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Structural analysis of chick-embryo cartilage proteoglycan by selective degradation with chondroitin lyases (chondroitinases) and endo-β-D-galactosidase (keratanase)

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Digestion of chick-embryo cartilage proteoglycan (type H) with chondroitin AC II lyase or keratanase, in the presence of EDTA, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride and pepstatin, resulted in the removal of the bulk of the chondroitin sulphate or keratan sulphate chains respectively, without altering the protein portion of the macromolecule. An exhaustive treatment of the proteoglycan with chondroitin AC II lyase followed by digestion with keratanase yielded a core fraction having the enzymically modified linkage oligosaccharides. Zonal sedimentation of this core preparation on a sucrose gradient in 0.5% SDS resulted in a single narrow band with a sedimentation coefficient of 6S. In 4M-guanidinium chloride, the core preparation showed a tendency to aggregate to multiple-molecular-weight forms which could dissociate in the presence of Triton X-100. The results indicate that the preponderance of glycosaminoglycans in the proteoglycan molecule is a main reason for both polydispersity and hydrophilicity of the proteoglycan preparation, and further suggest that the enzymic procedures could prove useful as a method to obtain new information about the structure and properties of proteoglycan core molecules.

Chondrogenic differentiation of chick-embryo limb-bud cells causes qualitative changes in cell morphology and synthesis of sulphated proteoglycans (Goetinck *et al.*, 1974; Okayama *et al.*, 1976; Kitamura & Yamagata, 1976; DeLuca *et al.*, 1977; Karasawa *et al.*, 1979). Our work (Kimata *et al.*, 1978) has shown that the proteoglycans synthesized by chick-embryo epiphyseal cartilages can be resolved into at least four components (termed H, Lb, Lt-I and Lt-II) by a series of fractionation procedures including zonal sedimentation, CsCl equilibrium density-gradient centrifugation and gel chromatography. In these studies as well as in a more recent study designed to elucidate the molecular organization of the mesoderm in chick-embryo limb buds at earlier developmental stages, attempts to isolate and purify proteoglycans from complex mixtures have been hampered by their high degree of polydispersity and heterogeneity. Only a small amount of information is available, therefore, as to whether the variable size of proteoglycan molecules is due to a true heterogeneity where several different protein core molecules are present or to a heterodispersity arising from variable length or number, or both, of the attached glycosaminoglycan chains.

Hascall *et al.* (1972) reported that treatment of bovine nasal cartilage proteoglycan with *P. vulgaris* chondroitin ABC lyase and *F. heparinum* chondroitin AC lyase removed the bulk of the chondroitin sulphate. Digestion with testicular hyaluronidase has also been shown to remove approx. 80% of the chondroitin sulphate of proteoglycans from bovine tracheal, bovine nasal and pig laryngeal cartilages (Heinegård, 1972; Baxter & Muir, 1975). In every case, the removal of the chondroitin sulphate reveals details of the molecular architecture of the region that might otherwise be masked by the preponderance of chondroitin sulphate. We present here new enzymic procedures suitable for

Abbreviations used: CS, chondroitin sulphate; ΔDi, unsaturated disaccharide; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; GalNAc(SO_4), *N*-acetylgalactosamine monosulphate; GlcA, glucuronic acid; GlcNAc(SO_4)-Gal, 3-*O*-(2-acetamido-2-deoxy-6-*O*-sulpho-β-D-glucose)-D-galactose; KS, keratan sulphate; PG-H, proteoglycan type H; PG-L, proteoglycan type L; SDS, sodium dodecyl sulphate.

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selective removal of the chondroitin sulphate or keratan sulphate, or both, of proteoglycans, without altering the protein portion of the macromolecules. A method is described which enables presently available preparations of chondroitinases and keratanase to be used for this purpose. The procedures are intended primarily for the study of radioactive-labelled proteoglycans produced by small embryonic tissues, but they are equally applicable to unlabelled proteoglycans from various other tissues if sufficient amounts of the materials are available for analysis by chemical means.

Experimental

Unless otherwise indicated, the materials and methods used are the same as those described in the Appendix (Oike *et al.*, 1980).

Materials

Fertile eggs (White Leghorn) were obtained from a local supplier on day 9 and were incubated in a moist atmosphere at 38°C until they were used on day 12. Tibiae and femurs were removed, placed in Eagle's minimal essential medium supplemented with 10% (v/v) foetal calf serum and 0.005% (w/v) ascorbic acid, freed of adhering non-cartilaginous tissues and rinsed (at room temperature).

Pronase was a product of Kaken Kagaku Co., Tokyo, Japan, and was kindly donated by Mr. K. Takamine of the company. A standard preparation of chondroitin 4-sulphate (mol.wt. 12000) was a gift from Dr. M. B. Mathews, University of Chicago, Chicago, IL, U.S.A. ΔDi-4S and ΔDi-6S were prepared by the method of Suzuki (1960). $Na_2{}^{35}SO_4$ (carrier-free) was purchased from Japan Radioisotope Association (Tokyo, Japan), [U-^{14}C]serine (150Ci/mol) and *Escherichia coli* [*pyrimidine*-2-^{14}C]RNA composed of 4S-, 16S- and 23S-RNA (50μCi/mg) from New England Nuclear (Boston, MA, U.S.A.), bovine serum albumin (fraction V) and phenylmethanesulphonyl fluoride from Sigma Chemical Co. (St. Louis, MO, U.S.A.), foetal calf serum from Grand Island Biological Co. (Grand Island, NY, U.S.A.), Eagle's minimal essential medium from Nissui Seiyaku Co. (Tokyo, Japan) and Sepharose CL-4B, Sephadex G-100 and Sephadex G-50 from Pharmacia Japan (Tokyo, Japan).

Incubation of cartilages in radioisotope-containing media

The medium was a modified Eagle's minimal essential medium, in which the amount of inorganic sulphate was decreased to give 0.1mM-$SO_4{}^{2-}$ and which contained 10% (v/v) foetal calf serum, 0.005% (w/v) ascorbic acid and either 133μCi/ml of carrier-free $Na_2{}^{35}SO_4$ or 10μCi/ml of [U-^{14}C]serine. For each incubation, about 3g of the cartilage tissue (from 100 embryos, see above) was placed in a 100-ml conical flask containing 15ml of warm (37°C) medium. The incubation was carried out in a humid atmosphere of air + CO_2 (95:5, v/v) at 37°C with gentle shaking. After 2h of incubation, the tissues were transferred to 27ml of warm (37°C) Eagle's minimal essential medium containing 10% (v/v) foetal calf serum and 0.005% (w/v) ascorbic acid, incubated for 5min and then replaced in the same volume of the washing medium for an additional incubation of 5min.

Preparation of proteoglycan (PG-H) from radioactive-labelled cartilages

From the labelled cartilages, proteoglycan type H (PG-H) was isolated by a modification of the procedure of Kimata *et al.* (1978) as follows. The labelled tissues (3g) were transferred to 27ml of cold extracting solution (solution A) composed of 4M-guanidium chloride, 0.05M-Tris/HCl, pH8.0, 10mM-disodium EDTA, 10mM-*N*-ethylmaleimide, 1mM-phenylmethanesulphonyl fluoride and 0.36mM-pepstatin (since it is difficult to dissolve phenylmethanesulphonyl fluoride and pepstatin directly in water the reagents were previously dissolved in methanol to give a concentration of 0.1M and 7.2mM respectively). The four proteinase inhibitors were included to avoid degradation of proteoglycans by endogenous proteinases [such degradation has been found to occur during the extraction of several tissues (Oegema *et al.*, 1975; Pearson & Mason, 1977; Roughley & Barrett, 1977)]. *N*-Ethylmaleimide may also serve as an alkylating agent to prevent non-specific disulphide exchange of polypeptides, found to occur with protein-denaturing reagents (cf. Parkhouse, 1971). The tissue suspension was frozen, kept at −20°C overnight and then thawed to 2°C. The freeze–thawing procedure was repeated twice to facilitate the penetration of the solvent and the final suspension was kept at 0°C for 14h with gentle agitation. The mixture was centrifuged (12000*g*, 30min, 2°C) and the precipitate (tissue residues) was re-extracted in the same way. The two supernatants were combined, diluted with 54ml of 0.05M-Tris/HCl, pH8.0, containing the four proteinase inhibitors and mixed with 243ml of 95% (v/v) ethanol containing 1.3% potassium acetate to precipitate proteoglycans. The precipitate was collected by centrifugation (12000*g*, 30min, 2°C), dissolved in 20ml of solution A and allowed to stand in the dark at room temperature for 2h (to ensure a complete alkylation of free SH groups with *N*-ethylmaleimide). The preparation thus obtained (termed crude PG/PR) contained approx. 100μmol of hexuronate (95% of the total hexuronate in the tissue) and 1.11×10^9c.p.m. of ^{35}S (98% of the total

^{35}S-labelled macromolecules in the tissue) or 4.4×10^7 c.p.m. of ^{14}C. ^{35}S- or ^{14}C-labelled PG-H was isolated from portions of the crude PG/PR preparations by zonal sedimentation on a glycerol gradient in 4 M-guanidinium chloride (to remove the minor proteoglycan component PG-L and the bulk of the non-proteoglycan proteins) followed by CsCl equilibrium density-gradient centrifugation (initial density 1.47 g/ml) to remove residual non-proteoglycan proteins, essentially as described before (Kimata *et al.*, 1978). From the bottom three-tenths of the CsCl gradient (the PG-H fraction), macromolecules were precipitated with 3 vol. of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate. The precipitate was dissolved in 1 vol. of 5 mM-Tris/HCl, pH 8.0, containing 1 mM-disodium EDTA, 1 mM-*N*-ethylmaleimide, 0.1 mM-phenylmethanesulphonyl fluoride and 0.036 mM-pepstatin (solution B). The precipitation with ethanol in solution B was repeated three times and the final precipitate was freeze–dried. Approximately 65, 4 or 83% of the total ^{35}S, ^{14}C or hexuronate respectively, in the crude PG/PR preparation, was recovered as a PG-H fraction with a specific radioactivity of 8.6×10^6 c.p.m. (^{35}S) or 2×10^4 c.p.m. (^{14}C) per μmol of hexuronate. On a per mg of Lowry protein basis (Lowry *et al.*, 1951), the specific radioactivity was 2.6×10^8 c.p.m. (^{35}S) or 6.3×10^5 c.p.m. (^{14}C). The data indicate that approx. 79% of the total hexuronate or 64% of the total ^{35}S-labelled macromolecules in the tissue was isolated in PG-H. The proportion of PG-H hexuronate is comparable to the value for proteoglycan monomer A1-D1 (64%) isolated from bovine tracheal cartilage (Heinegård, 1972).

Enzymic digestion of PG-H and related compounds

Various combinations of enzyme concentration, incubation time, and inhibitor composition were investigated to find satisfactory conditions for the selective degradation of the chondroitin sulphate and keratan sulphate chains of PG-H. The procedures described in the Appendix (Oike *et al.*, 1980) proved satisfactory for this purpose.

When $PG(-CS)_{ABC}$ was to be digested by chondroitin AC II lyase, a 30 μg (as Lowry protein) portion of the core fraction from the Sepharose CL-4B column (see below) was desalted by precipitation in solution B with ethanol (as described under the Preparation of proteoglycan (PG-H) section) and freeze-dried in a stainless-steel test tube before the addition of the enzyme reagent.

When $PG(-CS)_{AC}$ was to be digested by keratanase, a 30 μg (as Lowry protein) portion of the core fraction from the Sepharose CL-4B column (see the text) was desalted and freeze–dried as above before the addition of enzyme reagent.

Alkaline β-elimination

All sample solutions (the eluates of ^{35}S-labelled PG-H or its core derivatives from Sepharose CL-4B columns) were diluted with 2 vol. of 0.05 M Tris/HCl, pH 8.0, and then mixed with 3 vol. of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate. The resulting precipitates were collected by centrifugation (12000 *g*, 30 min), dissolved in 5 ml of 0.2 M-NaOH and allowed to stand at room temperature for 20 h. The reaction was terminated by addition of acetic acid to give the final pH of 8.0. To this solution (containing about 30 μg of Lowry protein) was added 250 μl of 1 M-Tris/HCl, pH 8.0, 250 μl of ethanol and 100 μg of Pronase, and the mixture was incubated at 50°C for 15 h. The reaction was terminated by placing the reaction tube in a boiling-water bath for 3 min. The entire reaction mixture was freeze-dried, dissolved in 0.5 ml of water and subjected to analyses by gel filtration (see below).

Gel chromatography

Sepharose CL-4B chromatography. A sample solution, 1 ml, containing radioactive-labelled PG-H or its core derivatives (about 15 μg as Lowry protein) in 4 M-guanidinium chloride/0.02 M-Tris/HCl (pH 8.0)/10 mM-disodium EDTA/1 mM-*N*-ethylmaleimide/0.1 mM-phenylmethanesulphonyl fluoride/0.036 mM pepstatin/0.2% (w/v) Triton X-100 (solution C) was applied to a column (79 cm × 0.95 cm diam.) of Sepharose CL-4B which had been equilibrated with solution C. The column was then eluted with solution C at 5 ml/h at 4°C. Fractions of 0.7 ml were collected and their radioactivity contents were determined.

Bio-Gel A-1.5 m chromatography. A sample solution, 0.4 ml, containing 5×10^4 c.p.m. of [^{14}C]non-proteoglycan proteins in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 6.0, was applied to a column (35 cm × 0.95 cm diam.) of Bio-Gel A-1.5 m which had been equilibrated with the same guanidinium chloride solution. The column was eluted with the guanidinium chloride solution at 5 ml/h at 4°C. Fractions of 0.5 ml were collected and their radioactivity contents were determined.

Sephadex G-100 chromatography. A sample solution, 0.3 ml, containing 1×10^4 c.p.m. of ^{35}S-labelled glycosaminoglycans in 0.4 M-ammonium formate, pH 5.8, was applied to a column (40 cm × 0.95 cm diam.) of Sephadex G-100 which had been equilibrated with the same salt solution. The column was eluted with the salt solution at 5 ml/h at room temperature. Fractions of 0.45 ml were collected and their radioactivity contents were determined.

Sephadex G-50 chromatography. A sample solution, 0.5 ml, containing 0.12×10^5–2×10^5 c.p.m. of ^{35}S-labelled oligosaccharides in 0.4 M-ammonium

formate, pH 5.8, was applied to a column (45 cm × 1.2 cm diam.) of Sephadex G-50 which had been equilibrated with the same salt solution. The column was eluted with the salt solution at 5 ml/h at room temperature. Fractions of 0.57 ml were collected and their radioactivity contents were determined.

SDS zonal sedimentation

Solutions containing 15% (w/w) or 30% (w/w) sucrose in 0.1 M-NaCl/5 mM-Tris/HCl (pH 7.5)/1 mM-disodium EDTA/1 mM-*N*-ethylmaleimide/0.1 mM-phenylmethanesulphonyl fluoride/0.036 mM-pepstatin/0.5% (w/w) SDS were prepared. The gradients (30–15% sucrose, 15 ml) were made at 22°C and stored overnight at room temperature. A test sample, 0.2 ml, containing 5000–10000 c.p.m. of ^{14}C-labelled proteoglycan or its core derivative (Fig. 4; the preparation of sample solutions) was layered on top of the gradient. In a control tube, *E. coli* [^{14}C]RNA (10000 c.p.m.) was layered in a similar way. The centrifugation was carried out in the rotor RPS-25-3A of a Hitachi model RP-65 preparative ultracentrifuge at 72500 *g* ($r_{av.}$, 11.1 cm) for 24 or 34.5 h at 25°C. Each tube was punctured at the bottom, 0.5 ml-fractions were collected and their radioactivity contents were determined. The established linear relationship between the distance of migration and the sedimentation coefficient (Martin & Ames, 1961) was used for determining the sedimentation coefficient of unknown samples of the proteoglycan.

Paper chromatography and paper electrophoresis

Paper chromatography was carried out on 60 cm-strips of Toyo no. 51A paper at room temperature by the descending method. The solvent used was isobutyric acid/0.5 M-ammonia (5:3, v/v); the solvent will be referred to as solvent A. Paper electrophoresis was carried out on 60 cm-strips of Toyo no. 51A paper at room temperature in the apparatus described by Markham & Smith (1952) at a potential gradient of 30 V/cm for 30 min. The buffer used was 0.05 M-ammonium acetate/acetic acid, pH 5.0.

Other methods

Hexuronate was determined by the procedure of Bitter & Muir (1962) with glucuronolactone as standard. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as standard. Analysis of chondroitin ABC lyase digestion products of chondroitin [^{35}S]sulphate was carried out as described by Saito *et al.* (1968). Radioactivity was measured by spotting a sample solution on a paper disc (2.4 cm diam.), the disc was then dried in an oven at 50°C and counted in an Aloka liquid-scintillation spectrometer (Aloka Co., Tokyo, Japan) with the solvent system recommended by the manufacturer.

Results

On the basis of the observations described in the Appendix (Oike *et al.*, 1980), the inhibition mixture (10 mM-disodium EDTA, 10 mM-*N*-ethylmaleimide, 5 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin) was routinely utilized in the experiments described below to avoid enzymic degradation of the protein in the core structure.

Selective degradation of PG-H with chondroitin ABC lyase, chondroitin AC II lyase and keratanase

With [^{35}S]PG-H and the mixture of proteinase inhibitors, different incubation conditions were tested for each enzyme to attain the most selective removal of the glycosaminoglycan moieties. The conditions described in the Appendix (Oike *et al.*, 1980) proved satisfactory for this purpose; chondroitin ABC lyase, chondroitin AC II lyase and keratanase released 92.4, 93.9 and 5.4% respectively, of the added ^{35}S as small size fragments, leaving in each case a residual ^{35}S on a core fraction (Fig. 1).

The following results indicate that degradation of [^{35}S]PG-H by the procedures with chondroitin ABC lyase (or chondroitin AC II lyase) and keratanase is limited to chondroitin sulphate and keratan sulphate chains respectively, attached to the core protein. (1) Upon paper chromatography in solvent A, the small-molecular-size component released by chondroitin ABC lyase (or chondroitin AC II lyase) digestion (Fig. 1*a*,*b*; ΔDi) was resolved into [^{35}S]ΔDi-4S and [^{35}S]ΔDi-6S, which accounted for 33 and 50% respectively of the applied radioactivity (the remainder of the radioactivity was found in several unidentified products). (2) Upon gel chromatography on Sephadex G-50, the small-molecular-size component released by keratanase digestion (Fig. 1*c*; Oligo) showed an elution profile very similar to that of a keratanase digest of chick-embryo corneal keratan [^{35}S]sulphate (Fig. 2); both digests gave a major peak in the position expected for disaccharide, GlcNAc(SO_4)-Gal, plus several minor peaks corresponding to higher oligosaccharides.

From the elution profiles in Fig. 1, it is clear that the hydrodynamic size of each core fraction reflects the proportion of enzymically removed ^{35}S; the higher the proportion of released ^{35}S, the smaller is the apparent molecular size of the resulting core fraction. Although the PG(−KS) sample was eluted in the void volume fractions where the starting proteoglycan sample was also eluted, they were readily distinguished by SDS zonal sedimentation on a sucrose density gradient (see below).

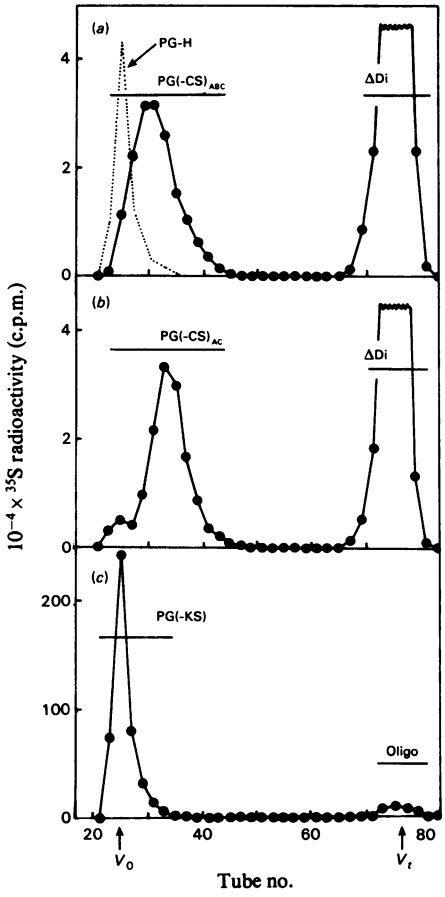

Fig. 1. *Elution profiles from Sepharose CL-4B of products obtained after digestion of [^{35}S]PG-H with (a) chondroitin ABC lyase, (b) chondroitin AC II lyase and (c) keratanase in the presence of proteinase inhibitors*
Portions (1 ml, 4.3×10^6 c.p.m.) of the digests were applied to the column and separated under conditions described in the Experimental section. Intact [^{35}S]PG-H was eluted in the void volume (V_0) as shown in (*a*) (····). Peaks PG(−CS)$_{ABC}$, PG(−CS)$_{AC}$, PG(−KS), ΔDi and Oligo were pooled as indicated by the horizontal bars and analysed as described in the text.

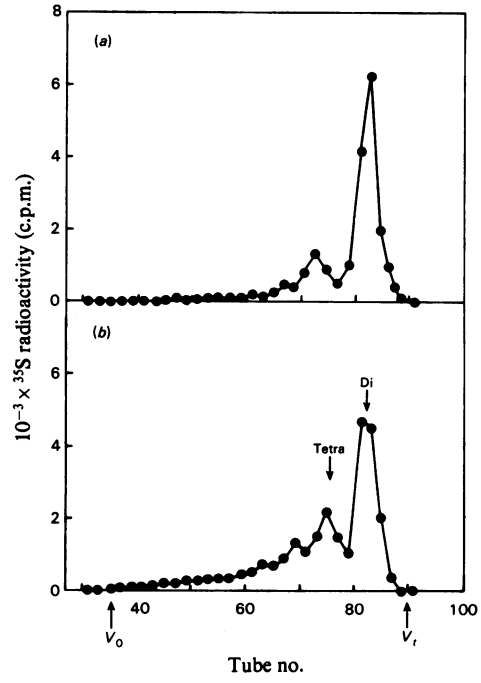

Fig. 2. *Elution from Sephadex G-50 of (a) components in peak Oligo from Sepharose CL-4B and (b) keratanase digest of chick-embryo corneal keratan [^{35}S]sulphate*
Fractions in the Oligo peak from Sepharose CL-4B were combined as indicated in Fig. 1, concentrated to a small volume in a vacuum and desalted by gel filtration on a small Bio-Gel P-2 column with water as eluent. The desalted sample (after concentration in a vacuum) was applied to the Sephadex G-50 column and separated under conditions described in the Experimental section. The data in (*b*) are those obtained by Dr. H. Habuchi in this laboratory during the course of study on keratan sulphate sulphatases (Habuchi *et al.*, 1979). The peaks designated as Di and Tetra correspond in position to the disaccharide monosulphate and tetrasaccharide trisulphate respectively, prepared from bovine corneal keratan sulphate (Nakazawa & Suzuki, 1975).

The small differences between PG(−CS)$_{ABC}$ and PG(−CS)$_{AC}$ in their ^{35}S contents and molecular sizes probably arise from the different specificities of the enzymes used, since Hascall *et al.* (1972) have presented data suggesting that the disaccharide closest to the linkage oligosaccharide, GlcA-Gal-Gal-Xyl (Rodén & Smith, 1966), resists digestion with chondroitin ABC lyase but is removed by *F. heparinum* chondroitin AC lyase. To see if PG(−CS)$_{ABC}$ contains such additional disaccharide residues, a PG(−CS)$_{ABC}$ sample (3.3×10^5 c.p.m.) was digested with chondroitin AC II lyase. Gel chromatography on Sepharose CL-4B of the digest showed a large size component (2.8×10^5 c.p.m.) with the K_D of PG(−CS)$_{AC}$ (0.19) and a small size component (5.0×10^4 c.p.m.) eluted in the position expected for disaccharides. Paper chromatography of the small size component yielded [^{35}S]ΔDi-4S and [^{35}S]ΔDi-6S in the ratio for radioactivity of 2:1. The predominance of the sulphate units at position 4 is in contrast with the chondroitin ABC lyase-susceptible region in which the sulphate units at position 4 can account for only 33% of the total ^{35}S (see above). As

will become evident, these residual disaccharides are recovered after alkaline β-elimination as a higher oligosaccharide corresponding in size to the linkage region between chondroitin sulphate and protein. These results are consistent with the view that digestion of the proteoglycan with chondroitin ABC lyase leaves a residual disaccharide (predominantly sulphated at position 4) on the chondroitin sulphate chains in the core preparation.

Sequential degradation of PG-H with chondroitin AC II lyase and keratanase

The results above suggest that a sequential degradation of PG-H with chondroitin AC II lyase and keratanase may remove the bulk of chondroitin sulphate and keratan sulphate chains, yielding a core fraction containing the intact protein moiety. To test this possibility, a PG(−CS)$_{AC}$ sample obtained from [^{35}S]PG-H (cf. Fig. 1*b*) was digested with keratanase in the presence of proteinase inhibitors, and then subjected to chromatography on Sepharose CL-4B with detergent-containing guanidinium chloride solution as eluent (Fig. 3*a*). Approximately 90% of the total ^{35}S radioactivity in PG(−CS)$_{AC}$ was released as a small-molecular-size fraction corresponding in position to oligosaccharides (Fig. 3*a*; Oligo), while the remainder was found in a macromolecular peak with a K_D value of 0.23 [Fig. 5*a*; PG(−CS,KS)]. The results indicate that the digestion of PG(−CS)$_{AC}$ with keratanase removes a large portion of the keratan sulphate chains, leaving a residual sulphated sugar unit on the keratan sulphate chains in the core fraction. The results also indicate that 99.4% of the total radioactivity in [^{35}S]PG-H can be removed by the sequential treatment with chondroitin AC II lyase and keratanase.

When a [^{14}C]serine-labelled PG(−CS)$_{AC}$ sample, prepared from [^{14}C]PG-H [cf. Appendix (Oike *et al.*, 1980), Fig. 1*e*], was digested in a similar way with keratanase, a ^{14}C-labelled core fraction was obtained which had a K_D value identical with that of [^{35}S]PG(−CS,KS) (Fig. 3*b*). There was no indication that ^{14}C-labelled small peptides were released during the digestion with keratanase.

The PG(−CS,KS) preparation is only partially soluble in 4 M-guanidinium chloride, which limits the conditions under which chromatographic separation may be carried out. For gel filtration studies, the inclusion of 0.2% (w/v) Triton X-100 in the eluent is essential to obtain a reliable result. Thus chromatography of the core preparation on Sepharose CL-4B with detergent-free eluent (Fig. 3*c*) did not result in a single peak but revealed a tendency to be eluted in a broad, irregular band. Only 45% of the applied radioactivity could be eluted and most of the remaining activity was recovered by washing the glassware (column and test tubes used for chromatography) with solution C.

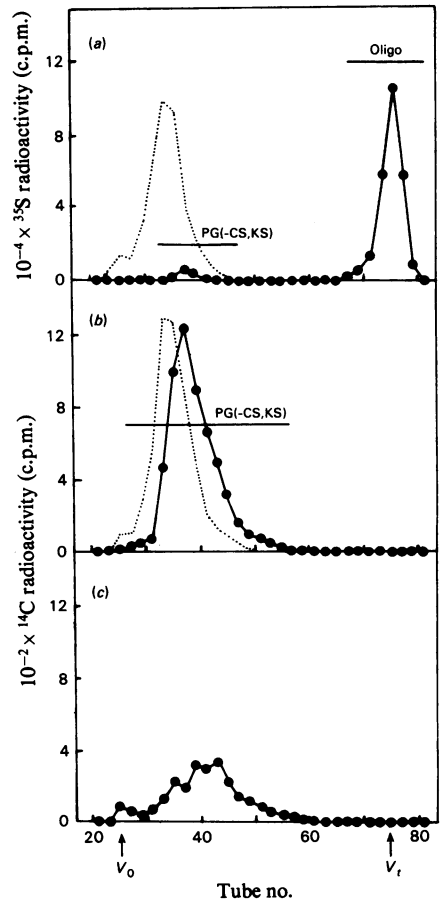

Fig. 3. *Elution profiles from Sepharose CL-4B of products obtained after digestion of ^{35}S- or ^{14}C-labelled PG(−CS)$_{AC}$ with keratanase*

[^{35}S]PG(−CS)$_{AC}$ and [^{14}C]PG(−CS)$_{AC}$ (isolated as in Fig. 1*b* and in the Appendix, Fig. 1*e* respectively) were digested with keratanase in the presence of proteinase inhibitors as outlined in the Experimental section. Unless otherwise indicated, portions (1 ml) of the digests were applied to the column and separated under conditions described in the Experimental section. (*a*) [^{35}P]PG(−CS)$_{AC}$ before (····) and after (——) digestion with keratanase. (*b*) [^{14}C]PG(−CS)$_{AC}$ before (····) and after (——) digestion with keratanase. (*c*) as in (*b*) but eluted with detergent-free solution C. Peaks PG(−CS,KS) and Oligo were pooled as indicated by the horizontal bars and analysed as described in the text.

Further characterization of PG(−KS), PG(−CS)$_{ABC}$, PG(−CS)$_{AC}$ and PG(−CS,KS)

Fig. 4 shows the sedimentation profiles observed

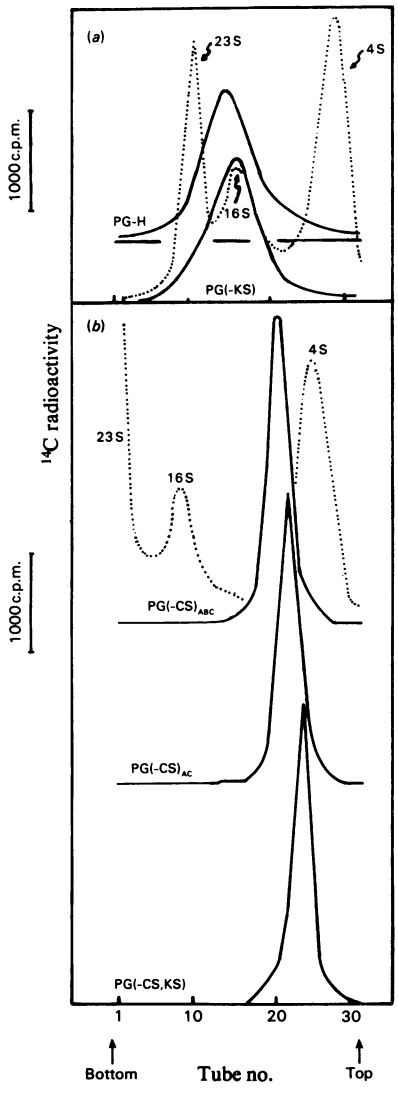

Fig. 4. *SDS zonal sedimentation of* ^{14}*C-labelled PG-H, PG(−KS), PG(−CS)*$_{ABC}$*, PG(−CS)*$_{AC}$ *and PG(−CS,KS)*

Proteoglycan and core molecules were recovered from the corresponding fractions from the CsCl gradient and Sepharose CL-4B columns respectively, by precipitation with ethanol. Each sample was dissolved in solution B and precipitated with ethanol (three times) to ensure complete removal of CsCl or guanidinium chloride. The final precipitate was dissolved in 0.1 M-NaCl/5 mM-Tris/HCl (pH 7.5)/1 mM-disodium EDTA/1 mM-*N*-ethylmaleimide/0.1 mM-phenylmethanesulphonyl fluoride/0.036 mM-pepstatin/0.5% (w/w) SDS/0.25% (w/v) bovine serum albumin to give a concn. of 25000–100000 c.p.m./ml. A 0.2 ml portion of this solution was heated at 90°C for 3 min. After cooling, the solution was layered on the top of the sucrose gradient and centrifugation was carried out as outlined in the Experimental section. Operation time: (*a*) 24 h; (*b*) 34.5 h.

when the core preparations from [^{14}C]PG-H were subjected to SDS zonal sedimentation on a sucrose gradient. In order to estimate sedimentation coefficients, *E. coli* 4S-, 16S- and 23S-RNA (standards) were run under the same conditions (····). As shown in Fig. 4(*a*), the intact PG-H preparation migrates in a broad band of a sedimentation coefficient of about 18S. The profiles of the core preparations indicate that their sedimentation rate decreases with the decrease in the glycosaminoglycan content: 16S for PG(−KS), 8S for PG(−CS)$_{ABC}$, 7S for PG(−CS)$_{AC}$ and 6S for PG(−CS,KS). The relative compactness of the band is also changed: PG(−KS) < PG(−CS)$_{ABC}$ < PG(−CS)$_{AC}$ < PG(−CS,KS). Since the compactness of a band could be a measure of the homogeneity of the molecules composing the group, the results indicate that PG(−CS,KS) has the highest degree of homogeneity and further suggest that the existence of glycosaminoglycan chains of various sizes is a main reason for the polydispersity of the proteoglycan molecules.

The behaviour of PG(−CS,KS) in sedimentation was not changed by pretreatment (90°C, 3 min) of the sample with 0.17 M-2-mercaptoethanol. It seems unlikely, therefore, that the core molecule contains disulphide-bonded subunits.

To characterize the residual ^{35}S-labelled chains in each core preparation, the core preparations from [^{35}S]PG-H were treated with 0.2 M-NaOH and Pronase as described in the Experimental section. The resulting mixtures were fractionated by gel chromatography on Sephadex G-50 (Fig. 5).

Fig. 5(*a*) shows the elution profile of ^{35}S-labelled fragments released from PG(−CS)$_{ABC}$ by β-elimination. A large-molecular-size fraction (KS), which accounts for 86% of the total ^{35}S, and three minor fractions (Oligo, X and Y) appeared. (1) Upon treatment with keratanase, all the ^{35}S-labelled material in peak KS was degraded to smaller sized components as observed by elution patterns on Sephadex G-50 (not shown; the patterns were essentially identical with those shown in Fig. 2). Peak KS should therefore represent keratan sulphate. When chromatographed on a Sephadex G-100 column, which had been calibrated with standard chondroitin 4-sulphate ($K_D = 0.23$, mol.wt. 12000), the keratan sulphate showed a disperse pattern with a K_D value of about 0.3 (Fig. 5*a*, inset). The average molecular weight of the keratan sulphate calculated from these data is approx. 8000. (2) Approximately 70% of the ^{35}S-labelled material in peak Oligo was susceptible to degradation with chondroitin AC II lyase, yielding [^{35}S]ΔDi-4S and [^{35}S]ΔDi-6S (identified by paper chromatography in solvent A; radioactivity ratio 2:1). The results suggest that peak Oligo represents mainly an oligosaccharide [presumably unsaturated GlcA-

GalNAc(SO)$_4$-GlcA-Gal-Gal-Xyl] derived from the chondroitin ABC lyase-resistant linkage portion on the chondroitin sulphate chains. Approximately 30% of the labelled material in peak Oligo was not degraded by chondroitin AC II lyase digestion. The chemical nature of this resistant material is not known. (3) Peak Y had the K_D of inorganic salt ions and, upon paper electrophoresis, showed a rapid mobility characteristic of SO_4^{2-}. (4) Retreatment of the Oligo fraction with 0.2M-NaOH at room temperature for 20h resulted in conversion of 40% of the radioactivity into fractions corresponding in gel filtration properties to X and Y, suggesting that X and Y may be degradation products arising, in part at least, from the oligosaccharide component during the β-elimination procedure.

Fig. 5(*b*) shows the elution profile of ^{35}S-labelled fragments released from PG(−CS)$_{AC}$ by β-elimination. More than 94% of the radioactivity was eluted near and in the void volume. This material was identified as keratan [^{35}S]sulphate by its susceptibility to keratanase (see above). No distinct peak appeared in the region for Oligo, X or Y, in contrast to the β-elimination products from PG(−CS)$_{ABC}$.

Fig. 5(*c*) shows the elution profile of ^{35}S-labelled fragments released from PG(−KS) by β-elimination. A large excluded peak (CS) and a minor retarded peak (KS-link) of a K_D value of 0.9 were observed. Upon treatment with chondroitin AC II lyase, essentially all of the ^{35}S-labelled material in peak CS was converted into [^{35}S]ΔDi-4S and

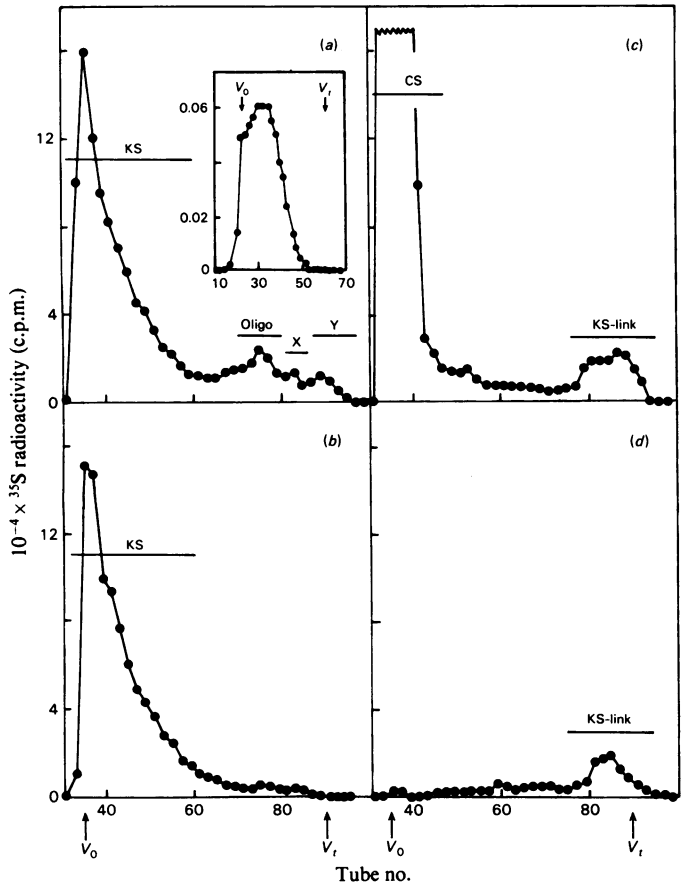

Fig. 5. *Elution profiles from Sephadex G-50 of products obtained by alkaline β-elimination of* (*a*) ^{35}*S-labelled PG(−CS)*$_{ABC}$, (*b*) *PG(−CS)*$_{AC}$, (*c*) *PG(−KS) and* (*d*) *PG(−CS,KS)*

The core preparations were treated successively with alkali and Pronase and subjected to gel filtration on the column as outlined in the Experimental section. Fraction KS in (*a*) gave the elution profile shown in the inset, when rechromatographed on a Sephadex G-100 column. Peaks CS, KS, Oligo, X, Y and KS-link were pooled as indicated by the horizontal bars and analysed as described in the text.

[^{35}S]ΔDi-6S (radioactivity ratio 2:3, determined by paper chromatography in solvent A), indicating that peak CS represents chondroitin sulphate. The material in peak KS-link, in contrast, was completely refractory to the action of chondroitin AC II lyase. Although no further information has been obtained about this component, it is a reasonable assumption that peak KS-link may represent a complex mixture of alkali degradation products (Hopwood & Robinson, 1974), derived from the residual linkage portion on the keratan sulphate chains which resists digestion with keratanase.

Fig. 5(*d*) shows the elution profile of ^{35}S-labelled fragments released from PG(−CS,KS) by β-elimination. No detectable radioactivity was eluted in the void volume fractions, indicating the absence of glycosaminoglycan chains of large size. Approximately 55% of the radioactivity in PG(−CS,KS) was recovered in the fraction corresponding to KS-link, a fraction which presumably involves alkali degradation products derived from the keratan sulphate linkage region (see above).

Discussion

In previous reports (Hopwood & Robinson, 1975; Hardingham *et al.*, 1976; Rosenberg *et al.*, 1976; Heinegård, 1977) it has been shown that the proteoglycans extracted from bovine nasal, bovine articular and pig laryngeal cartilages are extremely polydisperse. A large number of fractions have been isolated which have different buoyant densities, sizes and relative contents of chondroitin sulphate, keratan sulphate and protein. To account for this variability, Hardingham *et al.* (1976), Rosenberg *et al.* (1976) and Heinegård (1977) have proposed that the proteoglycans contain a non-variable polypeptide region (so-called hyaluronic acid-binding region and keratan sulphate-enriched region) and an extended region of variable length containing a variable degree of substitution with chondroitin sulphate chains. It is not known, however, whether this variation reflects, in all its aspects, a natural heterogeneity of proteoglycan composition introduced during its biosynthesis, or stems, in part at least, from cleavage of labile peptide bonds *in vivo* (during the growth and aging of the tissue) or *in vitro* (during the extraction of the tissue; e.g. see Oegema *et al.*, 1975; Pearson & Mason, 1977; Roughley & Barrett, 1977). In the work described here, we took several precautions to avoid these problems: (*1*) cartilage specimens were obtained fresh from chick-embryo epiphyses at the same developmental stage; (*2*) proteoglycans were labelled *in vitro* for a reasonably short period (2h) to minimize the effect of catabolic systems; (*3*) an appropriate mixture of proteinase inhibitors and an alkylating agent (*N*-ethylmaleimide) were included in the medium for tissue extraction to avoid both artificial degradation by endogenous proteinases and non-specific disulphide exchange of proteoglycans in 4M-guanidinium chloride; (*4*) several distinct proteoglycan species (type L; for their characteristics see Kimata *et al.*, 1978), that accounted for about 30% of the total ^{35}S in the tissue extract, were completely removed by zonal sedimentation followed by CsCl density-gradient centrifugation. Even after minimizing these sources of polydispersity, the final preparation of PG-H still contained a broad population of structures, as assessed by the sedimentation profile shown in Fig. 4(*a*). However, the dispersity could be diminished to a large extent by removal of the attached glycosaminoglycan chains, as shown by the high degree of compactness of the band of PG(−CS,KS) in SDS sucrose gradients (Fig. 4*b*). It appears therefore that the polydispersity of PG-H results mainly from a variable size or number (or both) of chondroitin sulphate and keratan sulphate chains attached to the protein core (see also Kato *et al.*, 1978; Lohmander *et al.*, 1979). Although the present study does not provide much information about the chemical nature of the core fraction, the above results are more in keeping with a single, non-variable core protein structure. PG-H appears to be different in this respect from the proteoglycans of adult cartilages described above (see also Kimura *et al.*, 1978).

Gel filtration analyses in 4M-guanidinium chloride showed that PG(−CS,KS) can be solubilized only in the form of protein–detergent complexes. A non-ionic detergent, Triton X-100, can be used for this purpose (ionic detergents such as SDS cannot be used because of their low solubility in 4M-guanidinium chloride). In detergent-free 4M-guanidinium chloride, gel chromatography of PG(−CS,KS) did not result in a single band but revealed a tendency to aggregate to multiple-molecular-weight forms and to adhere to the walls of glassware (Fig. 3*c*). To explain these properties, one may assume that the core molecule has preferred sites of association in the absence of detergents, which are disrupted by the detergent molecules. If this is so, the detergent could alter the hydrodynamic size of the core molecule. On the basis of buoyant density and keratan sulphate content, a molecular weight of 200000 was suggested by Hascall & Riolo (1972) for bovine nasal-cartilage core protein. Upholt *et al.* (1979) described experiments in which antibodies against hyaluronidase-digested proteoglycan were used to isolate nascent core polypeptide synthesized with mRNAs recovered from chick-embryo chondrocyte cultures. SDS/polyacrylamide-gel electrophoresis of the immunoprecipitated protein showed a single component migrating with a mol.wt. of 340000. Assuming that PG(−CS,KS) contains a protein of 340000, a rough estimate of the mol.wt. of

PG(−CS,KS) is approx. 600000, resulting from the addition of (*1*) residual linkage oligosaccharides of the glycosaminoglycan chains and (*2*) nonsulphated oligosaccharide chains of the glycoprotein type (DeLuca *et al.*, 1979, personal communication). It is difficult to ascertain whether this value is consistent with the behaviour of PG(−CS,KS) in Sepharose CL-4B gel (Fig. 3), because the effect of detergent on the molecular size is unknown and there is no protein or glycoprotein standard with such a high molecular weight. Attempts are in progress in this laboratory to determine the molecular weight of PG(−CS,KS) by physical means.

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References

Baxter, E. & Muir, H. (1975) *Biochem. J.* **149**, 657–668
Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 320–334
DeLuca, S., Heinegård, D. & Hascall, V. C. (1977) *J. Biol. Chem.* **252**, 6600–6608
DeLuca, S., Lohmander, S., Caputo, C., Kimura, J., Caplan, A. & Hascall, V. C. (1979) in *Glycoconjugates* (Schauer, R., Boer, E., Buddecke, E., Kramer, M. F., Vliegenthart, J. F. G. & Wiegandt, H., eds.), pp. 625–626, Georg Thieme Publishers, Stuttgart
Goetinck, P. F., Pennypacker, J. P. & Royal, P. D. (1974) *Exp. Cell Res.* **87**, 241–248
Habuchi, H., Tsuji, M., Nakanishi, Y. & Suzuki, S. (1979) *J. Biol. Chem.* **254**, 7570–7578
Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127–143
Hascall, V. C. & Riolo, R. L. (1972) *J. Biol. Chem.* **247**, 4529–4538
Hascall, V. C., Riolo, R. L., Hayward, J. Jr. & Reynolds, C. C. (1972) *J. Biol. Chem.* **247**, 4521–4528
Heinegård, D. (1972) *Biochim. Biophys. Acta* **285**, 193–207
Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980–1989
Hopwood, J. J. & Robinson, H. C. (1974) *Biochem. J.* **141**, 57–69
Hopwood, J. J. & Robinson, H. C. (1975) *Biochem. J.* **151**, 581–594
Karasawa, K., Kimata, K., Ito, K., Kato, Y. & Suzuki, S. (1979) *Dev. Biol.* **70**, 287–305
Kato, Y., Kimata, K., Ito, K., Karasawa, K. & Suzuki, S. (1978) *J. Biol. Chem.* **253**, 2784–2789
Kimata, K., Oike, Y., Ito, K., Karasawa, K. & Suzuki, S. (1978) *Biochem. Biophys. Res. Commun.* **85**, 1431–1439
Kimura, J. H., Osdoby, P., Caplan, A. I. & Hascall, V. C. (1978) *J. Biol. Chem.* **253**, 4721–4729
Kitamura, K. & Yamagata, T. (1976) *FEBS Lett.* **71**, 337–340
Lohmander, L. S., Hascall, V. C. & Caplan, A. I. (1979) *J. Biol. Chem.* **254**, 10551–10561
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Markham, R. & Smith, J. D. (1952) *Biochem. J.* **52**, 552–557
Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
Nakazawa, K. & Suzuki, S. (1975) *J. Biol. Chem.* **250**, 912–917
Oegema, T. R., Hascall, V. C. & Dziewiatkowski, D. D. (1975) *J. Biol. Chem.* **250**, 6151–6159
Oike, Y., Kimata, K., Shinomura, T. & Suzuki, S. (1980) *Biochem. J.* **191**, 203–207
Okayama, M., Pacifici, M. & Holtzer, H. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3224–3228
Parkhouse, R. M. E. (1971) *Biochem. J.* **123**, 635–641
Pearson, J. P. & Mason, R. M. (1977) *Biochim. Biophys. Acta* **498**, 176–188
Rodén, L. & Smith, R. (1966) *J. Biol. Chem.* **241**, 5949–5954
Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. & Strider, W. (1976) *J. Biol. Chem.* **251**, 6439–6444
Roughley, P. J. & Barrett, A. J. (1977) *Biochem. J.* **167**, 629–637
Saito, H., Yamagata, T. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536–1542
Suzuki, S. (1960) *J. Biol. Chem.* **235**, 3580–3588
Upholt, W. B., Vertel, B. M. & Dorfman, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4847–4851