Biochem. J. (1972) **130**, 729–738
Printed in Great Britain

Mode of Degradation of the Chondroitin Sulphate Proteoglycan in Rat Costal Cartilage

By ÅKE WASTESON, ULF LINDAHL and ANUND HALLÉN
Institute of Medical Chemistry, University of Uppsala, Box 551, S-751 22 Uppsala, Sweden

(*Received* 3 *July* 1972)

1. Chondroitin sulphate was isolated from different regions of rat costal cartilage after extensive proteolysis of the tissues. The molecular weight, determined by gel chromatography, of the polysaccharide obtained from an actively growing region (lateral zone) near the osteochondral junction was higher than that of the polysaccharide isolated from the remaining portion of the costal cartilage (medial zone). 2. In both types of cartilage the molecular weight of chondroitin sulphate, labelled with [^{35}S]sulphate, remained unchanged *in vivo* over a period of 10 days, approximately corresponding to the half-life of the chondroitin sulphate proteoglycan. The molecular-weight distribution of chondroitin [^{35}S]sulphate, labelled *in vivo* or *in vitro*, was invariably identical with that of the bulk polysaccharide from the same tissue. It is concluded that the observed regional variations in molecular-weight distribution were established at the time of polysaccharide biosynthesis. 3. In tissue culture more than half of the ^{35}S-labelled polysaccharide–proteins of the two tissues was released into the medium within 10 days of incubation. The released materials were of smaller molecular size than were the corresponding native proteoglycans. In contrast, the molecular-weight distribution of the chondroitin [^{35}S]sulphate (single polysaccharide chains) remained constant throughout the incubation period. 4. A portion (about 20%) of the total radioactive material released from ^{35}S-labelled cartilage in tissue culture was identified as inorganic [^{35}S]sulphate. No corresponding decrease in the degree of sulphation of the labelled polysaccharide could be detected. These findings suggest that a limited fraction of the proteoglycan molecules had been extensively desulphated. 5. It is suggested that the initial phase of degradation involves proteolytic cleavage of the proteoglycan, but the constituent polysaccharide chains remain intact. The partially degraded proteoglycan may be eliminated from the cartilage by diffusion into the circulatory system. An additional degradative process, which may occur intracellularly, includes desulphation of the polysaccharide, probably in conjunction with a more extensive breakdown of the polymer.

The metabolism of the glycosaminoglycans of the intercellular matrix has long been a subject of major interest in connective-tissue biochemistry. Although much information has been gained about the biosynthesis of the glycosaminoglycans (Silbert, 1966; Stoolmiller & Dorfman, 1969; Rodén, 1970), the degradative mechanisms operating *in vivo* remain obscure.

In an early study, Boström (1952) found a biological half-life of 16 days for the chondroitin sulphate of rat costal cartilage. A more rapid turnover, with a half-life of 8 days, was indicated by the results of Gross *et al.* (1960). The latter workers also concluded that although more than one metabolic pool is involved, the entire proteoglycan is degraded as a unit, the protein and carbohydrate constituents being eliminated at identical rates. Although the role of lysosomal proteases has since been amply verified (Lucy *et al.*, 1961; Ali, 1964; Morrison, 1970; Dingle *et al.*, 1971) it is still not known how polysaccharidases participate in the catabolism of cartilage. Although hyaluronidase-like enzymes occur in various mammalian tissues (Bollet *et al.*, 1963; Aronson & Davidson, 1965) attempts to demonstrate their presence in chondrocytes have been unsuccessful (Platt & Dorn, 1968). It is noteworthy that chondroitin sulphate isolated from human urine had apparently not been subjected to degradation by an endo-polysaccharidase (Wasteson & Wessler, 1971).

The aim of the present investigation was to characterize further the catabolism of the chondroitin sulphate proteoglycan in cartilage. Particular attention has been devoted to the eventual participation of polysaccharide-splitting enzymes. The results indicate that one phase of the turnover process involves partial proteolysis of the proteoglycan while the constituent polysaccharide chains remain intact. In addition, part of the polysaccharide molecules are desulphated, presumably in the course of an intracellular, more extensive degradation.

Materials

Sprague–Dawley rats of various ages were obtained from Anticimex, Norrviken, Sweden.

Papain (EC 3.4.4.10), obtained in crude form from Sigma Chemical Co., St. Louis, Mo., U.S.A., was purified by the procedure of Kimmel & Smith (1954) and was used as a suspension (0.1 M-sodium acetate buffer, pH4.5), containing approx. 40mg of protein/ml.

DEAE-cellulose (microgranular, pre-swollen DE-52) was a product of Whatman Biochemicals Ltd., Maidstone, Kent, U.K.

Dextran gels (Sephadex G-25 and G-200) and agarose gel (Sepharose 2B) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Chondroitinase ABC (EC 4.2.–.–), chondro-4-sulphatase (EC 3.1.6.–) and the monosulphated, unsaturated disaccharide from chondroitin 6-sulphate, Δ-di-6-sulphate (Yamagata *et al.*, 1968) were purchased from Miles Laboratories Inc., Kankakee, Ill., U.S.A.

The glycosaminoglycan preparations used as references have been described previously (Wasteson, 1971*a*; Wasteson & Lindahl, 1971); in addition, a heparan sulphate preparation from human aorta was generously given by Dr. P.-H. Iverius of this Institute.

[^{35}S]Sulphate (carrier free) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Analytical methods

Uronic acid was determined by the method of Bitter & Muir (1962) or by an automated modification of this method (Balazs *et al.*, 1965). Glucuronolactone was used as standard.

Molar ratios of glucosamine to galactosamine were determined by g.l.c. (Radhakrishnamurthy *et al.*, 1966) after hydrolysis of saccharides in 4 M-HCl at 100°C for 14h (see also Lindahl, 1970).

Electrophoresis of glycosaminoglycans was done on strips of cellulose acetate in 0.1 M-barium acetate, pH6.6 (2.7V/cm for 6h) (Wessler, 1968), or in 0.1 M-HCl, pH1.2 (1.9V/cm for 2h) (Wessler, 1971).

Radioactivity was determined with a Beckman model LS-250 liquid-scintillation counter. The scintillation liquid contained 5g of 2,5-diphenyloxazole and 100g of naphthalene/litre of dioxan.

The relative amounts of chondroitin 4-[^{35}S]-sulphate and chondroitin 6-[^{35}S]sulphate in ^{35}S-labelled polysaccharide preparations were determined as described by Yamagata *et al.* (1968). The polysaccharide was treated with chondroitinase ABC and chondro-4-sulphatase and the digest was subjected to paper electrophoresis on Whatman 3MM paper in 0.08 M-pyridine–0.05 M-acetic acid, pH5.3, at 85V/cm for 20min. The unsaturated disaccharide Δ-di-6-sulphate (see the Materials section), and free [^{35}S]sulphate were used as reference compounds. The areas on the sample strip corresponding to these markers were eluted with water and their radioactivities were measured. The ratio of chondroitin 4-[^{35}S]sulphate/chondroitin 6-[^{35}S]sulphate was assumed to equal that of [^{35}S]sulphate/Δ-di-6-[^{35}S]sulphate.

Average molecular weights and molecular-weight distributions of chondroitin sulphates were determined by gel chromatography in 0.2 M-NaCl on calibrated columns of Sephadex G-200 by the method of Wasteson (1971*b*).

Ion-exchange chromatography of chondroitin sulphate (Hallén, 1972) was done on a column (1cm × 5cm) of DE-52 DEAE-cellulose, equilibrated with 0.15 M-NaCl. Samples (1–5mg) were applied to the column, and were then eluted at 60°C with a LiCl gradient produced by the method of Hinton & Dobrota (1969). The reservoir contained 3 M-LiCl, and the mixing vessel 100ml of 0.2 M-LiCl in 0.05 M-sodium acetate buffer, pH4. Effluent fractions of 1.25ml were collected at a rate of 5ml/h and analysed for uronic acid and ^{35}S radioactivity.

Isolation of chondroitin sulphate

Lateral and medial zones of rat costal cartilage (Fig. 1) were dissected free of non-cartilaginous tissue. Portions of cartilage (<100mg wet weight) were suspended in 3ml of 0.05 M-sodium acetate buffer (pH5.5) – 0.3 M-NaCl – 0.01 M-EDTA – 0.01 M-cysteine–HCl. After addition of 50μl of papain suspension, digestion was allowed to proceed at 65°C for 20h. The digest was passed through a Celite pad equilibrated with 0.3 M-NaCl and was then mixed with an equal volume of 1% cetylpyridinium chloride in

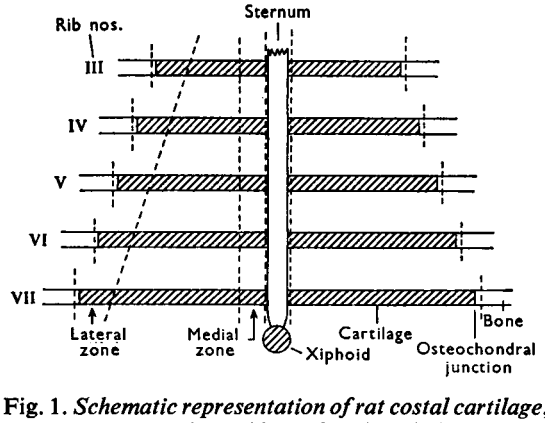

Fig. 1. *Schematic representation of rat costal cartilage, showing sampling of lateral and medial zones*

0.3M-NaCl (Scott, 1960). The precipitated polysaccharide was dissolved in 1ml of 2.0M-KCl and reprecipitated by the addition of 4ml of water and 1ml of 1% cetylpyridinium chloride; the latter step was repeated twice. The polysaccharide was finally precipitated from 2.0M-NaCl by the addition of 3vol. of ethanol.

Precipitations of radioactively labelled material with cetylpyridinium chloride were done in the presence of 0.01M unlabelled sulphate. Occasionally (see below), unlabelled chondroitin sulphate (1.5mg) from rat costal cartilage was added as a carrier, before precipitation with cetylpyridinium chloride.

Preparation of chondroitin sulphate from incubation mixtures *in vitro* generally included proteolysis of the incubation medium along with the tissue specimen (Herbai & Lindahl, 1970).

Labelling of chondroitin sulphate in vitro

Lateral and medial zones (approx. 25mg) of costal cartilage from freshly killed 9-week-old female rats were dissected free of adherent tissue, cut into 1mm-thick pieces and transferred to 25-ml Erlenmeyer flasks containing 3ml of Krebs–Ringer phosphate buffer, pH7.4, containing 0.25% (w/v) glucose [Umbreit *et al.* (1964) as modified by Herbai & Lindahl (1970)]. All operations were carried out in the cold. After thermal equilibration at 37°C for about 2min 200μl of buffer, containing 100μCi of [^{35}S]sulphate, was added, and incubation was allowed to proceed at 37°C for 15, 30 or 60min. Each incubation mixture was then inactivated at 100°C on a water bath for 10min, and chondroitin sulphate was isolated as described above.

The rate of polysaccharide synthesis, as reflected by the increase in the ratio of ^{35}S/μg of uronic acid, was higher in the lateral than in the medial zone of costal cartilage (Fig. 2; see also Herbai & Lindahl, 1970), and remained constant for at least 1h of incubation.

Labelling of chondroitin sulphate in vivo

Female 9-week-old rats were injected subcutaneously three times at intervals of 5h with 0.3ml of 0.9% NaCl, containing 40μCi of [^{35}S]sulphate. After various periods of time the animals were killed and the lateral and medial zones of costal cartilage were collected. Cartilage specimens, labelled *in vivo* for subsequent tissue-culture experiments, were prepared 30h after the last injection was given.

Tissue culture experiments

Tissue-culture experiments were carried out at 37°C with Eagle's (1959) MEM, containing 10% (v/v) of baby calf serum (inactivated by heating to 56°C for 30min), and antibiotics [100 units of penicillin (Glaxo), 50μg of streptomycin (Glaxo), 1.25μg of amphotericin/ml (Squibb)]. Lateral and medial zones (generally about 25mg) were cultured in 3-ml portions of medium. The media were changed every 6 days, unless otherwise stated.

Viability of cartilage. Explants of costal cartilage retained their histological appearance over a 2-week period in tissue culture, under the conditions described above. The presence in the culture medium of calf serum was obviously essential for the viability of the tissue, as necrotic areas developed in the absence of this component.

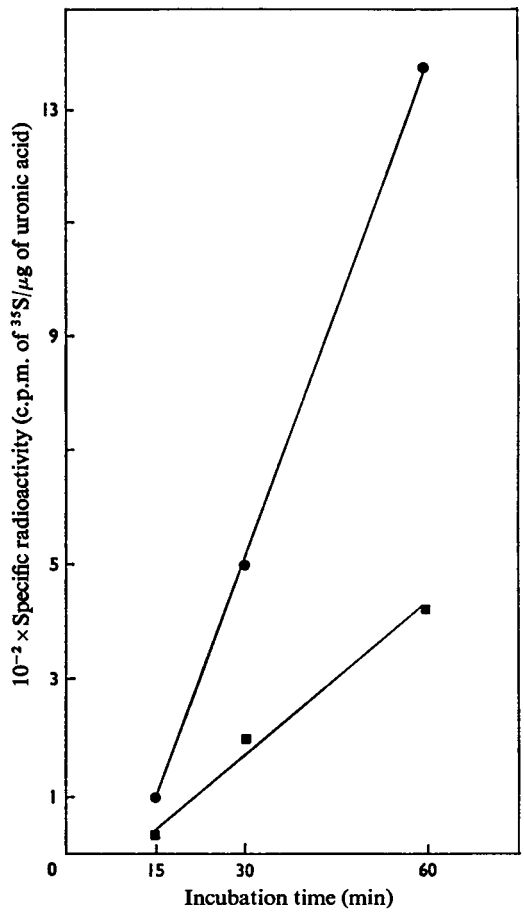

Fig. 2. *Incorporation in vitro of [^{35}S]sulphate into chondroitin sulphate in lateral (●) and medial (■) zones of rat costal cartilage*

The specific radioactivity (c.p.m. of ^{35}S/μg of uronic acid) is plotted against the time of incubation. The lag phase probably reflects the time required for diffusion of [^{35}S]sulphate into the cartilage.

Table 1. *Effect of preincubation on the rate of incorporation of [^{35}S]sulphate into cultured rat costal cartilage*

Change of culture medium (sixth day of incubation) is indicated by +. For experimental details see the text.

Type of cartilage	Time of preincubation (days)	Change of medium	Incorporation rate (c.p.m./h per μg of uronic acid)
Lateral zone	0	—	266
Medial zone	0	—	198
Lateral zone	1	—	174
Medial zone	1	—	178
Medial zone	4	—	82
Lateral zone	8	+	174
Medial zone	8	+	224
Lateral zone	8	—	33
Medial zone	8	—	50

In addition, the ability of costal cartilage specimens to incorporate [^{35}S]sulphate under tissue-culture conditions was investigated. [^{35}S]Sulphate (25μCi in 100μl of 0.9% NaCl) was added to the culture medium after various periods of preincubation, and incorporation of the label was allowed to proceed for 20h. After removal of the tissue samples from the media, chondroitin sulphate was isolated as described above, and the specific radioactivity (c.p.m./μg of uronic acid) of the polysaccharide was determined. The results showed a progressive decrease in the rate of polysaccharide synthesis (Table 1); after 8 days of tissue culture the rate of ^{35}S incorporation was only 15–20% of the initial value. However, the ability of the cartilage cells to synthesize chondroitin sulphate was restored after renewal of the culture medium (Table 1), thus further demonstrating the retained viability of the tissue.

Rate of elimination of chondroitin sulphate from cartilage. Samples of costal cartilage, labelled *in vivo* with [^{35}S]sulphate, were transferred to culture dishes. After various periods of incubation (2–13 days), the tissue specimens were removed, and polysaccharide was isolated, with carrier chondroitin sulphate, both from the media and from the explants. The distribution of ^{35}S-labelled polysaccharide between tissue and medium was calculated.

Characterization of ^{35}S-labelled metabolites

Proteoglycans. Samples of lateral and medial zones of costal cartilage were cultured for 10 days. The media collected from each culture were combined and concentrated by freeze-drying to a final volume of about 2ml. Control samples were obtained from non-cultured portions of lateral (210mg) and medial (350mg) zones. These were finely divided and were then extracted for 30h at room temperature with 2.5ml of 3.0M-$MgCl_2$, with continuous stirring (Sajdera & Hascall, 1969). By this procedure approx. 90% of the labelled polysaccharides of the tissues were solubilized. Proteoglycan was precipitated from the clarified extracts by the addition of cetylpyridinium chloride, followed by dilution to an ionic strength of 0.6. The solution obtained on dissolving the precipitate in 2M-NaCl was centrifuged and proteoglycan was precipitated from the supernatant by the addition of 3 vol. of ethanol. The resulting products were analysed, along with the freeze-dried culture media, by chromatography on Sepharose 2B. The column (1.2cm × 63cm) was eluted with 0.2M-NaCl at a rate of 4ml/h. Effluent fractions (2–3ml) were collected and analysed for radioactivity.

Single polysaccharide chains. ^{35}S-labelled costal cartilage was cultured for 10 days under the conditions described above. Control cultures, containing homologous cartilage specimens from the opposite half of the same rib cage, were kept frozen. Chondroitin sulphate was isolated from the entire cultures, including explant and medium. The resulting preparations were subjected to gel chromatography on Sephadex G-200 or to ion-exchange chromatography on DEAE-cellulose.

Low-molecular-weight products. Portions (1–2ml) of culture media were directly applied to a column (0.8cm × 100cm) of Sephadex G-25 (superfine grade) and were then eluted with 0.2M-NaCl at a rate of 3ml/h. Effluent fractions (1.5ml) were collected and analysed for radioactivity.

Results

Characterization of glycosaminoglycan from rat costal cartilage

The polysaccharide isolated from lateral and medial zones of costal cartilage migrated like chondroitin sulphate on electrophoresis in barium acetate (Wessler, 1968) and was clearly separated

Table 2. *Weight-average ($\bar{M}_w$) and number average ($\bar{M}_n$) molecular weights of chondroitin sulphate from rat costal cartilage*

For experimental details see the text.

Type of cartilage	Yield (mg of uronic acid/ 100mg of dried cartilage)	Age of animal (weeks)	$\bar{M}_w$	$\bar{M}_n$
Lateral zone	3.1	3	19500	15500
Medial zone	2.8	3	15000	12500
Lateral zone	2.2	9	16600	13200
Medial zone	2.2	9	13500	11400

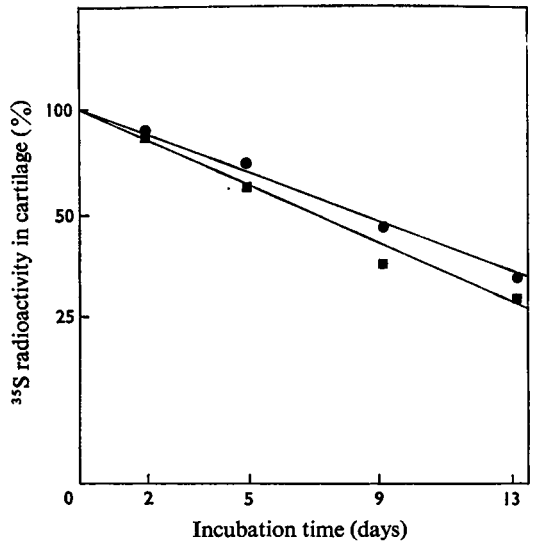

Fig. 3. *Elimination of radioactivity from ^{35}S-labelled lateral (●) and medial (■) zones of rat costal cartilage in tissue culture*

The amount of [^{35}S]polysaccharide remaining in the tissue, related to that of the entire culture, is plotted against time of incubation.

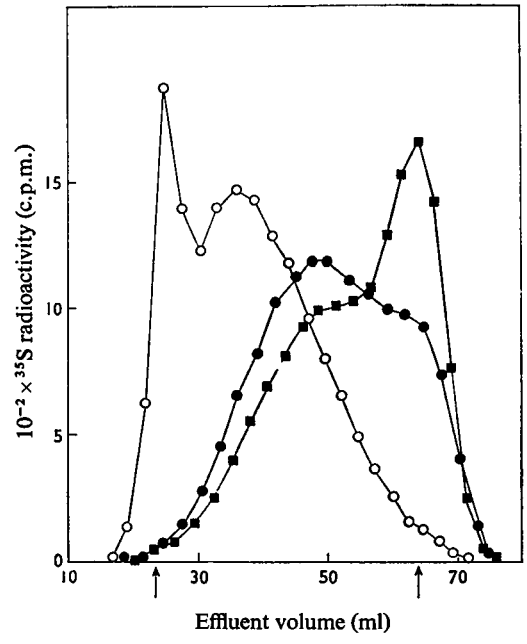

Fig. 4. *Gel chromatography on Sepharose 2B of culture media from tissue culture for 10 days of lateral (●) and medial (■) zones of ^{35}S-labelled rat costal cartilage*

The elution pattern of proteoglycan obtained by extraction of non-cultured cartilage (lateral zone, o) is also included. (The proteoglycan obtained from the corresponding medial zone showed an essentially similar pattern.) Effluent fractions were analysed for ^{35}S. The two arrows indicate V_0 and V_t of the column respectively.

from reference standards of heparin, heparan sulphate, hyaluronic acid and dermatan sulphate. Electrophoresis in HCl (Wessler, 1971) revealed approximately one residue of sulphate per disaccharide unit. Galactosamine was the only hexosamine detected by g.l.c. The yields of chondroitin sulphate (Table 2) were in good agreement with those reported in the literature (Gross *et al.*, 1960; Rosenberg *et al.*, 1965).

Chondroitin 4-[^{35}S]sulphate constituted 75–80% of the total polysaccharide labelled *in vivo* isolated from either zone of cartilage 30h after the last of three injections of [^{35}S]sulphate (see the Methods section).

The remainder migrated as Δ-di-6-sulphate on electrophoresis after digestion with chondroitinase ABC and chondro-4-sulphatase, and was therefore probably identical with chondroitin 6-sulphate.

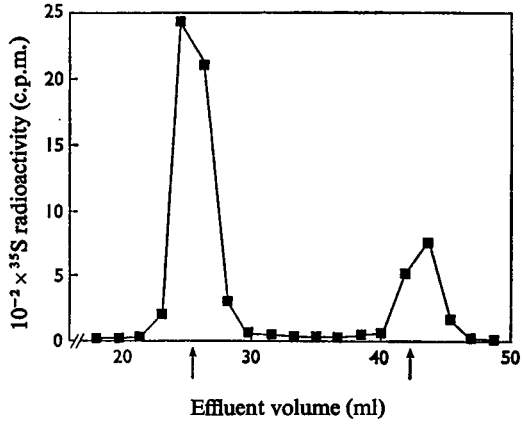

Fig. 5. *Gel chromatography on Sephadex G-25 of products released from explant of ^{35}S-labelled rat costal cartilage (medial zone) during 4 days in tissue culture*

See the Methods section for experimental details. The two arrows indicate V_0 and V_t respectively of the column.

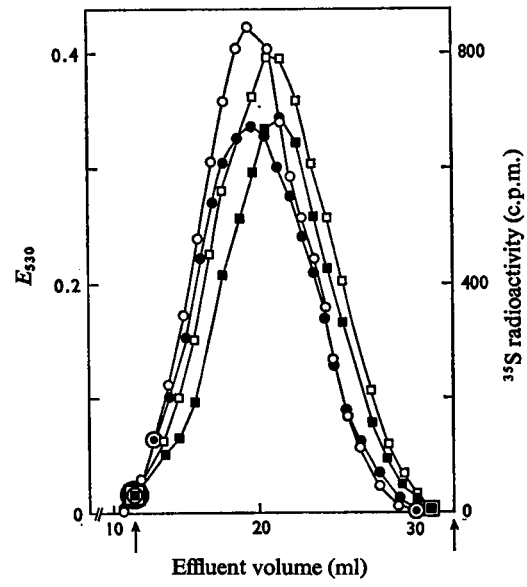

Fig. 6. *Gel chromatography on Sephadex G-200 of chondroitin sulphate, isolated from medial zones of rat costal cartilage, incubated in vitro with [^{35}S]-sulphate for 15 min (26 c.p.m./μg of uronic acid), and of chondroitin sulphate from lateral zones labelled in vivo with [^{35}S]sulphate and isolated 10 days after administration of the label (38 c.p.m./μg of uronic acid)*

Effluent fractions were analysed for uronic acid (E_{530}; medial zone, ■; lateral zone, ●) and for ^{35}S (medial zone, □; lateral zone, ○). The two arrows indicate V_0 and V_t respectively of the column.

The average molecular weights of chondroitin sulphates, prepared from the lateral and medial zones of costal cartilage from male rats of different ages (3 and 9 weeks respectively) are listed in Table 2. Apparently, chondroitin sulphate from lateral zones is of higher molecular weight than that isolated from medial zones. Further, the average molecular weight of polysaccharide from either zone, and particularly the lateral one, was higher in young (3 weeks) than in adult (9 weeks) animals.

Effect of turnover on the chondroitin [^{35}S]sulphate proteoglycan

The effects of turnover on labelled proteoglycan were studied, with particular regard to the macromolecular properties, the degree of sulphation and the polysaccharide chain length of the resulting products. Such products were obtained by tissue culture of rat costal cartilage, labelled *in vivo* with [^{35}S]sulphate. In addition, polysaccharide was isolated from cartilage that had been labelled either *in vitro* for short periods of time, or *in vivo* at various times before sampling of the tissue.

Macromolecular properties. The release of ^{35}S-labelled polysaccharide from cartilage in tissue culture into the medium is illustrated in Fig. 3. For both types of cartilage the semilogarithmic plot of the proportion of ^{35}S remaining in the cartilage against time is essentially linear. The half-life of ^{35}S-labelled polysaccharide in the lateral and medial zones of costal cartilage were estimated as 8.5 days and 7 days respectively. These values may be too high, as they do not account for the inorganic [^{35}S]sulphate released into the medium (see below).

The molecular size of material released during a 10-day period from ^{35}S-labelled cartilage in tissue culture is illustrated by the gel chromatograms shown in Fig. 4. For comparison, the elution pattern of a control preparation obtained by extraction of non-cultured cartilage is also included. The labelled components of the culture media were essentially smaller than the corresponding native proteoglycans, thus suggesting that the material released had been subjected to partial degradation. The difference in size between the products obtained from cultured and from non-cultured cartilage, respectively, was too large to be explained by passive and preferential extraction of low-molecular-weight components during tissue culture.

Gel chromatography of culture media on Sephadex G-25 showed that approx. 20% of the released

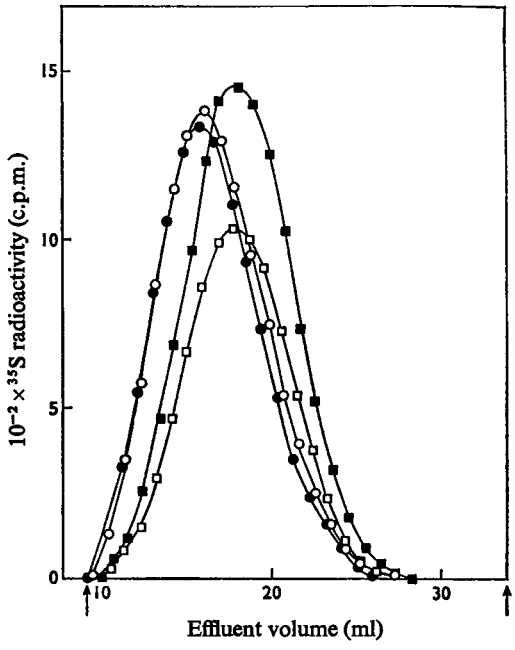

Fig. 7. *Gel chromatography on Sephadex G-200 of chondroitin sulphate isolated from lateral* (○) *and medial* (□) *zones of* ^{35}S*-labelled rat costal cartilage after incubation in tissue culture for* 10 *days, and from the corresponding controls* (●, *lateral zone;* ■, *medial zone*)

Effluent fractions were analysed for ^{35}S. The two arrows indicate V_0 and V_t respectively of the column.

radioactivity emerged as a distinct retarded peak, separated from the excluded material (Fig. 5). After desalting on Sephadex G-10, the retarded component migrated as inorganic sulphate on high-voltage paper electrophoresis at pH5.3 (see the Methods section). Gel chromatograms of products liberated from lateral and medial zones of cartilage, respectively, showed essentially equal proportions of inorganic [^{35}S]-sulphate. Further, the labelled material released between day 4 and day 8 of tissue culture contained about the same relative amounts of inorganic [^{35}S]-sulphate as that collected during the preceding 4-day period. This finding indicates the presence in rat costal cartilage of a sulphatase which remains active under tissue-culture conditions for at least 8 days.

Polysaccharide chain-length. Costal cartilage was labelled, *in vivo* or *in vitro*, with [^{35}S]sulphate. After various periods of time the tissues were subjected to extensive proteolysis and the liberated polysaccharide was isolated. The molecular-weight distributions of chondroitin [^{35}S]sulphate from lateral and medial

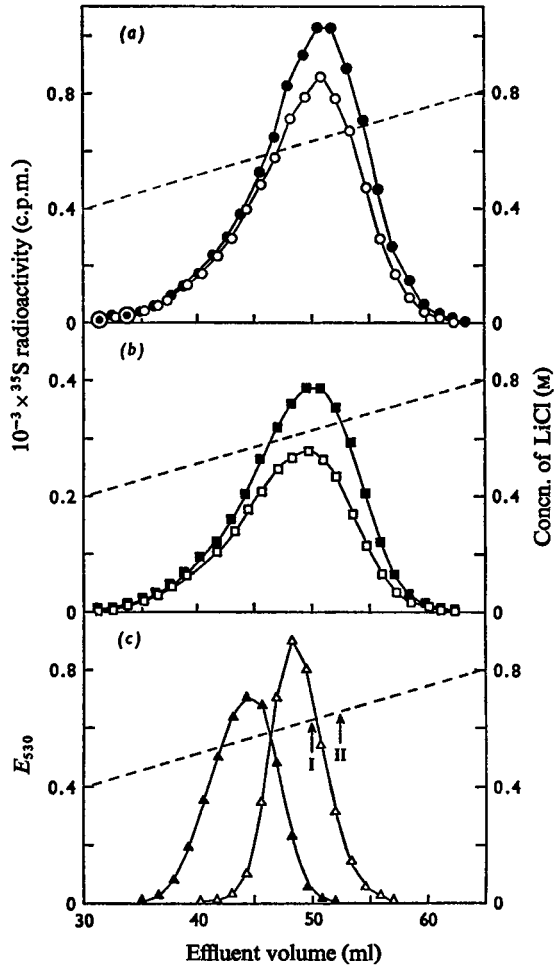

Fig. 8. *Chromatography on DEAE-cellulose of chondroitin sulphate isolated from cultures of* ^{35}S*-labelled rat costal cartilage after* 10 *days of incubation*

(*a*) Lateral zone; ○, cultured material; ●, non-cultured control; (*b*) medial zone; □, cultured material; ■, non-cultured control; fractions analysed for radioactivity. (*c*) Reference fractions of chondroitin sulphate; ▲, mol.wt. 8600, molar ratio sulphate/disaccharide 0.90; △, mol.wt. 8600, molar ratio sulphate/disaccharide 1.14 (Wasteson & Lindahl, 1971); arrow I (position of peak fraction), mol.wt. 12300, molar ratio sulphate/disaccharide 0.96; arrow II (position of peak fraction), mol.wt. 19700, molar ratio sulphate/disaccharide 0.95 (Wasteson, 1971*a*); fractions analysed for uronic acid (E_{530}; carbazole reaction). ----, Concn. of LiCl (M). The distribution of uronic acid (carbazole reaction; not indicated in *a* and *b*) invariably coincided with that of the radioactivity.

zones invariably conformed to those of the corresponding bulk polysaccharides (indicated by the uronic acid peaks of the gel chromatograms; Fig. 6). Although the labelled chondroitin sulphate recovered after short incubations *in vitro* was newly synthesized, the chondroitin [^{35}S]sulphate, labelled *in vivo*, had remained in the cartilage for as long as 20 days before isolation. Nevertheless, within each of the two types of cartilage studied, the chondroitin sulphate labelled *in vivo* showed the same molecular-weight distribution as did the polysaccharide labelled *in vitro*, irrespective of the time passed between administration of isotope and isolation of the chondroitin sulphate. For comparison, the gel-chromatography patterns of chondroitin sulphate isolated from medial and lateral zones 15min and 10 days, respectively, after administration of [^{35}S]sulphate, are shown in Fig. 6.

In tissue-culture experiments polysaccharide was isolated after proteolysis of the entire culture mixtures, including both explant and medium. After a 10-day period of incubation more than half of the total [^{35}S]polysaccharide had been released into the medium (Fig. 3); yet the gel chromatograms of homologous polysaccharide preparations isolated from such cultures and from non-cultured controls, respectively, showed identical distributions of both ^{35}S (Fig. 7) and uronic acid.

Degree of sulphation. The effect of turnover on the degree of sulphation was studied by chromatography on DEAE-cellulose of ^{35}S-labelled polysaccharide preparations isolated from cultures of costal cartilage. In this chromatographic system the behaviour of a polysaccharide depends both on the charge density and on the size of the molecule, as illustrated by the chromatograms of standard preparations shown in Fig. 8(*c*). A slight difference in degree of sulphation is thus readily detected, provided that the preparations are of equal molecular weight.

Figs. 8(*a*) and 8(*b*) show the chromatograms of polysaccharide preparations derived from lateral and medial zones, respectively, of costal cartilage, after tissue culture for 10 days. In both cases the elution patterns coincided with those of the non-cultured control materials. It is concluded that the chondroitin sulphate isolated from the cultures had not been desulphated to any appreciable extent.

Discussion

The molecular weight of chondroitin sulphate, isolated after proteolysis of rat costal cartilage, varied with the site of origin of the cartilage specimen and with the age of the animal. Polysaccharide isolated from lateral zones was of higher molecular weight than that obtained from medial zones, and in both cases the molecular weights showed a decrease with age (Fig. 3). Similar variation has been observed by a number of authors (Loewi, 1953; Hjertquist & Engfeldt, 1967; Wasteson, 1971*b*; Hjertquist & Wasteson, 1972).

Differences in the molecular-weight distribution of various polysaccharide preparations may be due to a variability in chain length of the newly synthesized chondroitin sulphate molecule. Alternatively, such differences could arise as a result of turnover of the proteoglycan; in this case the involvement of a polysaccharidase would be mandatory. It was therefore believed that some information about the mode of degradation of the proteoglycan could be acquired by comparing the molecular-weight distributions of chondroitin [^{35}S]sulphate, isolated from lateral and from medial zones at various times after administration of [^{35}S]sulphate.

Analysis of labelled chondroitin sulphate by gel chromatography failed to show any effect of turnover on the chain length of the polysaccharide *in vivo*. The molecular-weight distribution of chondroitin [^{35}S]-sulphate, isolated 10 days after administration of the label, was thus similar to that of a preparation obtained from homologous tissue after incorporation *in vitro* of [^{35}S]sulphate for 15min. Further, contrary to the preferential labelling of high-molecular-weight glycosaminoglycan observed by other workers (Kleine & Hilz, 1968; Olsson, 1968; Hardingham & Muir, 1970; Rokosova & Bentley, 1972), all polysaccharide preparations of the present study yielded ^{35}S peaks on gel chromatography coinciding with those of the bulk polysaccharide (uronic acid peak) of the respective tissue (Fig. 6). A continuous degradation of the entire pool of chondroitin sulphate chains should be expressed by a shift with time of the molecular-weight distribution of the labelled polysaccharide in the lateral zone towards that of the newly synthesized chondroitin sulphate in the medial zone. Obviously, the results illustrated in Fig. 6 do not conform to this concept. It seems reasonable to conclude that the observed regional variations in the molecular-weight distribution of chondroitin sulphate (Table 2) were established at the time of polysaccharide biosynthesis.

It is recognized that chondroitin [^{35}S]sulphate isolated from cartilage labelled *in vivo* does not account for degradation products eliminated from the tissues in the course of turnover. Hence, any degradation occurring in close association with the elimination process might escape detection. The experimental approach to this problem was therefore extended to include tissue culture, by which technique components released from the cartilage could also be recovered.

The rates of elimination of chondroitin [^{35}S]-sulphate from lateral and medial zones of costal cartilage in tissue culture (Fig. 3) were in fair agreement with the half-life values determined by experiments *in vivo* (Boström, 1952; Gross *et al.*, 1960; Å. Wasteson & U. Lindahl, unpublished work). The

released materials differed from the corresponding native proteoglycans with regard to molecular size (Fig. 4), but not with regard to the molecular-weight distribution of the polysaccharide constituent (Fig. 7). Apparently, the degradation process had not affected the polysaccharide chains but only the protein core of the proteoglycan molecule, in agreement with the postulated role of cathepsin D in proteoglycan turnover (Morrison, 1970; Dingle *et al.*, 1971).

Although the present results indicate that the chain length of chondroitin sulphate in costal cartilage was unchanged during most of the turnover period, the possibility remains of a polysaccharidase-mediated degradation mechanism, operating in close association with the biosynthesis of the proteoglycan (Fratantoni *et al.*, 1968). Further, the techniques employed in the present work were unsuitable for the study of degradation mechanisms too rapid to permit accumulation of partially degraded polysaccharide fragments. The operation of such a mechanism in rat costal cartilage is in fact suggested by the finding that a considerable proportion of inorganic [^{35}S]sulphate was released from labelled cartilage explants in tissue culture (Fig. 5). No sulphated oligosaccharides were detected. The occurrence of inorganic [^{35}S]sulphate could be due either to partial desulphation of a large fraction of the total ^{35}S-labelled polysaccharide or to an extensive desulphation of a more limited fraction of the molecules. The former possibility appears to be excluded, as partially desulphated chondroitin sulphate chains were not formed to any significant extent under tissue-culture conditions (Figs. 8*a* and 8*b*). The remaining alternative would seem to imply an intracellular process, probably occurring in conjunction with further degradation of the polymer. A similar conclusion was presented by Morrison (1970), who studied the catabolism of the chondroitin sulphate proteoglycan of embryonic chick cartilage.

As a conclusion to the present and other studies it may be suggested that elimination of the chondroitin sulphate proteoglycan from cartilage may proceed by two different routes: (*a*) by diffusion into the circulatory system, which contains a mixture of single polysaccharide chains and proteoglycans (Calatroni *et al.*, 1969); or (*b*) by intracellular degradation after incorporation into cartilage cells. It seems likely that the proteoglycan, before final elimination from the tissue, is mobilized through partial proteolysis by cathepsin D, which thus initiates degradation by either route.

This work was supported by grants from the Swedish Medical Research Council (13P-3431; 13X-4; 13X-2309), the Swedish Cancer Society (53), Konung Gustaf V:s 80-årsfond, Svenska Sällskapet för Medicinsk Forskning and the Faculty of Medicine, University of Uppsala.

Thanks are due to Professor J. Pontén for helpful advice and for tissue-culture equipment, placed at our disposal. The excellent technical assistance of Miss B. Hiort, Miss C. Sellin, Mrs. A. Minnberg and Miss M. Lindström is gratefully acknowledged.

References

Ali, S. Y. (1964) *Biochem. J.* **93**, 611–618
Aronson, N. N. & Davidson, E. A. (1965) *J. Biol. Chem.* **240**, 1322–1326
Balazs, E. A., Berntsen, K. O., Karossa, J. & Swann, D. A. (1965) *Anal. Biochem.* **12**, 547–558
Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
Bollet, A. J., Bonner, W. M. & Nance, J. L. (1963) *J. Biol. Chem.* **238**, 3522–3527
Boström, H. (1952) *J. Biol. Chem.* **196**, 477–481
Calatroni, A., Donnelly, P. V. & DiFerrante, N. (1969) *J. Clin. Invest.* **48**, 332–343
Dingle, J. T., Barrett, A. J. & Weston, P. D. (1971) *Biochem. J.* **123**, 1–13
Eagle, H. (1959) *Science* **130**, 432–437
Fratantoni, J. C., Hall, C. W. & Neufeld, E. F. (1968) *Proc. Nat. Acad. Sci. U.S.* **60**, 699–706
Gross, J., Mathews, M. B. & Dorfman, A. (1960) *J. Biol. Chem.* **235**, 2889–2892
Hallén, A. (1972) *J. Chromatogr.* **71**, 83–91
Hardingham, T. E. & Muir, H. (1970) *FEBS Lett.* **9**, 145–148
Herbai, G. & Lindahl, U. (1970) *Acta Physiol. Scand.* **80**, 502–509
Hinton, R. H. & Dobrota, M. (1969) *Anal. Biochem.* **30**, 99–110
Hjertquist, S.-O. & Engfeldt, B. (1967) *Acta Pathol. Microbiol. Scand. Suppl.* **187**, 40–41
Hjertquist, S.-O. & Wasteson, Å. (1972) *Calcif. Tissue Res.* **10**, 31–37
Kimmel, J. R. & Smith, E. L. (1954) *J. Biol. Chem.* **207**, 515–531
Kleine, T. O.& Hilz, H. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1027–1036
Lindahl, U. (1970) *Biochem. J.* **116**, 27–34
Loewi, G. (1953) *J. Pathol. Bacteriol.* **65**, 381–388
Lucy, J. A., Dingle, J. T. & Fell, H. T. (1961) *Biochem. J.* **79**, 500–508
Morrison, R. I. G. (1970) in *The Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), pp. 1683–1706, Academic Press, New York
Olsson, I. (1968) *Biochim. Biophys. Acta* **165**, 324–334
Platt, D. & Dorn, M. (1968) *Clin. Chim. Acta* **21**, 333–345
Radhakrishnamurthy, B., Dalferes, E. R. & Berenson, G. S. (1966) *Anal. Biochem.* **17**, 545–550
Rodén, L. (1970) *Metab. Conjugation Metab. Hydrolysis* **2**, 345–442
Rokosova, B. & Bentley, J. P. (1972) *Biochim. Biophys. Acta* **264**, 98–102
Rosenberg, L., Johnson, B. & Schubert, M. (1965) *J. Clin. Invest.* **44**, 1647–1656
Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87
Scott, J. E. (1960) *Methods Biochem. Anal.* **8**, 145–197

Silbert, J. E. (1966) in *Glucuronic Acid, Free and Combined* (Dutton, G. J., ed.), pp. 385–453, Academic Press, New York
Stoolmiller, A. C. & Dorfman, A. (1969) *Compr. Biochem.* **17**, 241–275
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) *Manometric Techniques*, pp. 131–133, Burgess Publishing Co., Minneapolis
Wasteson, Å. (1971*a*) *Biochem. J.* **122**, 477–485
Wasteson, Å. (1971*b*) *J. Chromatogr.* **59**, 87–97
Wasteson, Å. & Lindahl, U. (1971) *Biochem. J.* **125**, 903–908
Wasteson, Å. & Wessler, E. (1971) *Biochim. Biophys. Acta* **252**, 13–17
Wessler, E. (1968) *Anal. Biochem.* **26**, 439–444
Wessler, E. (1971) *Anal. Biochem.* **41**, 67–69
Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535