linking process producing larger from smaller molecules does not occur outside the cell and that both large and small molecules were formed at the same time.

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