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The Effect of β-D-Xylosides on Chondroitin Sulphate Biosynthesis in Embryonic Chicken Cartilage in the Absence of Protein Synthesis Inhibitors

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Incorporation of [^{35}S]sulphate, [^{3}H]glucose and [^{3}H]serine into glycosaminoglycans and proteoglycans of embryonic-chicken sternum was measured *in vitro* in incubation medium containing 4-methylumbelliferyl β-D-xyloside or *p*-nitrophenyl β-D-xyloside at low concentrations, and in the absence of inhibitors of protein synthesis. Incorporation of sulphate was decreased by 80% in incubations in which 1 mM-4-methylumbelliferyl β-xyloside or 2.5 mM-*p*-nitrophenyl β-xyloside was present; under these conditions, serum factors stimulated incorporation to only a small extent. When the concentration of the xyloside was decreased tenfold, incorporation of sulphate was inhibited by 60–70%, but when normal human serum or L-3,3',5-tri-iodothyronine or both were also added to the incubation medium, incorporation was markedly stimulated. Experiments in which [^{35}S]sulphate and [^{3}H]glucose were incorporated simultaneously, and enzymic analysis of glycosaminoglycans formed in such experiments, indicated that chondroitin sulphate formed in the presence of 0.1 mM-4-methylumbelliferyl β-xyloside contained 30–40% less sulphate than did chondroitin sulphate synthesized in the absence of xylosides. Similar experiments, with [^{3}H]serine instead of [^{3}H]glucose, suggested also a 20–30% decrease in chain length of the chondroitin sulphate; this was confirmed by direct gel filtration of labelled glycosaminoglycans on a calibrated column. Incorporation of [^{3}H]glucose or [^{3}H]serine was stimulated by serum and tri-iodothyronine in parallel with incorporation of sulphate. The changes seen in the total chondroitin sulphate were mirrored in the major proteoglycan fraction, purified by isopycnic centrifugation of salt-extracted proteoglycans. The labelling pattern of chondroitin sulphate from this proteoglycan indicated that decreased sulphation of chondroitin sulphate was largely due to the inferior ability of short polysaccharide chains to accept sulphate, with some direct interference with transfer of sulphate to all chains. The results also suggested that the action of serum factors on synthesis of proteochondroitin sulphate is exercised at the level of either protein synthesis or transport to the sites of initiation of polysaccharide synthesis.

Chondroitin sulphate is the major glycosaminoglycan in chick-embryo sternum (Kosher *et al.*, 1973). *In vivo*, chondroitin sulphate occurs complexed with protein, with each polysaccharide chain linked to the polypeptide chain through a tetrasaccharide whose structure is GlcA1→4Gal*p*1→3Gal*p*1→4Xyl*p*1→Ser (Rodén & Smith, 1966; Helting & Rodén, 1968). Biosynthesis of chondroitin sulphate occurs by stepwise addition of monosaccharide units from the appropriate UDP-glycosides to the growing chains; this process is catalysed by at least six enzymes, some of which have been highly purified (Rodén, 1970; Schwartz & Rodén, 1974, 1975). Chick-embryo cartilage incubated *in vitro* incorporates sulphate and other precursors into chondroitin sulphate; this incorporation can be stimulated by addition of normal serum from several species, including man (Hall, 1970; Audhya & Gibson, 1974). Thyroid hormones added at physiological concentrations *in vitro* stimulate incorporation by themselves, and also increase the stimulation by serum (Audhya & Gibson, 1975); the increased incorporation of sulphate represents increased synthesis of at least the major proteochondroitin sulphate fraction in the tissue (Audhya *et al.*, 1976). Normal serum also contains a growth-hormone-dependent factor that stimulates incorporation of sulphate; the effect of this factor is additive with that of thyroid hormone (Gibson *et al.*, 1975). We have found that the growth-hormone-dependent factor in serum can be replaced by the peptides responsible for non-suppressible insulin-like activity of human serum, provided that a third unidentified serum factor is also present (Froesch *et al.*, 1976).

Inhibitors of protein synthesis, such as puromycin or cycloheximide, inhibit synthesis of chondroitin

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sulphate in cartilage, presumably by preventing the formation of suitable protein acceptors for the transfer of the monosaccharide residues (Telser *et al.*, 1965). Addition of xylose at high concentrations, or of certain β-xylosides at much lower concentrations, restores, at least partially, the ability of the tissue to synthesize chondroitin sulphate (Okayama *et al.*, 1973; Robinson *et al.*, 1975). The extent to which synthesis is restored depends on the nature of the aglycone moiety of the β-xyloside; in general, the more non-polar the aglycone, the greater the amount of chondroitin sulphate synthesized (Robinson *et al.*, 1975). β-Xylosides can also act as acceptors for chondroitin sulphate synthesis in cells and tissues that do not normally synthesize large amounts of proteochondroitin sulphate (Schwartz *et al.*, 1974; Galligani *et al.*, 1975). When 4-methylumbelliferyl β-D-xyloside was used as acceptor, the product contained 4-methylumbelliferone in glycosidic linkage with the terminal xylose residue (Fukunaga *et al.*, 1975). In all these studies normal proteochondroitin sulphate was not synthesized to a great extent, either because protein synthesis was blocked, or because the tissue was incapable of forming much proteoglycan in any case.

In this report we present studies of proteochondroitin sulphate synthesis by chick-embryo cartilage *in vitro*, with β-xylosides added to the incubation medium but without protein-synthesis inhibitors. Our main conclusions are that chondroitin sulphate, synthesized in incubation mixtures containing low concentrations of 4-methylumbelliferyl β-xyloside, is shorter than normal and undersulphated, and that in such incubations, serum factors stimulate the synthesis of proteochondroitin sulphate without causing a major change in the chain length or degree of sulphation.

Experimental

Materials

Chick embryos, aged 9–11 days, from an inbred flock of White Leghorns, were obtained from Spring Lake Farms, Wyckoff, NJ, U.S.A. Embryos were maintained in a humidified incubator at 38°C until they were 12 days old. Sterna were rapidly dissected free from other cartilages and adhering tissues and placed in incubation medium at room temperature (approx. 20°C).

Sources of chemicals were as follows: Alcian Blue 8GX, J. T. Baker Co., Phillipsburg, NJ, U.S.A., and Polysciences, Warrington, PA, U.S.A.; Manoxol 1B (dibutyl ester of sodium sulphosuccinic acid), Accurate Chemical Corp., Hicksville, NY, U.S.A.; 4-methylumbelliferyl β-D-xyloside, N.K. Laboratories, Jersey City, NJ, U.S.A.; *p*-nitrophenyl β-D-xyloside and puromycin hydrochloride, Sigma Chemical Co., St. Louis, MO, U.S.A.; Sepharose 6B, Pharmacia Fine Chemicals, Uppsala, Sweden; Aquasol, New England Nuclear, Boston, MA, U.S.A. Sources of other chemicals are listed elsewhere (Audhya & Gibson, 1974; Audhya *et al.*, 1976). Reagents whose sources are not given were of analytical grade.

Pooled normal human serum was purchased from Flow Laboratories, Rockville, MD, U.S.A., and stored at −20°C. Bovine serum albumin (crystallized and freeze-dried) was from Sigma. Chondroitin ABC lyase (EC 4.2.2.4) was purchased from Seikagaku Kogyo Co., Tokyo, Japan, and papain (twice crystallized) was from Worthington Biochemicals, Freehold, NJ, U.S.A.

Carrier-free $H_2{}^{35}SO_4$, [3-^{3}H]glucose and [6-^{3}H]-glucose, and NaB^3H_4 were obtained from New England Nuclear; [3-^{3}H]serine was from The Radiochemical Centre, Amersham, Bucks., U.K. Appropriate mixtures of the radiochemicals were diluted with incubation medium to the desired specific radioactivities before use.

Spectrapor no. 3 dialysis tubing, purchased from Spectrum Medical Industries, Los Angeles, CA, U.S.A., was heated in 1% disodium EDTA at 100°C for 15min, and washed several times with glass-distilled water, before use.

Methods

Incorporation of radioactive precursors. Sterna from 12-day chick embryos were incubated individually under air in 1ml of a synthetic medium, as described by Audhya & Gibson (1974). The incubation medium used in all experiments contained 0.1M-NaCl, 5mM-KCl, 1mM-$MgSO_4$, 50mM-Tris/HCl, pH7.4, 5.6mM-D-glucose, bovine serum albumin (0.5mg/ml) and 21 amino acids, whose concentrations are listed by Audhya *et al.* (1976). Unless otherwise stated, all additions to the medium were present from the start of the experiment. After preincubation, for 30min unless otherwise indicated, radioactive precursors were added as described in the Tables and Figures, in a total volume of 25μl, and incubation was continued, usually for a further 6h. The sterna were then washed rapidly with 2×5ml of water, and total incorporation, or incorporation into glycosaminoglycans or proteoglycans, was determined.

For determination of total incorporation, sterna were first soaked in 5ml of satd. aq. Na_2SO_4 for 16–24h at room temperature (Hall, 1970). They were then washed with 4×5ml of water and digested in 0.5ml of a solution of papain (0.5mg/ml) in 0.1M-sodium phosphate (pH6.5)/4mM-EDTA/3mM-cysteine for 16h at 57°C. Radioactivity of the digests was taken to represent total incorporation. Incorporation into glycosaminoglycans was also measured, after precipitation with Alcian Blue as described in the next section.

When incorporation into glycosaminoglycans only

was to be determined, washed sterna from incubations were digested with papain without prior soaking in Na_2SO_4.

Precipitation of glycosaminoglycans. This was accomplished as described previously (Audhya & Gibson, 1974), with slight modifications. Samples (0.1ml) of papain digests of sterna were mixed with 5ml of 0.05% Alcian Blue 8GX in 0.05M-$MgCl_2$/0.05M-sodium acetate, pH5.8 (Whiteman, 1973). The precipitates were collected after 2h, washed with 2×5ml of 95% (v/v) ethanol and redissolved in 5ml of 40% (w/v) Manoxol 1B. After removal of 2.5ml for determination of radioactivity, the A_{622} of the remainder of the solutions was determined. The amount of glycosaminoglycan in each tube was estimated by comparison with a standard curve, obtained by subjecting samples containing between 20 and 70μg of chondroitin sulphate A+C to the above procedure.

Preparation of glycosaminoglycans. Papain digests of sterna were combined and precipitated with trichloroacetic acid (5%, w/v). The supernatant solutions were exhaustively dialysed against water, in dialysis tubing with a nominal 3500-mol.wt. cut-off (Spectrapor no. 3), and then freeze-dried.

Analysis of glycosaminoglycans. The proportions of galactosamine residues sulphated at the 4 or 6 positions, or at neither, were estimated after digestion with chondroitin lyase of glycosaminoglycans labelled with $^{35}SO_4^{2-}$ and [^{3}H]glucose. Solutions of glycosaminoglycans in 'enriched' Tris buffer (Saito *et al.*, 1968) were digested with chondroitin lyase (0.1–0.2 unit/ml) for 16h at 37°C. The digestion products were separated by chromatography on 50cm strips of Whatman no. 1 paper, with butan-1-ol/acetic acid/1M-NH_3 (2:3:1, by vol.) as solvent. Chromatograms were developed for 36h and cut into 1cm strips, which were analysed for radioactivity. Proportions of Δ-di-OS,* Δ-di-4S and Δ-di-6S were estimated graphically as described elsewhere (Audhya *et al.*, 1976). Results for Δ-di-4S and Δ-di-6S were not considered meaningful unless the estimates from both radioactive labels agreed within 15%; this guards against the possibility of error caused by the presence of inorganic sulphate, which is not completely separated from Δ-di-6S by the chromatographic technique used.

Approximate estimates of relative amounts of hyaluronic acid and chondroitin sulphate were obtained by adsorption–desorption (Pal & Nath, 1974). Solutions (0.5ml, containing approx. 250μg of uronic acid) of glycosaminoglycans were mixed with $BaSO_4$ (200mg), and centrifuged (15min at 3500*g*) after 10min. Desorption was accomplished by mixing the precipitate with 0.5ml of the appropriate solution and centrifuging in the same way after 10min. Solutions used for desorption were, in sequence, water (twice), 0.1M-HCl (three times) and 0.05M-H_2SO_4 (three times). Radioactivity was determined on portions of each solution; in each case, desorption with a particular solution was continued until no further radioactivity was eluted.

Amounts of heparin and heparan sulphate were measured by treating solutions of glycosaminoglycans with 1.67% (w/v) $NaNO_2$ and 11% (v/v) acetic acid at room temperature for 2h (Lagunoff & Warren, 1962). The reaction mixtures were applied to Whatman no. 1 paper and chromatographed in 2-methylpropanoic acid/0.5M-NH_3 (5:3, v/v) for 36h (Shapiro & Poon, 1976). The papers were cut into 1cm strips and analysed for radioactivity. Radioactivity that was more than 1cm from the origin was used to estimate the amount of heparin or heparan sulphate.

Gel filtration of glycosaminoglycans. This was performed on a column (0.9cm×101cm) of Sepharose 6B equilibrated with 0.2M-NaCl (Wåsteson, 1971). The column was operated at 4°C at a flow rate of 2ml/h; fractions (approx. 0.75ml) were collected and their volumes determined gravimetrically. The column was calibrated with end-labelled chondroitin sulphate, prepared from purified sternal proteoglycan by the method of Hopwood & Robinson (1973), with two important modifications as described elsewhere (Audhya *et al.*, 1976). These changes were a 10-fold increase in the specific radioactivity of the NaB^3H_4, and a more reliable estimate of this radioactivity by reduction of glucose rather than cyclohexanone.

Extraction of proteoglycans. Groups of ten sterna were incubated with radioactive precursors as described above. After washing with water, the combined sterna from each group (wet weight approx. 220–280mg) were extracted with 3ml of 0.5M-$LaCl_3$, adjusted to pH5.8, for 16h at room temperature (Mason & Mayes, 1973). The mixture was centrifuged at 12000*g* for 20min, and proteoglycans were precipitated by addition of 57ml of water (Mason & Roughley, 1974) to the supernatant. After 24h or more at 4°C, the precipitate was collected and dissolved as far as possible in 4M-guanidinium chloride/0.05M-sodium acetate, pH5.8 (usually 0.5–1ml). The small amount of insoluble material remaining was discarded.

In some experiments, the sterna were extracted directly with 2.5ml of 4M-guanidinium chloride/0.05M-sodium acetate, under the same conditions as above. Extracts obtained by these two procedures are compared below, in the Results section.

Isopycnic centrifugation of proteoglycans. This was performed essentially as described by Hascall & Sajdera (1969), under both 'associative' and 'dissociative' conditions. For 'associative' centrifugation,

* Abbreviations: Δ-di-OS, 3-*O*-β-(gluc-4-enuronosyl)-*N*-acetylgalactosamine or 3-*O*-β-(gluc-4-enuronosyl)-*N*-acetylglucosamine; Δ-di-4S, 3-*O*-β-(gluc-4-enuronosyl)-4-*O*-sulpho-*N*-acetylgalactosamine; Δ-di-6S, 3-*O*-β-(gluc-4-enuronosyl)-6-*O*-sulpho-*N*-acetylgalactosamine.

solutions of proteoglycan in 4M-guanidinium chloride/0.05M-sodium acetate were diluted with 0.05M-sodium acetate to adjust the concentration of guanidinium chloride to 0.5M. Solid CsCl (1.19g/g of solution) was added, and the solutions were centrifuged at 40000rev/min, 25°C, in a SW56 rotor in a Spinco L2-65B ultracentrifuge. The contents of the tubes were frozen and chopped into five equal sections. Densities were determined by weighing portions of the fractions. For subsequent analysis, the contents of the lower three-fifths of the gradients were combined; this preparation is referred to below as the major proteoglycan fraction. In some experiments, the material in the top fifth of the gradients was also retained; this is referred to as the minor proteoglycan fraction. The preparations were dialysed exhaustively against water and freeze-dried before further use.

Centrifugation under 'dissociative' conditions was also performed as described by Hascall & Sajdera (1969); details are given in the legend to Fig. 2.

Zone sedimentation of proteoglycans. Solutions of proteoglycan were subjected to zone sedimentation on 5–20% (w/v) linear gradients of sucrose in 4M-guanidinium chloride (Kimata *et al.*, 1974) as described previously (Audhya *et al.*, 1976). Details are given in the legend to Fig. 1.

Analytical methods. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, and uronic acid by a modified carbazole reaction (Galambos, 1967) with glucuronic acid as standard. Glucose, amino acids and inorganic sulphate in serum were determined as described previously (Audhya & Gibson, 1974; Audhya *et al.*, 1976).

Determination of radioactivity. All samples were mixed with Aquasol and counted for radioactivity in a Beckman LS250 scintillation counter. Aqueous solutions were counted for radioactivity as single-phase liquids or gels. Solutions containing Manoxol 1B (2.5ml) were mixed with 10ml of Aquasol and sufficient water (1.0–2.0ml) to produce a clear solution or gel. Double-labelled samples were counted for radioactivity with automatic quench correction; radioactivity from each radioisotope was determined by comparison with appropriate standards counted at the same time under identical conditions. By these methods, the maximum quench observed, even in samples containing Alcian Blue, was 2% for ^{35}S and 6% for ^{3}H.

Incorporation of radioisotopes is expressed in all cases as pmol or nmol, on the basis of the estimated specific radioactivity of inorganic sulphate, glucose or serine in the incubation medium. This procedure automatically corrects for decay of radioactive isotopes, and allows meaningful comparison between experiments conducted at wide intervals of time. Experimental justification for the procedure with inorganic sulphate is presented by Audhya & Gibson (1974); detailed discussion or experimental justification, with glucose and serine, is given by Audhya *et al.* (1976).

Table 1. *Incorporation of sulphate into glycosaminoglycans of chick-embryo sterna in the presence or absence of 4-methylumbelliferyl β-xyloside*

Sterna were incubated in medium containing [^{35}S]sulphate (1.5mCi/mmol) for 6h, and glycosaminoglycans were isolated with Alcian Blue, as described under 'Methods'. Results are expressed as means ± S.E.M. for groups of five sterna.

Additions to the incubation medium			
Human serum (%)	Tri-iodothyronine (nM)	4-Methylumbelliferyl β-xyloside (mM)	[^{35}S]Sulphate incorporated (pmol/μg of chondroitin sulphate)
—	—	—	87.8±1.0
—	2	—	130.9±1.4
20	—	—	160.2±2.3
20	10	—	206.4±3.3
—	—	0.1	25.6±0.7
—	2	0.1	49.4±1.0
20	—	0.1	49.8±1.4
20	10	0.1	95.2±3.5
—	—	0.25	20.1±0.6
—	2	0.25	33.9±1.5
20	—	0.25	36.0±1.0
20	10	0.25	65.3±1.9
—	—	1.25	7.7±0.4
—	2	1.25	9.9±0.3
20	—	1.25	13.9±0.4
20	10	1.25	18.3±1.2

Results

In the absence of added β-xylosides, incorporation of sulphate in control incubations proceeds linearly with time for at least 6h (Audhya & Gibson, 1974), and the amount of sulphate incorporated in this period represents an increase of approx. 5% in the total chondroitin sulphate. Addition of 2nM-tri-iodothyronine to the incubation medium stimulates incorporation of sulphate by about 40%; addition of 20% (v/v) normal human serum stimulates by 50–60%; and 20% serum supplemented with 10nM-tri-iodothyronine stimulates by 90–100% (Audhya *et al.*, 1976; see also Table 1). When 1.25mM-4-methylumbelliferyl β-xyloside was added to the incubation medium, incorporation in incubations with no further addition was decreased by 88%, and little stimulation was observed when either tri-iodothyronine or serum or both was added (Table 1). When the concentration of the xyloside was lowered to 0.1mM, incorporation in the absence of serum or tri-iodothyronine was 29% of the control value. Addition of 2nM-tri-iodothyronine increased the incorporation to 56% of the control value, and addition of 20% serum supplemented with 10nM-tri-iodothyronine restored the incorporation to 108% of the control value (Table 1). At an intermediate concentration, 0.25mM, the effect of adding the xyloside to the incubation medium lay between these extremes.

Very similar results were obtained when *p*-nitrophenyl β-xyloside was added to the incubation medium in place of 4-methylumbelliferyl β-xyloside, except that the concentrations required to produce similar effects were about 2.5 times as high with the former xyloside as with the latter (Table 2). Thus 2.5mM-*p*-nitrophenyl β-xyloside decreased incorporation of sulphate by 80% when added to the incubation medium in the absence of serum factors, and addition of serum and tri-iodothyronine led only to a small stimulation of incorporation. When the concentration of this xyloside was lowered to 0.25mM, incorporation in incubations with no further addition was still 60% lower than that in control incubations, but serum factors appeared to be highly stimulatory (Table 2).

In these experiments, incorporation was measured after precipitation of glycosaminoglycans with Alcian Blue. When sterna are incubated in the absence of xylosides, 90–95% of the total sulphate incorporated is found as Alcian Blue-precipitable glycosaminoglycans (Audhya & Gibson, 1974). When 0.1mM-4-methylumbelliferyl β-xyloside was included in the incubation medium, the percentage of total ^{35}S incorporated that was precipitated with Alcian Blue decreased to 80–85%. This change was the result of a large decrease in the amount of Alcian Blue-precipitable ^{35}S, with no significant change in the amount of non-precipitable ^{35}S (Table 3). The results suggest that synthesis of glycosaminoglycans only was inhibited, and that other metabolic pathways, which utilize and incorporate sulphate into other components of the tissue, were unaffected. Table 3 also indicates that incorporation of ^{35}S into those other tissue components was changed very little when sterna were incubated in the presence of serum and tri-iodothyronine.

To gain information about the mechanism of action of the xyloside, various properties of the product were investigated. When sterna are incubated with [^3H]glucose and [^{35}S]sulphate in the absence of xylosides, the two radioisotopes are incorporated in a

Table 2. *Incorporation of sulphate into glycosaminoglycans of chick-embryo sterna in the presence or absence of p-nitrophenyl β-xyloside*

Sterna were incubated in medium containing [^{35}S]sulphate (1.5mCi/mmol) for 6h, and glycosaminoglycans were isolated with Alcian Blue, as described under 'Methods'. Results are expressed as means ± S.E.M. for groups of five sterna.

Additions to the incubation medium			
Human serum (%)	Tri-iodothyronine (nM)	*p*-Nitrophenyl β-xyloside (mM)	[^{35}S]Sulphate incorporated (pmol/μg of chondroitin sulphate)
—	—	—	128.2±5.5
—	2	—	167.7±2.3
20	—	—	159.5±4.8
20	10	—	209.5±7.5
—	—	0.25	42.8±1.6
—	2	0.25	80.2±3.3
20	—	0.25	56.2±1.6
20	10	0.25	109.3±3.2
—	—	2.5	25.8±1.1
—	2	2.5	35.2±1.6
20	—	2.5	33.0±1.7
20	10	2.5	47.4±1.3

Table 3. *Total incorporation of sulphate into embryonic sterna, and incorporation into glycosaminoglycans, in the presence or absence of 4-methylumbelliferyl β-xyloside*

Sterna were incubated in medium containing [^{35}S]sulphate (2mCi/mmol) for 6h. They were then soaked in saturated aq. Na_2SO_4 overnight, digested with papain, and used for determination of total radioactivity and radioactivity in glycosaminoglycans as outlined under 'Methods'. Results are mean values ± S.E.M. for groups of five sterna, and have been converted into pmol/μg of chondroitin sulphate in each sternum.

Additions to the incubation medium			[^{35}S]Sulphate incorporated (pmol/μg of chondroitin sulphate)		
Human serum (%)	Tri-iodothyronine (nM)	4-Methylumbelliferyl β-xyloside (mM)	Total	Glycosaminoglycans	Difference
—	—	—	106.9±5.6	96.8±3.8	10.0±2.9
—	2	—	151.4±4.1	138.4±2.6	12.6±3.2
20	—	—	164.8±6.5	155.3±5.0	12.8±3.9
20	10	—	206.5±8.4	198.5±5.3	11.4±3.4
—	—	0.1	51.4±2.3	40.2±2.5	11.2±2.1
—	2	0.1	77.9±3.2	60.6±2.0	17.3±1.4
20	—	0.1	56.6±5.2	45.1±4.4	11.5±0.9
20	10	0.1	105.4±3.7	89.5±4.0	15.9±1.0

characteristic molar ratio that depends on the carbon atom to which the ^{3}H label is attached. This ratio is only slightly altered when chondroitin sulphate synthesis is increased up to twofold by addition of serum or tri-iodothyronine or both (Audhya *et al.*, 1976). When sterna were incubated in the presence of 0.1mM-4-methylumbelliferyl β-xyloside, the ratio was increased significantly (Table 4), and addition of serum and tri-iodothyronine to the incubation medium did not restore the ratio to its original value. Quite similar results were obtained when 0.25mM-*p*-nitrophenyl β-xyloside was substituted for 4-methylumbelliferyl β-xyloside (Table 4). The results suggest that chondroitin sulphate formed in the presence of the xylosides was undersulphated.

Further analysis of the glycosaminoglycans formed in the presence of 0.1mM-4-methylumbelliferyl β-xyloside confirmed the view that they consist largely of undersulphated chondroitin sulphate. Chondroitin lyase digestion of crude glycosaminoglycans labelled with [^{35}S]sulphate and [^{3}H]glucose released more than 90% of the ^{35}S as Δ-di-4S and Δ-di-6S, in a molar ratio of 1.0–1.2 (Table 5). This ratio does not differ from the ratio observed in chondroitin sulphate synthesized in the absence of xylosides (Audhya *et al.*, 1976). However, 30–40% of the ^{3}H liberated by chondroitin lyase migrated with an R_F characteristic of Δ-di-OS (Table 5). This is at least twice as much Δ-di-OS as is found in digests of glycosaminoglycans synthesized by sterna that are not exposed to xylosides (Audhya *et al.*, 1976). The chromatographic technique used may not distinguish between Δ-di-OS from chondroitin sulphate and Δ-di-OS from hyaluronic acid, which is also digested by chondroitin lyase (Yamagata *et al.*, 1968). The amount of hyaluronic acid synthesized was estimated independently by the absorption–desorption technique of Pal & Nath (1974), in which glycosaminoglycans are adsorbed to $BaSO_4$, and hyaluronic acid and chondroitin sulphate are sequentially eluted with 0.1M-HCl and 0.05M-H_2SO_4 respectively. When this technique was applied to glycosaminoglycans labelled with [^{35}S]sulphate and [^{3}H]glucose, 76–88% of the applied ^{35}S was eluted by 0.05M-H_2SO_4, and less than 2% was eluted by 0.1M-HCl. The amount of ^{3}H eluted by 0.1M-HCl was in all cases less than 10% of the amount eluted by 0.05M-H_2SO_4, indicating that the cartilage synthesized at least 10 times as much chondroitin sulphate as hyaluronic acid. Combining this with the data in Table 4, we conclude that less than one-quarter of the Δ-di-OS released by digestion with chondroitin lyase arose from hyaluronic acid, and hence that chondroitin sulphate synthesized in the presence of 4-methylumbelliferyl β-xyloside was undersulphated.

Crude glycosaminoglycans synthesized in the presence or absence of 0.1mM-4-methylumbelliferyl β-xyloside were treated with HNO_2 and subjected to chromatography, as described under 'Methods'. Less than 3% of the total ^{35}S incorporated was released as inorganic sulphate or sulphated oligosaccharides, in all cases. Thus trivial amounts of heparan sulphate or heparin were formed in these incubations, whether xyloside was present or not. Sterna incubated in the absence of xylosides do not synthesize keratan sulphate (Audhya *et al.*, 1976). When [6-^{3}H]glucose and [^{35}S]sulphate were used as precursors, the ^{3}H/^{35}S molar ratio was increased from 1.0–1.1 in the absence of xylosides (Audhya *et al.*, 1976), to 1.3–1.5 when 0.1mM-4-methylumbelliferyl β-xyloside was present (Table 4). Similarly, when the precursors were [3-^{3}H]glucose and [^{35}S]sulphate, the ^{3}H/^{35}S ratio

Table 4. *Simultaneous incorporation of [^{35}S]sulphate and [^{3}H]glucose into glycosaminoglycans of chick-embryo sterna in the presence or absence of β-xylosides*

In Expts. 1 and 3, sterna were incubated in medium containing [6-^{3}H]glucose (3.0mCi/mmol) and [^{35}S]sulphate (0.67mCi/mmol) for 6h. In Expt. 2, incubation was for the same period, in medium containing [3-^{3}H]glucose (3.0mCi/mmol) and [^{35}S]sulphate (0.7mCi/mmol). Incorporation into glycosaminoglycans, determined as described under 'Methods', is expressed as mean values ± S.E.M. for groups of five sterna. Apparent ^{3}H/^{35}S molar ratios are given as mean values ± S.D. for the same groups.

		Additions to the incubation medium				Incorporation into glycosaminoglycans (pmol/μg of chondroitin sulphate)		
Expt. no.	^{3}H-labelled precursor	Human serum (%)	Tri-iodothyronine (nM)	4-Methylumbelliferyl β-xyloside (mM)	*p*-Nitrophenyl β-xyloside (mM)	[^{3}H]Glucose	[^{35}S]Sulphate	Molar ratio ^{3}H/^{35}S
1	[6-^{3}H]Glucose	—	—	—	—	113.2± 4.4	113.6±2.2	1.00±0.06
		20	10	—	—	208.9±10.7	186.3±8.9	1.12±0.03
		—	—	0.1	—	50.6± 2.6	35.5±1.3	1.47±0.18
		—	2	0.1	—	63.8± 4.7	46.2±3.0	1.42±0.04
		20	—	0.1	—	56.2± 2.9	44.9±2.1	1.29±0.04
		20	10	0.1	—	109.4± 2.9	86.3±1.5	1.36±0.04
2	[3-^{3}H]Glucose	—	—	—	—	151.9± 5.0	92.2±3.8	1.66±0.13
		—	—	0.1	—	96.2± 4.3	45.0±2.5	2.15±0.07
		—	2	0.1	—	121.3± 4.8	56.4±1.6	2.14±0.05
		20	—	0.1	—	130.8± 4.7	53.4±1.6	2.18±0.08
		20	10	0.1	—	221.9± 1.7	100.4±1.0	2.21±0.04
3	[6-^{3}H]Glucose	—	—	—	—	73.7± 2.9	74.9±2.9	0.99±0.07
		—	—	—	0.25	35.3± 1.6	24.2±0.3	1.46±0.12
		20	10	—	0.25	166.8± 9.2	86.1±4.5	1.94±0.05

Table 5. *Chondroitin lyase digestion of glycosaminoglycans formed by embryonic sterna in the presence of 4-methylumbelliferyl β-xyloside*

Sterna were incubated in medium containing 0.1mM-4-methylumbelliferyl β-xyloside, [^{35}S]sulphate (0.5 mCi/mmol) and [3-^{3}H]glucose (4.0mCi/mmol) for 6h. Papain digests of groups of five sterna were combined, deproteinized with 5% trichloroacetic acid, dialysed exhaustively and freeze-dried; the residues were then dissolved in 0.2ml of water and digested with chondroitin lyase, as described under 'Methods'. The products of digestion were separated by paper chromatography, and the proportions of Δ-di-4S, Δ-di-6S and Δ-di-OS were estimated as outlined under 'Methods'. The ratio, Δ-di-4S/Δ-di-6S, was estimated from the ^{35}S radioactivity; the proportion of [^{3}H]glucose liberated as Δ-di-OS was estimated from the ^{3}H radioactivity associated with Δ-di-OS, Δ-di-4S and Δ-di-6S.

Additions to the incubation medium			
Human serum (%)	Tri-iodothyronine (nM)	Δ-di-4S/Δ-di-6S	[^{3}H]Glucose as Δ-di-OS (%)
—	—	1.20	40
—	2	1.23	40
20	—	1.24	47
20	10	0.95	34

rose from 1.6–1.7 (Audhya *et al.*, 1976) to 2.1–2.2 (Table 4). The fact that there was a proportional rise in both ^{3}H/^{35}S ratios whenever incubations were performed in the presence of xyloside suggests that there was no change in the amount of keratan sulphate formed.

If embryonic cartilage is incubated in the presence of an inhibitor of protein synthesis and an exogenous β-xyloside, the chondroitin sulphate that is formed is largely, if not exclusively, covalently linked to the exogenous β-xyloside and not to the natural peptidyl-xyloside. This has been clearly demonstrated for 4-methylumbelliferyl β-xyloside (Fukunaga *et al.*, 1975), and is supported by less direct evidence for other β-xylosides (Robinson *et al.*, 1975). However, most exogenous β-xylosides appear to be less efficient acceptors for synthesis of chondroitin sulphate by cartilage than is the natural protein acceptor, since less radioactivity is usually incorporated into glycosaminoglycans in these experiments than in control incubations. When sterna from 12-day chick embryos are incubated in the presence of 1mM-puromycin, incorporation of sulphate is decreased by more than 98% (Audhya & Gibson, 1976). Addition of 1mM-*p*-nitrophenyl β-xyloside restores incorporation to about 10% of control values; however, neither serum nor tri-iodothyronine produces any increase in this

incorporation. Thus synthesis of chondroitin sulphate linked to this exogenous acceptor is much less efficient than synthesis of proteochondroitin sulphate, and does not appear to be stimulated by serum factors.

Qualitatively similar results were obtained when the exogenous acceptor was 0.1 mM-4-methylumbelliferyl β-xyloside rather than *p*-nitrophenyl β-xyloside. In incubations in which 1 mM-puromycin was added to the medium, incorporation of sulphate during a 6h period was decreased to 3% of that observed in control incubations, and simultaneous addition of xyloside restored the incorporation to 19% of control values (Table 6, Expt. 1). To test the effect of serum factors, the experimental procedure was modified so as to allow the serum factors to act on the sterna for a 3h period before addition of [^{35}S]sulphate for 3h; this period is probably sufficient for the full expression of stimulation (Audhya & Gibson, 1975). Puromycin was added at the end of the preincubation period, before addition of radioactive sulphate. As seen in Table 6, Expt. 2, incorporation of sulphate in the presence of puromycin and 4-methylumbelliferyl β-xyloside was 71% lower than incorporation in the presence of the β-xyloside alone, and whereas the serum factors stimulated incorporation by 75% when puromycin was absent from the incubation medium, they did not stimulate at all when puromycin was added to the incubation medium during the incubation with [^{35}S]sulphate. Assuming that puromycin has no direct effect on the enzymes that synthesize chondroitin sulphate, these results lead us to conclude that: (1) at least 70% of the chondroitin sulphate that is formed by sterna, incubated in the presence of 4-methylumbelliferyl β-xyloside without inhibitors of protein synthesis, is covalently linked to the natural peptide acceptor; (2) serum factors do not stimulate the polymerization of chondroitin sulphate itself, but exert their effect at some step in the biosynthetic pathway before the addition of the first galactose residue in the linkage region.

The first of these conclusions can be tested by incubating sterna with [^{35}S]sulphate and [^{3}H]serine. Chondroitin sulphate isolated from papain digests of sterna incubated with [^{35}S]sulphate and [^{3}H]serine in the absence of β-xylosides contains ^{35}S and ^{3}H in a molar ratio of approx. 50:1 (Audhya *et al.*, 1976). When proteoglycan synthesis is stimulated twofold by addition of serum and tri-iodothyronine, the ratio falls to about 42:1. This change is the result of a 15% decrease in number-average molecular weight of the chondroitin sulphate synthesized. When similar incubations were performed in the presence of 0.1 mM-4-methylumbelliferyl β-xyloside, both ^{35}S and ^{3}H were incorporated into Alcian Blue-precipitable glycosaminoglycans, and the molar ratio of the two radioisotopes was close to 25:1 (Table 7). This indicates that the tissue did indeed synthesize proteochondroitin sulphate. The twofold change in the ^{3}H/^{35}S ratio, compared with control incubations, also suggests that the chondroitin sulphate chains synthesized in the presence of the xyloside are not only undersulphated, but also shorter than usual.

Further analysis necessitated extraction of the proteoglycans from the sterna. A comparison was made between extraction with 4M-guanidinium chloride (Hascall & Sajdera, 1969) and extraction with 0.5M-$LaCl_3$ (Mason & Mayes, 1973). A single extraction with 4M-guanidinium chloride, as described under 'Methods', solubilized 80–82% of the total uronic acid. Extraction with 0.5M-$LaCl_3$ was

Table 6. *Incorporation of [^{35}S]sulphate into glycosaminoglycans of chick-embryo sterna in the presence of puromycin and 4-methylumbelliferyl β-xyloside*

In Expt. 1, sterna were incubated in medium containing [^{35}S]sulphate (1.0mCi/mmol) for 6h. Puromycin and 4-methylumbelliferyl β-xyloside were present from the start of the incubation. In Expt. 2, sterna were preincubated, in medium containing serum, tri-iodothyronine and 4-methylumbelliferyl β-xyloside as indicated, for 2.8h. Puromycin was then added, as indicated, in a total volume of 20μl; after a further 0.2h, [^{35}S]sulphate (final specific radioactivity 5.0mCi/mmol) was added, and incubation was continued for 3h. Incorporation into glycosaminoglycans, determined as described under 'Methods', is expressed as mean values ± S.E.M. for groups of five sterna.

	Additions to the incubation medium				
Expt. no.	Human serum (%)	Tri-iodothyronine (nM)	Puromycin (mM)	4-Methylumbelliferyl β-xyloside (mM)	Sulphate incorporated (pmol/μg of chondroitin sulphate)
1	—	—	—	—	106.5 ± 6.9
	—	—	1.0	—	3.4 ± 0.2
	—	—	1.0	1.0	20.6 ± 1.8
2	—	—	—	0.1	25.4 ± 2.2
	20	10	—	0.1	44.6 ± 2.2
	—	—	1.0	0.1	8.5 ± 0.5
	20	10	1.0	0.1	8.2 ± 0.5

Table 7. *Incorporation of [^{35}S]sulphate and [^{3}H]serine into glycosaminoglycans of chick-embryo sterna in the presence of 4-methylumbelliferyl β-xyloside*

Sterna were incubated in medium containing [^{35}S]sulphate (0.22mCi/mmol) and L-[3-^{3}H]serine (90.0mCi/mmol) for 6h, and glycosaminoglycans were isolated with Alcian Blue, as described under 'Methods'. Incorporation of each radioisotope is expressed as mean values ± S.E.M. for groups of five sterna. Apparent $^{3}H/^{35}S$ molar ratios are given as mean values ± S.D. for the same groups.

Additions to the incubation medium			Incorporation into glycosaminoglycans (pmol/μg of chondroitin sulphate)		
Human serum (%)	Tri-iodothyronine (nM)	Xyloside (mM)	[^{3}H]Serine	[^{35}S]Sulphate	Molar ratio, $^{3}H/^{35}S$
—	—	—	1.76±0.06	97.0±4.4	0.018±0.001
—	—	0.1	1.57±0.13	34.5±3.0	0.046±0.004
—	0.2	0.1	1.90±0.11	55.0±4.6	0.035±0.002
20	1.0	0.1	2.94±0.13	80.2±8.4	0.042±0.004

Table 8. *Specific radioactivities of proteoglycans extracted with $LaCl_3$ or guanidinium chloride*

Sterna were incubated in medium containing 0.1mM-4-methylumbelliferyl β-xyloside, [^{35}S]sulphate (0.5mCi/mmol) and [3-^{3}H]glucose (3.0mCi/mmol) for 6h. Groups of ten sterna were extracted with 4M-guanidinium chloride; duplicate groups were extracted with 0.5M-$LaCl_3$, as described under 'Methods'. Major and minor proteoglycan fractions, separated by isopycnic centrifugation under 'associative' conditions, were analysed for radioactivity and uronic acid content.

Additions to the incubation medium	Proteoglycan extractant	Incorporation into major proteoglycan fraction (pmol/μg of uronic acid)		Incorporation into minor proteoglycan fraction (pmol/μg of uronic acid)	
		^{3}H	^{35}S	^{3}H	^{35}S
None	$LaCl_3$	475	265	589	278
	Guanidinium chloride	505	285	517	286
20% Human serum+10nM-tri-iodothyronine	$LaCl_3$	1000	563	776	471
	Guanidinium chloride	960	526	705	398

somewhat less efficient, since it removed only 67–75% of the uronic acid. In the latter case, a further 6–8% of the uronic acid could be extracted by a subsequent treatment with 4M-guanidinium chloride. The remaining uronic acid, which constituted up to 20% of the total, could not be released except by proteolytic digestion of the cartilage. Proteoglycans were extracted with $LaCl_3$ or guanidinium chloride from sterna incubated with [^{3}H]glucose and [^{35}S]sulphate in the presence of 0.1mM-4-methylumbelliferyl β-xyloside and separated into major and minor fractions by isopycnic centrifugation. The specific radioactivities of proteoglycans extracted with $LaCl_3$ differed in nearly all cases by less than 10% from those of proteoglycans extracted with guanidinium chloride (Table 8). Thus although $LaCl_3$ was a slightly less efficient extractant of uronic acid, it did not appear to discriminate against any species of proteoglycan in the tissue. Since $LaCl_3$ solubilized less than half as much protein from the tissue as did guanidinium chloride, subsequent experiments were performed with proteoglycans extracted with $LaCl_3$ and precipitated by addition of water, as described under 'Methods'.

[In pulse-chase and other experiments extending over periods of 5–30min, no evidence was obtained for the existence of a pool of glycosaminoglycans serving as a precursor for the salt-extractable glycosaminoglycans. Also, no evidence was obtained that radioactivity incorporated into non-extractable glycosaminoglycans could be chased into salt-extractable ones. This situation contrasts with cartilage from pig larynx, where a precursor–product relation seems to exist between non-extractable and salt-extractable proteoglycans (Hardingham & Muir, 1972).]

Chick-embryo sternum contains two classes of proteoglycan, which differ in their proportions of protein and chondroitin sulphate (Kimata *et al.*, 1974; Palmoski & Goetinck, 1972). The major proteoglycan, which accounts for 70–80% of the total uronic acid, has a carbohydrate/protein (w/w) ratio close to 10:1, whereas this ratio is probably much lower for the minor proteoglycan (K. D. Gibson & B. J. Segen, unpublished work). Proteoglycans of the two classes can be separated by zone sedimentation on a sucrose gradient (Kimata *et al.*, 1974), and the major proteoglycan can be isolated by isopycnic centrifugation in a CsCl gradient (Audhya *et al.*,

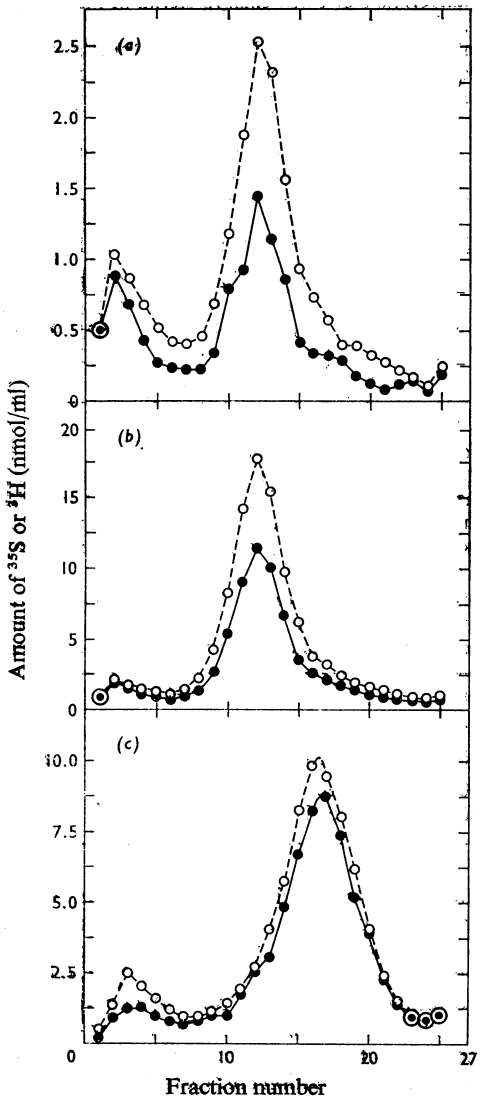

Fig. 1. *Zone sedimentation of proteoglycans synthesized in the presence or absence of 4-methylumbelliferyl β-xyloside*

Sterna were incubated in medium containing [^{35}S]-sulphate (0.67mCi/mmol) and [3-^{3}H]glucose (2.6 mCi/mmol) for 6h. Proteoglycans were extracted with 0.5M-$LaCl_3$ from groups of ten sterna, precipitated by dilution and redissolved in 4M-guanidinium chloride, as described under 'Methods'. A portion (0.15ml) was layered on a 12.5ml gradient of sucrose (5–20% in 4M-guanidinium chloride/0.05M-sodium acetate, pH5.8) and centrifuged in a SW40 rotor in a Beckman L2-65B ultracentrifuge at 38000rev./min, 4°C, for approx. 18h. Integrated $\omega^2 t$, measured with an $\omega^2 t$ Integrator Accessory, was 9.03×10^{11} rad^2-s. The contents of the tubes were collected by upward displacement with 60% sucrose and separated into 0.5ml fractions. Amounts of ^{35}S (●) and ^{3}H (○) in each fraction are expressed as nmol/ml, based on the calculated specific radioactivities of inorganic sulphate and glucose in the incubation medium. (*a*) Proteoglycans from sterna incubated in the presence of 0.1mM-4-methylumbelliferyl β-xyloside; (*b*) proteoglycans from sterna incubated in medium containing 0.1mM-4-methylumbelliferyl β-xyloside, 20% human serum and 10nM-tri-iodothyronine; (*c*) proteoglycans from sterna incubated with no addition to the medium.

1976). Proteoglycans extracted from sterna that had been incubated with [^{3}H]glucose and [^{35}S]sulphate in the presence of 0.1mM-4-methylumbelliferyl β-xyloside were subjected to zone sedimentation on a sucrose gradient. As with sterna that were incubated in the absence of the xyloside, two peaks were seen (Fig. 1). However, the peaks migrated less far with proteoglycan from sterna that had been exposed to the xyloside. This is particularly evident for the major faster-moving peak in the profiles in Fig. 1; the apparent sedimentation rate of this peak was decreased by 30% in the extracts from sterna exposed to the xyloside (Figs. 1*a* and 1*b*), compared with extracts from sterna that were incubated in its absence (Fig. 1*c*). However, the sedimentation rate of this peak was the same in all extracts of sterna exposed to the xyloside, whether the incubation was performed in the absence (Fig. 1*a*) or in the presence of tri-iodothyronine or serum or both (Fig. 1*b*).

The major proteoglycan fraction was isolated by isopycnic centrifugation in 'associative' CsCl gradients, as described under 'Methods', and analysed further by centrifugation in 'dissociative' CsCl gradients. When the proteoglycan was labelled with [^{3}H]glucose and [^{35}S]sulphate, the distribution of the two labels in the 'dissociative' gradients was virtually identical (Fig. 2). This implies that there was little or no 'link glycoprotein' (Hascall & Sajdera, 1969) remaining in the preparation after the first, 'associative' CsCl gradient. Similar results were obtained with proteoglycan labelled with [^{3}H]serine and [^{35}S]sulphate. Taken together, the observations indicate that the major proteoglycan fraction isolated from a single 'associative' CsCl gradient was at least 90% pure, and was essentially free from contamination with other carbohydrate-containing material. Presumably, any complexes that may exist between the major embryonic sternal proteoglycan and other components of the cartilage can be disrupted by high salt alone, in contrast with complexes that occur in adult cartilage, which are not disrupted unless a more drastic treatment is used (Hascall & Sajdera, 1969).

Incorporation of [^{3}H]glucose, [^{3}H]serine and [^{35}S]sulphate into the major proteoglycan in incubations containing 0.1mM-4-methylumbelliferyl β-xyloside was stimulated in a parallel fashion by serum

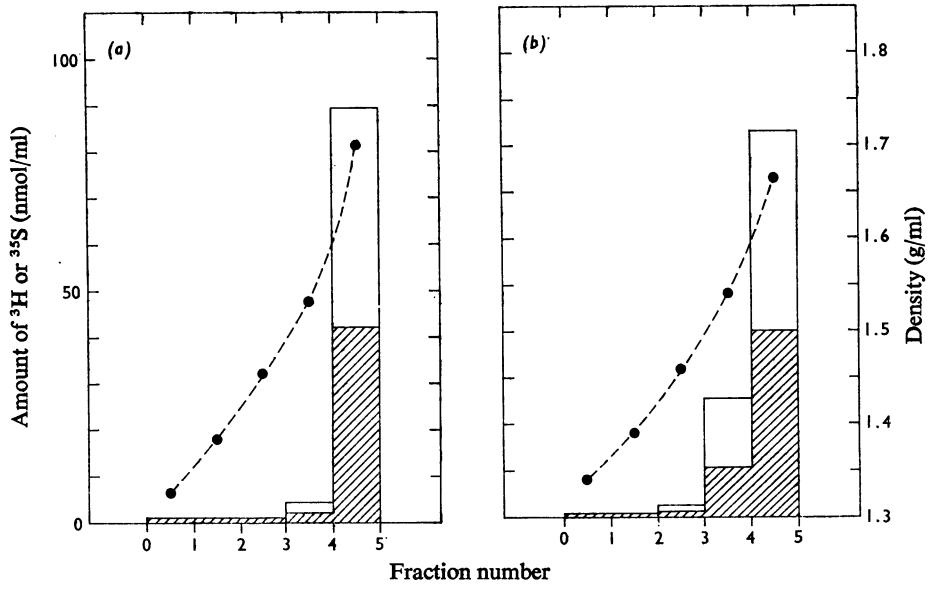

Fig. 2. *Isopycnic centrifugation of the major proteoglycan fraction under 'dissociative' conditions*
Sterna were incubated in medium containing [^{35}S]sulphate (0.67mCi/mmol) and [6-^{3}H]glucose (3.0mCi/mmol) for 6h; proteoglycans were extracted from groups of ten sterna with 0.5M-$LaCl_3$, precipitated by dilution, redissolved in 4M-guanidinium chloride and subjected to isopycnic centrifugation under 'associative' conditions, as described under 'Methods'. Material from the lower three-fifths of the gradients was dialysed against 0.5M-guanidinium chloride/0.05M-sodium acetate, pH5.8, and mixed with an equal volume of 7.5M-guanidinium chloride/0.05M-sodium acetate, pH5.8. Solid CsCl (0.59g/g) was added and the mixtures were centrifuged for 48h at 40000rev/min in a SW56 rotor in a Beckman L2-65B ultracentrifuge operated at 20°C. The contents of the tubes were frozen and cut into five equal sections. Density (●) was determined by weighing portions of the fractions. Amounts of ^{35}S (cross-hatched symbols) and ^{3}H (open symbols) are expressed as nmol/ml, on the basis of the estimated specific radioactivities of inorganic sulphate and glucose in the incubation medium. (*a*) Major proteoglycan fraction from incubations with no further addition to the medium; (*b*) major proteoglycan fraction from incubations in medium containing 20% human serum and 10mM-tri-iodothyronine.

and tri-iodothyronine (Table 9). This indicates that synthesis of both the protein and the carbohydrate moieties of the proteoglycan was stimulated co-ordinately. The ratio of [^{3}H]serine to [^{35}S]sulphate in Table 9 is 50–100% higher than the value of 0.05–0.07 observed in experiments in which no xyloside was present (Audhya *et al.*, 1976). When this proteoglycan fraction was digested with papain, and glycosaminoglycans were isolated with Alcian Blue, the ^{3}H/^{35}S ratio was approx. threefold lower (Table 9); this suggests that about one in every three serine residues bore a glycosaminoglycan side chain, and agrees with our earlier observations on proteoglycan from sterna incubated in the absence of xylosides (Audhya *et al.*, 1976). In the experiment with [3-^{3}H]glucose and [^{35}S]sulphate in Table 9, the ^{3}H/^{35}S ratio was nearly 50% higher than the value of 1.3–1.4 observed in this proteoglycan fraction in the absence of xylosides (K. D. Gibson & B. J. Segen, unpublished work). All these data are consistent with a decrease in chain length and some undersulphation of the glycosaminoglycan side chains, with no change in the number of side chains attached to each peptide molecule. Digestion of the proteoglycan, labelled with [^{3}H]glucose and [^{35}S]sulphate, with chondroitin lyase gave rise to Δ-di-6S, Δ-di-4S and Δ-di-OS in proportions close to those in Table 5, and accounting for more than 95% of the ^{35}S.

Direct evidence for a decrease in chain length of the chondroitin sulphate was obtained when the major proteoglycan was digested with papain, and the digest was subjected to chromatography on a calibrated column of Sepharose 6B. Fig. 3 shows elution profiles of chondroitin sulphate labelled with [3-^{3}H]-glucose and [^{35}S]sulphate, which was obtained by digestion of the major proteoglycan from sterna that had been incubated with or without serum and tri-iodothyronine, in either the presence or the absence of 0.1mM-4-methylumbelliferyl β-xyloside. The distribution was shifted towards higher $K_{av.}$ values whenever the xyloside was present in the incubation medium (Figs. 3*c* and 3*d*). The peak, number-average

Table 9. *Incorporation of [^{35}S]sulphate and [^{3}H]serine into the major proteoglycan of embryonic sterna in the presence of 4-methylumbelliferyl β-xyloside*

In Expt. 1, sterna were incubated in medium containing [^{35}S]sulphate (0.25mCi/mmol) and [3-^{3}H]serine (107.0mCi/mmol) for 6h; in Expt. 2, the incubation was for 6h in medium containing [^{35}S]sulphate (0.35mCi/mmol) and [3-^{3}H]glucose (3.0mCi/mmol). In all experiments, the incubation medium also contained 0.1mM-4-methylumbelliferyl β-xyloside. Groups of ten sterna were combined and extracted with $LaCl_3$; proteoglycans were precipitated by dilution, redissolved in 4M-guanidinium chloride and separated by isopycnic centrifugation under 'associative' conditions, as described under 'Methods'. The major proteoglycan fraction was collected from the bottom three-fifths of the gradients. In Expt. 1, incorporation into glycosaminoglycans from this fraction was measured after digestion with papain and precipitation with Alcian Blue. Incorporation into glycosaminoglycans was not measured in Expt. 2.

			Incorporation into proteoglycan			Incorporation into glycosaminoglycan		
Expt. no.	^{3}H-labelled precursor	Addition to the incubation medium	^{3}H (pmol/μg of uronic acid)	^{35}S (pmol/μg of uronic acid)	Molar ratio $^{3}H/^{35}S$	^{3}H (pmol/μg of chondroitin sulphate)	^{35}S (pmol/μg of chondroitin sulphate)	Molar ratio $^{3}H/^{35}S$
1	Serine	None	8.7	80.5	0.107	0.87	25.0	0.034
		20% Serum+10nM-tri-iodothyronine	25.5	270	0.094	3.06	86.4	0.035
2	Glucose	None	229	110	2.08	—	—	—
		2nM-Tri-iodothyronine	304	144	2.14	—	—	—
		20% Serum+10nM-tri-iodothyronine	605	330	1.83	—	—	—

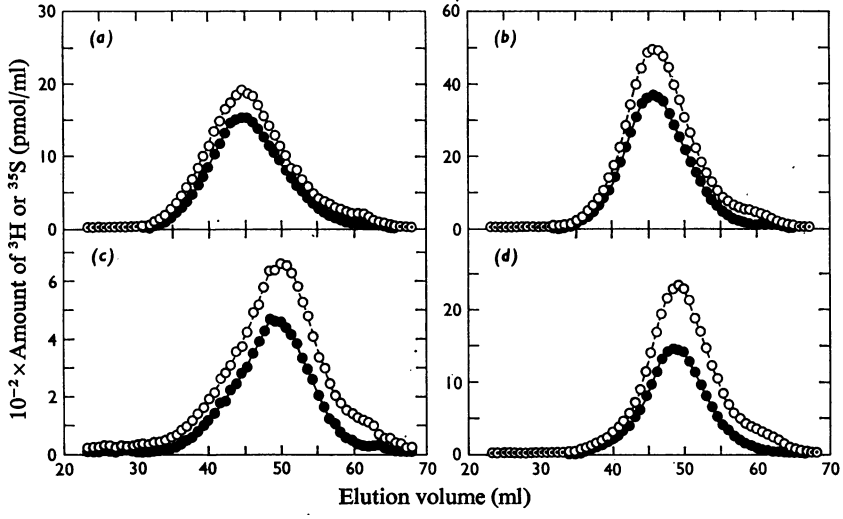

Fig. 3. *Gel filtration of chondroitin sulphate from the major proteoglycan fraction*

Sterna were incubated in medium containing [^{35}S]sulphate (0.67mCi/mmol) and [3-^{3}H]glucose (3.0mCi/mmol) for 6h; proteoglycans were extracted from groups of ten sterna with $LaCl_3$ and the major proteoglycan fraction was isolated by isopycnic centrifugation under 'associative' conditions, as described under 'Methods'. The fractions were digested with papain, and portions of the digests (0.3ml) were chromatographed on a calibrated Sepharose 6B column, as outlined under 'Methods'. The void volume and total volume of the column were 27.7 and 64.4ml respectively. Amounts of ^{35}S (●) and ^{3}H (○) in the eluates are expressed as nmol/ml, on the basis of the estimated specific radioactivities of the inorganic sulphate and glucose in the incubation medium. (*a*) Chondroitin sulphate from sterna incubated in medium with no further addition; (*b*) chondroitin sulphate from sterna incubated in medium containing 20% human serum and 10nM-tri-iodothyronine; (*c*) chondroitin sulphate from sterna incubated in medium containing 0.1mM-4-methylumbelliferyl β-xyloside; (*d*) chondroitin sulphate from sterna incubated in medium containing 0.1mM-4-methylumbelliferyl β-xyloside, 20% human serum and 10nM-tri-iodothyronine.

and weight-average molecular weights, calculated from the distributions of ^{35}S in Fig. 3, were all 20–30% lower when the xyloside was present during incubation than when it was absent (Table 10).

Since the major proteoglycan isolated by isopycnic centrifugation appears to be free from contamination by other carbohydrate-containing material, the ratio of ^{3}H to ^{35}S in each fraction eluted from the agarose column in the experiment described in Fig. 3 should reflect the molar ratio of glucose to sulphate incorporated into the chondroitin sulphate in that fraction. In Fig. 4, the $^{3}H/^{35}S$ molar ratio of each of these fractions is plotted against its apparent molecular weight. In all cases, the ratio had a broad minimum, extending over the molecular-weight range 15000–40000. Outside these limits, the ratio rose sharply, indicating that long or short chains of chondroitin sulphate are much less efficiently sulphated than those of intermediate size. The shape of the curve was modified slightly when the incubations were performed in the presence of serum supplemented with tri-iodothyronine (compare the two curves in Fig. 4*a*); however, chains of mol.wt. 15000–40000 were still more efficiently sulphated than longer or shorter chains. Somewhat greater changes were seen when 4-methylumbelliferyl β-xyloside was also present in the incubation medium. The $^{3}H/^{35}S$ ratio was increased by 10–15% at all mol.wt. values below 40000 (compare the corresponding curves in Figs. 4*a* and 4*b*). Addition of serum and tri-iodothyronine to incubations containing xyloside again caused a slight shift in the shape of the curve (Fig. 4*b*); this shift was very similar to the shift seen in the absence of xyloside. The major difference between the curves in Fig. 4(*a*) and those in Fig. 4(*b*) occurs in the region corresponding to mol.wts. above 40000, where there was an apparent sharp decrease in the degree of sulphation; since, however, there was not much chondroitin sulphate in this mol.wt. region, the estimates of the $^{3}H/^{35}S$ ratio in this region may be subject to some error.

Analysis of the minor proteoglycan fraction, obtained by collecting the upper portion of 'associative' CsCl gradients, gave results that were qualitatively similar to those obtained with the major proteoglycan. Since this fraction was contaminated with other proteins and carbohydrates, data on incorporation of serine or glucose have little meaning. Incorporation of sulphate, in incubations in the presence or absence of 0.1 mM-4-methylumbelliferyl β-xyloside, was stimulated by serum and tri-iodothyronine, in general to a lesser degree than in the case of the major proteoglycan. Digestion with chondroitin lyase released more than 90% of incorporated [^{35}S]sulphate as Δ-di-6S and Δ-di-4S, in essentially the same proportions as those in Table 5. Chromatography of papain digests on Sepharose 6B

Table 10. *Molecular weights of chondroitin sulphate from proteoglycan fractions*

Sterna were incubated in medium containing [^{35}S]sulphate (0.33 mCi/mmol) and [3-^{3}H]glucose (3.0 mCi/mmol) for 6 h. Total chondroitin sulphate was prepared from pooled papain digests of five sterna, as outlined under 'Methods'. Major and minor proteoglycan fractions were isolated by isopycnic centrifugation under 'associative' conditions, of proteoglycans extracted with $LaCl_3$, from groups of ten sterna, as described under 'Methods'; the proteoglycans were then digested with papain. Each preparation (0.3 ml, containing 80–100 μg of uronic acid) was separated by gel filtration on Sepharose 6B, on a column that had previously been calibrated by a modification of the method of Hopwood & Robinson (1973), as outlined under 'Methods'. Elution patterns of chondroitin sulphate from the major proteoglycan fractions are shown in Fig. 3. Number-average and weight-average molecular weights were calculated as described by Wåsteson (1971) from the distribution of ^{35}S.

Additions to the incubation medium				Molecular weights		
Human serum (%)	Tri-iodothyronine (nM)	4-Methylumbelliferyl β-xyloside (mM)	Proteoglycan fraction	Number-average	Weight-average	Peak
—	—	—	Total	23000	25300	24100
			Major	22300	25400	24300
			Minor	22600	26100	24900
20	10	—	Total	20100	22100	21500
			Major	21700	24100	23800
			Minor	19800	22400	20900
—	—	0.1	Total	16300	18500	16000
			Major	17900	20400	17700
			Minor	15900	18100	15400
20	10	0.1	Total	17800	19800	19000
			Major	18100	20000	18600
			Minor	17000	18800	18900

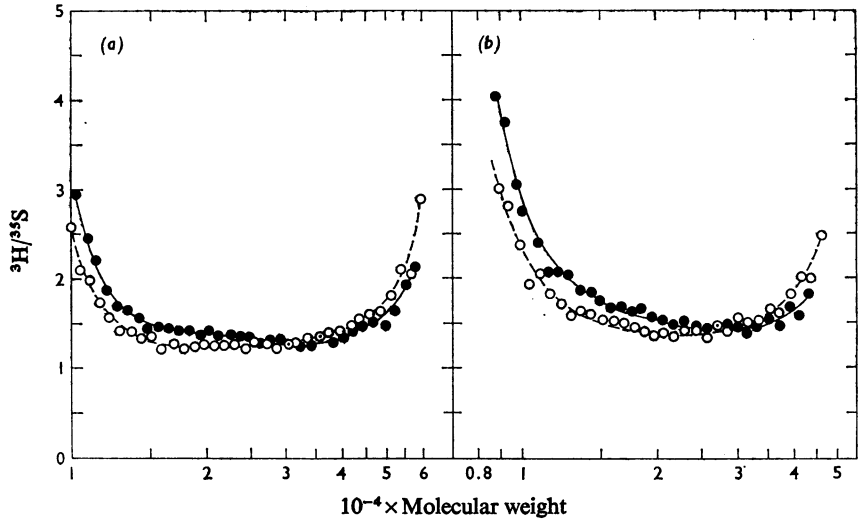

Fig. 4. *Extent of sulphation and molecular weight of chondroitin sulphate from the major proteoglycan fraction*
Apparent molar ratios, [^{3}H]glucose/[^{35}S]sulphate, were calculated for each fraction eluted from the Sepharose 6B column in the experiment described in Fig. 3. The molecular weight of chondroitin sulphate in each fraction was estimated from the elution volume, by using data derived from chromatography of a sample of end-labelled chondroitin sulphate (Audhya *et al.*, 1976; see under 'Methods'). (*a*) Chondroitin sulphate from sterna incubated in medium with no further addition (●), or in medium containing 20% human serum and 10nM-tri-iodothyronine (○); (*b*) chondroitin sulphate from sterna incubated in medium containing 0.1mM-4-methylumbelliferyl β-xyloside (●), or in medium containing 0.1mM-4-methylumbelliferyl β-xyloside, 20% human serum and 10nM-tri-iodothyronine (○).

gave distributions similar to those obtained with the major proteoglycan. Peak and average molecular weights, calculated from the distributions, were in general slightly lower than those of the major proteoglycan (Table 10). Table 10 also shows average molecular weights for samples of total chondroitin sulphate from another experiment, prepared by papain digestion of whole cartilages without prior extraction of proteoglycans. With one exception, average molecular weights of these samples were between the values for the separate major and minor proteoglycan fractions, and in all cases, inclusion of 4-methylumbelliferyl β-xyloside in the incubation medium led to a decrease in chain length of the chondroitin sulphate, amounting to 20–30%.

Discussion

4-Methylumbelliferyl β-xyloside has been used by several groups to investigate the ability of various cells or tissues to synthesize glycosaminoglycans when little or no proteoglycan can be formed (Okayama *et al.*, 1973; Schwartz *et al.*, 1974; Galligani *et al.*, 1975; Robinson *et al.*, 1975; Fukunaga *et al.*, 1975), but there does not seem to have been any investigation of its effect on synthesis of native proteoglycans. Our results show that, at concentrations in the range used by others, this β-xyloside has a rather profound effect on the proteochondroitin sulphate that is synthesized by chick-embryo sterna *in vitro*. There was a marked concentration-dependent decrease in the amount of sulphate incorporated into glycosaminoglycans, with no obvious effect on the incorporation of sulphate into other components of the tissue. This decrease was the result of changes in the chondroitin sulphate synthesized by the sterna, with little alteration in the spectrum of total glycosaminoglycans. There was apparently no increase in the very small amount of heparin or heparan sulphate formed, and no evidence for the synthesis of keratan sulphate. The failure of 4-methylumbelliferyl β-xyloside to promote synthesis of heparin or heparan sulphate may be an instance of a general phenomenon, since Schwartz *et al.* (1974) could find no increase in the formation of these glycosaminoglycans when several cell lines were incubated with this xyloside. Our results do not rule out the possibility of increased synthesis of hyaluronate when β-xylosides are present; however, this question has not been pursued further.

Several lines of evidence support the view that both chain length and degree of sulphation of the chondroitin sulphate synthesized by the cartilage were affected when incubations were carried out in the presence of 0.1mM-4-methylumbelliferyl β-xyloside.

A decrease in sulphation is suggested by the 30–40% increase over control values in the molar ratio of [^{3}H]glucose and [^{35}S]sulphate in incubations with both labels (Table 4), and also by the nearly threefold increase in the amount of Δ-di-OS liberated by chondroitin lyase (Table 5). Results of both types of experiments indicate that whereas chondroitin sulphate synthesized in incubations without xylosides is approx. 85% sulphated (Audhya *et al.*, 1976), when 0.1 mM-4-methylumbelliferyl β-xyloside was present the degree of sulphation fell to about 60%. The decrease in sulphation occurred at the expense of both the 4-sulphate ester and the 6-sulphate ester of *N*-acetylgalactosamine, and the ratio of the amounts of these esters remained the same as in control incubations (Table 5). A 20–30% decrease in chain length of the chondroitin sulphate is immediately evident from the results of column chromatography (Fig. 3 and Table 10). Further evidence for decreased chain length can be adduced from the results of simultaneous incorporation of [^{3}H]serine and [^{35}S]sulphate (Table 7). The ^{3}H/^{35}S ratios in such experiments would be affected by the average chain length of the chondroitin sulphate as well as by the degree of sulphation. The ^{3}H/^{35}S ratio in Table 7 was close to twice the ratio observed in the absence of the xyloside (Audhya *et al.*, 1976); this is too large a change to be accounted for solely by a decrease in sulphation, particularly since some of the newly synthesized chondroitin sulphate probably contained 4-methylumbelliferone rather than serine as its aglycone. However, the data are consistent with a 30% fall in the degree of sulphation and a simultaneous 25% decrease in average chain length. Thus the results of all these experiments are in reasonable agreement on the extent of the changes in the chondroitin sulphate synthesized in the presence of the xyloside.

The changes observed in the chondroitin sulphate isolated from papain digests of whole cartilage were also evident in the proteoglycan extracted from the tissue. In double-labelling experiments, with [^{35}S]-sulphate and either [^{3}H]glucose or [^{3}H]serine, the presence of 4-methylumbelliferyl β-xyloside in the incubation medium led to increases in the ^{3}H/^{35}S ratio in the major proteoglycan fraction (Table 9). These increases were essentially parallel to the increases in the ratios observed in the purified glycosaminoglycans. The results in Table 9, Expt. 1, also suggest rather strongly that one-third of the serine residues in each newly synthesized peptide chain were esterified with chondroitin sulphate, and that this number did not change when synthesis of proteoglycan was stimulated by addition of serum and tri-iodothyronine. The sedimentation rate of the major carbohydrate-rich proteoglycan peak was significantly decreased (Fig. 1), as would be expected if there were a decrease in chain length and degree of sulphation of the chondroitin sulphate in this fraction, with no change in the number of chains attached to the polypeptide. Characterization of the chondroitin sulphate in the major and minor proteoglycan fractions gave results that agreed satisfactorily with the findings for the total unfractionated glycosaminoglycans, and suggested that the changes that occur in the presence of the xyloside apply equally to all proteochondroitin sulphate synthesized by the tissue.

Chondroitin sulphate from the major proteoglycan, synthesized in the presence of 4-methylumbelliferyl β-xyloside, showed nearly the same relation between degree of sulphation and molecular weight as chondroitin sulphate synthesized in control incubations (Fig. 4). A small decrease in the apparent degree of sulphation at all mol.wts. below 40000 was evident in the chondroitin sulphate from incubations in the presence of the xyloside, as compared with incubations in its absence; this suggests some direct interference with the mechanism of sulphation. However, an equally important cause of the decreased sulphation of chondroitin sulphate synthesized in the presence of the xyloside is the overall decrease in chain length. Nearly half the chondroitin sulphate formed under these conditions had a mol.wt. less than 15000. Chondroitin sulphate whose molecular weight was below this limit was less sulphated in all incubations, in either the presence or the absence of the xyloside. The results suggest that a major effect of the xyloside in chick-embryo cartilage is to cause a significant decrease in the length of the polysaccharide side chains of all proteoglycans, and that much of the decrease in sulphation follows from the inferior ability of short polysaccharide chains to accept sulphate.

The mechanism by which 4-methylumbelliferyl β-xyloside exerts this effect is at present unknown. Although the action of *p*-nitrophenyl β-xyloside in this system has been examined only superficially, it seems probable that similar changes occur in the presence of this xyloside also; hence, the effect is likely to be fairly general. Preliminary results indicate that other 4-methylumbelliferyl β-glycosides, and 4-methylumbelliferone itself, do not affect synthesis of chondroitin sulphate (K. D. Gibson & B. J. Segen, unpublished work). Thus the effect is probably brought about only by β-xylosides and may involve the xylosides directly or require their prior conversion into other compounds. One possibility is that the effect is produced by the unnatural chondroitin sulphate formed by polymerization of carbohydrate residues on to the exogenous β-xyloside. There is both indirect (Table 6) and direct (B. J. Segen & K. D. Gibson, unpublished work) evidence to support the formation of such chondroitin sulphate, even in incubations in which protein synthesis is not blocked. However, possible mechanisms by which the presence of such chondroitin sulphate might lead to decrease

in chain length of newly synthesized proteochondroitin sulphate are a matter for speculation.

In spite of the alterations in the chondroitin sulphate formed in the presence of 0.1 mM-4-methylumbelliferyl β-xyloside, incorporation of sulphate into proteoglycan was effectively stimulated by serum and tri-iodothyronine. Indeed, the effect of tri-iodothyronine was particularly marked. Thus addition of 2 nM-tri-iodothyronine to the incubation medium alone frequently caused an increase of 60–90% (e.g. Table 1), and when added with 20% human serum, 10 nM-tri-iodothyronine usually increased sulphation by 150–300% (cf. Tables 1–4). These increases are somewhat illusory, since other parameters were not affected to the same extent. For instance, in the experiment in Table 7, incorporation of sulphate was stimulated 130% by addition of serum and tri-iodothyronine, but incorporation of serine into glycosaminoglycans was stimulated only by 90%. In a similar experiment, a 3.5-fold increase in incorporation of sulphate into the major proteoglycan was accompanied by a threefold increase in incorporation of serine (Table 9). The discrepancies are probably due to the fact that the chondroitin sulphate produced under conditions of stimulation had a slightly higher average molecular weight than that formed in the presence of the β-xyloside alone (Table 10). The results of these double-labelling experiments also illustrate the point that incorporation of sulphate does not always accurately reflect proteoglycan synthesis. Incorporation of sulphate into glycosaminoglycans was decreased by 60% in incubations containing 0.1 mM-4-methylumbelliferyl β-xyloside as compared with control incubations, but incorporation of serine was decreased only by 10% (Table 7). Similarly, comparison of the data in Table 9 with results presented elsewhere (Audhya *et al.*, 1976) shows that in the presence of the xyloside, incorporation of sulphate into the major proteoglycan was decreased much more than incorporation of serine. Thus synthesis of the protein moiety of the proteoglycan was inhibited considerably less at this concentration of the xyloside than was sulphation.

When allowance is made for the considerations outlined in the previous paragraph, the effect of the serum factors in the present work is found to be in reasonable accord with results obtained earlier (Audhya & Gibson, 1976). From experiments in which components of the medium were omitted, or metabolic inhibitors were added, we previously concluded that tri-iodothyronine stimulated sulphation in embryonic-chicken sterna only under conditions which were otherwise nearly optimal for proteoglycan synthesis. The work here supports that conclusion. Synthesis of chondroitin sulphate in the presence of puromycin, with 4-methylumbelliferyl β-xyloside as exogenous acceptor, was not stimulated at all by tri-iodothyronine and other serum factors, even though these factors were allowed to act on the cartilages for 3 h before the addition of the protein-synthesis inhibitor. In the absence of puromycin, at high concentrations of 4-methylumbelliferyl β-xyloside, which strongly inhibit sulphation, the serum factors again did not stimulate much, whereas at lower concentrations, which affect the synthesis of chondroitin sulphate but do not greatly inhibit the synthesis of the peptide moiety of the proteoglycan, there was marked stimulation. Perhaps the most significant implication of the work presented here is that stimulation by serum factors can occur in spite of quite considerable variations in the polysaccharide side chains of the proteoglycan that is synthesized. This point has relevance to the general problem of control of proteoglycan synthesis. Stoolmiller *et al.* (1972) proposed that transfer of the xylose residue from UDP-xylose to the protein moiety of the proteoglycan is the rate-limiting step in proteochondroitin sulphate synthesis. The data presented in this paper and elsewhere (Audhya *et al.*, 1976) strongly suggest that there is no alteration in the number of chondroitin sulphate chains attached to each protein molecule when proteochondroitin sulphate synthesis is stimulated twofold or more by serum factors; this is true whether the length and degree of sulphation of the chondroitin sulphate chains is 'normal', as in incubations without β-xylosides, or less than usual, as in the present work. Although it is possible to reconcile this observation with the hypothesis of Stoolmiller *et al.* (1972), it seems more reasonable to conclude that control is exerted at the level of protein synthesis, or at the level of intracellular transport of the peptide to the sites of initiation of polysaccharide synthesis, and that once these processes have taken place subsequent reactions, including the polymerization and sulphation of the chondroitin sulphate side chains, have little influence on the rate of formation or eventual fate of the product, however important they may be in determining its physiological function.

One final inference can be drawn from the work reported here; this concerns the order in which certain enzymic reactions involved in biosynthesis of chondroitin sulphate occur, and their possible localization within the cell. In particulate cell-free systems derived from chick-embryo cartilage, formation of the sulphate ester groups in chondroitin sulphate seems to occur by direct transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to polysaccharide chains during or immediately after polymerization (DeLuca *et al.*, 1973). However, the result presented in Fig. 4 supports a somewhat different mechanism, in which sulphation occurs at a site separate from polymerization (Horwitz & Dorfman, 1968), as the major one in chick-embryo sternum *in vivo*. There is only one sequence of reactions that could produce the relation between degree of sulphation and molecular weight of chondroitin sulphate

shown in Fig. 4; this is for sulphation to occur after polymerization of the carbohydrate chains is complete, with no temporal overlap of the two processes. If this is so, then the enzymes that transfer sulphate to the polysaccharide must be physically separated within the cell from the enzymes involved in polymerization. The primary intracellular site for sulphation in chondrocytes appears to be the Golgi apparatus (Godman & Lane, 1964), and there is evidence to support the view that incorporation of carbohydrates occurs both in this organelle (Freilich *et al.*, 1975) and in the endoplasmic reticulum (Horwitz & Dorfman, 1968; Stoolmiller *et al.*, 1972). Sequestration of the enzymes responsible for sulphation from those responsible for polymerization requires either that the two groups of enzymes occur in separate parts of the Golgi apparatus, or that the polymerizing enzymes are located in the endoplasmic reticulum. Homogenization and subsequent fractionation of the tissue would be expected to rupture and mix fragments of the organelles responsible for these two functions, especially since complete separation of membranes of the Golgi apparatus from other membranous components of the cell has probably not yet been achieved with any tissue (Bergeron *et al.*, 1973). Thus particulate preparations that catalyse the synthesis of chondroitin sulphate in cell-free incubations are likely to contain both the enzyme system responsible for polymerization of the carbohydrate chain, and the enzymes that catalyse sulphation, in close proximity, even though these enzyme systems may be physically separated *in vivo*. It therefore seems possible that synthesis of chondroitin sulphate in cell-free systems does not mirror accurately the sequence of events that prevails *in vivo*, and that inferences drawn from studies with cell-free systems should be treated with some reserve.

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