The Effect of D-Xylose, β -D-Xylosides and β -D-Galactosides on Chondroitin Sulphate Biosynthesis in Embryonic Chicken Cartilage

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The incorporation of [^3H]acetate into chondroitin sulphate was used as a measure of the rate of synthesis of this polysaccharide in whole tibias and femurs of embryonic chicken cartilage *in vitro*. The incorporation is inhibited by puromycin and by cycloheximide, but the inhibition is relieved by the addition of D-xylose, β -D-xylosides and β -D-galactosides to the incubation medium. β -D-Xylosides can stimulate the incorporation to 300% of that of controls incubated in the absence of cycloheximide or puromycin. D-Xylose, β -D-xylosides and β -D-galactosides appear to act as artificial initiators of chondroitin sulphate synthesis and enable polysaccharide-chain synthesis to be studied as an event separate from the synthesis of intact proteoglycan.

Inhibitors of protein synthesis such as puromycin and cycloheximide cause a marked inhibition of incorporation of label from [^{14}C]acetate, [^{14}C]glucosamine and [^{35}S]sulphate into chondroitin sulphate by intact cartilage preparations (Telser *et al.*, 1965; Cole & Lowther, 1969). This has been interpreted as an inhibition of synthesis of a protein essential for initiation of the polysaccharide chains, since puromycin has no marked effect on the incorporation of glucuronic acid or *N*-acetylgalactosamine from the corresponding UDP-sugars into chondroitin sulphate catalysed by cell-free preparations (Telser *et al.*, 1965). In embryonic chicken cartilage, puromycin does not inhibit the incorporation of [^{14}C]acetate into UDP-*N*-acetylgalactosamine (Telser *et al.*, 1965) or the incorporation of [^{14}C]galactose into UDP-glucuronic acid (Telser, 1968), so this inhibitor does not appear to have any direct effect on chondroitin sulphate biosynthesis.

Helting & Rodén (1969a) have demonstrated that cell-free preparations of embryonic chicken cartilage catalyse the transfer of [^{14}C]galactose from UDP-galactose to xylosylserine, xylose, β -methyl xyloside, and several β -galactosides. The same preparation also catalyses transfer of [^{14}C]glucuronic acid from UDP-glucuronic acid to certain β -galactosides (Helting & Rodén, 1969b). These reactions are believed to represent the enzymic steps leading to the biosynthesis of the oligosaccharide segment that links chondroitin sulphate to protein and which has the structure: $\text{GlcAp}\beta 1\text{--}3\text{Galp}\beta 1\text{--}3\text{Galp}\beta 1\text{--}4\text{Xylp}1\text{--}$ serine. In the present paper we report that D-xylose, β -D-xylosides and β -D-galactosides can relieve the inhibition caused by puromycin and cycloheximide

of acetate incorporation into chondroitin sulphate catalysed by intact cartilage. These results confirm that these protein-synthesis inhibitors have no direct effect on chondroitin sulphate chain elongation. D-Xylose, β -D-xylosides and β -D-galactosides appear to act as artificial initiators of chondroitin sulphate synthesis.

A preliminary communication of some of these results has been published (Brett & Robinson, 1971).

Experimental

Materials

Freshly laid fertile eggs of White Leghorn or of commercial broiler-chicken breeding stock (generously supplied by Home Pride Poultry Pty. Ltd., Melbourne, Vic., Australia) were incubated for 14 days in an electric incubator (Multiplo Incubator and Brooder Pty. Ltd., Sydney, N.S.W., Australia). Whole tibias and femurs were dissected from the embryonic chickens from 72 eggs and were cleaned of adhering tissues. During the dissection and before incubation the limb bones were kept immersed in incubation medium at room temperature (approx. 22°C).

Eagle's Basal Medium 10X Concentrate was purchased from the Commonwealth Serum Laboratories (Melbourne, Vic., Australia). Sephadex G-25 (fine grade) and Sephadex G-200 (fine grade) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), Whatman Chromedia ET-II cellulose (ECTEOLA) was from W. and R. Balston Ltd., Maidstone, Kent, U.K., and Bio-Gel P4 (50–100 mesh) was from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid] buffer, D-xylose,

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D-ribose, D-lyxose, L-arabinose, L-glutamine, *p*-nitrophenyl, β -D-glucuronide, methyl α -D-galactoside, methyl α -D-xyloside, methyl β -D-xyloside, methyl β -D-thiogalactoside, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide, *p*-nitrophenyl *N*-acetyl- β -D-galactosaminide, *N*-acetylgalactosamine, phenyl β -D-galactoside and phenethyl β -D-galactoside were all purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Papain (EC 3.4.22.2), prepared from crude papaya latex by the method of Kimmel & Smith (1958), was recrystallized twice before use. Chondroitin sulphate ABC-lyase (EC 4.2.2.4) from *Proteus vulgaris* was prepared as described by Robinson & Dorfman (1969). All other chemicals were of analytical grade.

Imidazole-HCl buffer solutions were prepared by dilution of a stock solution. Approximately 1.25M-HCl solution was adjusted to pH 7 by the addition of solid imidazole and then diluted with water to give a solution 1.0M in chloride. The pH values reported in the text are those measured on the solutions at the final concentration.

Sodium [$1\text{-}^{14}\text{C}$]acetate and sodium [3H]acetate were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and were diluted with unlabelled sodium acetate to the desired specific radioactivity before use. [$U\text{-}^{14}\text{C}$]Serine was also purchased from The Radiochemical Centre.

Synthesis of β -xylosides. Tri-*O*-acetyl- α -D-xylopyranosyl chloride was prepared by the action of acetyl chloride on D-xylose as described by Mattok & Phillips (1958). Alkyl tri-*O*-acetyl- β -D-xylopyranosides were prepared by the reaction of tri-*O*-acetyl- α -D-xylopyranosyl chloride (5.9g) with the appropriate anhydrous alcohol by the method of Lindberg (1949). The acetyl groups were removed by treatment with sodium methoxide (Thompson *et al.*, 1963) and the β -D-xylosides were recrystallized from ethanol-benzene (1:1, v/v). In this way, the following known compounds (De Bruyne & Loontjens, 1966) were prepared: ethyl β -D-xylopyranoside, yield 0.93g, m.p. 92°C , $[\alpha]_D^{18} -62 \pm 2^\circ$ (*c* 1.78 in water) (Found: C, 47.3; H, 7.82; Calc. for $\text{C}_7\text{H}_{14}\text{O}_5$: C, 47.2; H, 7.92%); *n*-butyl β -D-xylopyranoside, yield 1.49g, m.p. 87°C , $[\alpha]_D -58 \pm 2^\circ$ (*c* 2.06 in water) (Found: C, 52.5; H, 8.78; Calc. for $\text{C}_9\text{H}_{18}\text{O}_5$: C, 52.41; H, 8.80%); *n*-octyl β -D-xylopyranoside, yield 0.85g of oily solid; benzyl β -D-xylopyranoside, yield 1.51g, m.p. 112°C , $[\alpha]_D -68 \pm 2^\circ$ (*c* 2.4 in water) (Found: C, 57.9; H, 6.70; Calc. for $\text{C}_{12}\text{H}_{16}\text{O}_5$: C, 60.0; H, 6.71%).

Benzyl α -D-xylopyranoside was prepared by reaction of xylose (10g) with 50ml of anhydrous benzyl alcohol and 2.5g of dry HCl in 500ml of benzene for 12h at approximately 22°C . The reaction was stopped by the addition of 10ml of pyridine and the reaction mixture was concentrated to about 70ml by rotary evaporation. Crude benzyl xyloside

was precipitated by addition of 500ml of *n*-hexane and was collected by filtration. Pure benzyl α -D-xylopyranoside was isolated by repeated crystallization from water: yield approx. 2g, m.p. 128°C , $[\alpha]_D +140 \pm 2^\circ$ (*c* 2.4 in water).

Phenyl β -D-thioxylopyranoside was prepared by condensation of tri-*O*-acetyl- α -D-xylopyranosyl chloride (5.9g) with thiophenol as described by Purves (1929). Acetyl groups were removed with sodium methoxide as described above and the phenyl thioxyloside was recrystallized from benzene: yield 1.4g, m.p. $130\text{--}135^\circ\text{C}$, $[\alpha]_D -67 \pm 2^\circ$ (*c* 2.42 in water) (Found: C, 54.7; H, 5.93; S, 13.3; Calc. for $\text{C}_{11}\text{H}_{14}\text{O}_4\text{S}$: C, 54.5; H, 5.82; S, 13.2%).

Phenyl β -D-xyloside was a generous gift to Dr. M. Okayama from Dr. H. Kushida (Kyoto General Medicochemical Laboratory, Japan).

Methods

Incubation of chicken cartilage. Tissue (15–20 embryonic chicken bones) was incubated in 25ml conical flasks with 5.0ml of incubation medium. Incubations were performed at 37°C in a Gallenkamp metabolic incubator with a gassing manifold. The flasks were shaken at 80 cycles/min and were maintained in an atmosphere of O_2 or of $\text{O}_2 + \text{CO}_2$ (95:5) throughout the incubation as indicated below. Incubations were performed in one of the following media.

Medium A was Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) supplemented with 5.5mm-glucose, 1.0mm-L-glutamine and 0.002% Phenol Red. The solution was buffered at pH 7.4 by equilibration with $\text{O}_2 + \text{CO}_2$ (95:5) at 38°C .

Medium B was Eagle's Basal Medium supplemented with Hepes-KOH buffer, pH 7.4 (12.5mm in Hepes), and 23.7mm- NaHCO_3 . The solution was equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5) at 38°C . Stock Hepes buffer was prepared at 0.25M and was titrated with saturated KOH solution until a sample diluted to 12.5mm was pH 7.4.

Medium C was Eagle's Basal Medium supplemented with 1.0mm-L-glutamine and Hepes-KOH buffer, pH 7.4 (25mm in Hepes). The solution was equilibrated with O_2 . Stock Hepes buffer was prepared at 0.25M and was titrated with saturated KOH solution until a sample diluted to 25mm was pH 7.4. The flasks plus tissue were preincubated for 30min with inhibitor and other added compounds before the addition of the labelled acetate or serine. At the end of the 2h incubation period, the reactions were stopped by chilling each flask in crushed ice and by the addition of 0.25ml of 2.0M-KOH containing 0.5M- NaBH_4 for the subsequent isolation of chondroitin sulphate or by the addition of 0.25ml of 6.1M-trichloroacetic acid

solution for the subsequent isolation of protein and proteoglycan.

Isolation of chondroitin sulphate. Chondroitin sulphate was isolated from reactions stopped by the addition of KOH-NaBH₄. The flasks were kept at 4°C for 24h and then the excess of borohydride was decomposed by the addition of 0.05ml of acetone to each flask. After 30min at room temperature, the flask contents were neutralized with 0.5ml of 2.0M-acetic acid solution and 0.6ml of 1.0M-sodium acetate buffer, pH 5.5, containing 0.05M-EDTA and 10mg of cysteine hydrochloride/ml was added. The flasks were then incubated at 65°C for 24h with 0.5mg of papain. After digestion, the solutions were centrifuged to remove insoluble matter and chondroitin sulphate was precipitated from the supernatant solution by dropwise addition of 10% (w/v) cetylpyridinium chloride solution at 37°C until flocculation was complete. The precipitate was collected by centrifugation and the clear supernatant solution was discarded. The precipitate was washed with 3×10ml of 0.1% cetylpyridinium chloride in 0.2M-NaCl. The chondroitin sulphate was finally isolated as the calcium salt (Telser *et al.*, 1966). The dry calcium salt was dissolved in 5.0ml of water and samples were taken for assay of hexuronic acid content and radioactivity. Radioactivity was determined in a liquid-scintillation analyser model PW 4510/01 (Philips, Eindhoven, The Netherlands). Aqueous samples (0.5ml) were mixed with 10ml of scintillation mixture prepared as described by Bruno & Christian (1961). Hexuronate was determined by the carbazole method of Dische (1947).

Isolation of proteoglycan. Reactions were stopped by the addition of 0.25ml of 6.1M-trichloroacetic acid. After 1h at 4°C, the flask contents were adjusted to about pH 7.0 by dropwise addition of 2.0M-Tris solution with constant stirring. The addition was judged complete when the Phenol Red indicator turned light pink in colour. The entire flask contents were then homogenized in an all-glass Duall tissue grinder (Kontes Glass Co., Vineland, N.J., U.S.A.) with 6.0ml of 10.0M-LiCl containing imidazole-HCl buffer, pH 7.2 (0.02M in HCl). The homogenates were centrifuged for 30min at 35000g at 4°C and the supernatant solution was subjected to gel filtration on a Bio-Gel P4 column (60cm×2.5cm diam.) in pyridine-acetic acid buffer, pH 5.2 (0.2M in acetic acid; 0.2M in pyridine). The polysaccharide was eluted at the void volume of the column, well separated from the main peak of [³H]acetate. The hexuronate-positive fractions were combined, concentrated to approx. 5.0ml and applied to a column (37cm×2.8cm diam.) of Sephadex G-200 equilibrated with 5.0M-LiCl containing imidazole-HCl buffer, pH 7.2, 1.02mol/l. Fractions (6.0ml) were collected at a rate of 15ml/h and were assayed for hexuronate content and radioactivity.

Isolation of protein. Reactions were stopped by the addition of 0.25ml of 6.1M-trichloroacetic acid. The tissue was then transferred to an all-glass Duall tissue grinder and homogenized in 10ml of 0.30M-trichloroacetic acid containing 10mM-serine. The protein precipitate was collected by centrifugation, washed thoroughly with 0.3M-trichloroacetic acid solution, heated for 15min at 80°C in 0.3M-trichloroacetic acid containing 0.5M-HCl, and finally washed with acetone, ethanol and diethyl ether and dried in air at 37°C. The dry product was dissolved in 5.0ml of concentrated formic acid solution and assayed for protein content and radioactivity. Protein was determined by the biuret reaction (Layne, 1957) with bovine serum albumin as standard.

Molecular-weight determination of chondroitin sulphate by gel filtration on Sephadex G-200. Chondroitin sulphate, released from the tissue by treatment with alkaline borohydride, was isolated as the calcium salt after precipitation with cetylpyridinium chloride as outlined above. The sample (approx. 5.0mg) dissolved in 5.0ml of water was mixed with 1.0ml of 0.02% Phenol Red solution and applied to a column (90cm×2.2cm diam.) of Sephadex G-200 (fine grade) equilibrated with imidazole-HCl buffer, pH 7.2 (1.05mol/l) containing 5.0mM-NaF and 0.1% sodium dodecyl sulphate. Fractions (8.0ml) were collected at a flow rate of 8.0ml/h and each fraction was assayed for radioactivity and hexuronic acid content. Molecular-weight values for chondroitin sulphate were calculated from the elution profile by the method of Hopwood & Robinson (1973). The void volume of the column was determined with Blue Dextran (Pharmacia Fine Chemicals) and the effective total volume of the column was determined with Phenol Red indicator and with sodium [³H]acetate.

Results

Whole femurs and tibias of 14-day-old embryonic chickens, incubated *in vitro*, catalyse the incorporation of acetate into chondroitin sulphate. The extent of incorporation of acetate can conveniently be expressed as the specific radioactivity (d.p.m./mg of hexuronate) of the isolated chondroitin sulphate, because the hexuronate content of the tissue (35mg of hexuronic acid/g dry wt. of tissue) is relatively constant from one preparation to the next and does not change significantly during the course of an incubation *in vitro*.

When the tissue is incubated with labelled acetate, the specific radioactivity of the chondroitin sulphate increases linearly with time up to 5h (Fig. 1). No significant lag time (i.e. less than 5min) in the incorporation was detected, which suggests that the labelled acetate must be incorporated into and

Fig. 1. Effect of incubation time on the incorporation of $[^3\text{H}]$ acetate into chondroitin sulphate

Cartilage was incubated in medium B containing 1.0mM-L-glutamine and 0.9mM-sodium $[^3\text{H}]$ acetate (10mCi/mmol) and chondroitin sulphate was isolated as outlined under 'Methods'.

Table 1. Rate of incorporation of $[^3\text{H}]$ acetate into chondroitin sulphate in embryonic chicken cartilage *in vitro*

Tibias and femurs from 14-day-old embryonic chickens were incubated with medium containing 1.0mM-sodium $[^3\text{H}]$ acetate (10mCi/mmol) for 2h and labelled chondroitin sulphate was isolated as outlined under 'Methods'. Medium A was Krebs-Ringer bicarbonate buffer saturated with $\text{O}_2 + \text{CO}_2$ (95:5). Medium B was a modified Eagle's medium (see under 'Methods') containing 23.7mM- NaHCO_3 and 12.5mM-Hepes-KOH buffer, pH 7.4, also equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5). Medium C was a modified Eagle's medium (see under 'Methods'), containing 25mM-Hepes-KOH buffer, pH 7.4, equilibrated with 100% O_2 . Values are mean values ($\pm$ s.d.) with the number of determinations shown in parentheses. Each value represents results of duplicate determinations from 8-15 different batches of chick embryos.

Incubation medium	L-Glutamine concn. added (mM)	$[^3\text{H}]$ Acetate incorporation into chondroitin sulphate (nmol/h per mg of hexuronate)
A	1.0	8.68 ± 1.32 (20)
B	—	4.43 ± 1.23 (28)
B	1.0	10.42 ± 1.10 (20)
C	1.0	9.36 ± 2.59 (15)

equilibrate with intracellular UDP-N-acetylhexosamine very rapidly. This result is rather surprising since the turnover time of UDP-N-acetylhexosamine in neonatal rat cartilage (Handley & Phelps, 1972) is considerably longer (approx. 10min). This difference requires further study.

In all of the experiments described below, the cartilage was incubated with labelled acetate (or serine) for 2h after an initial incubation for 30min without any labelled substrate. The specific radioactivity of chondroitin sulphate, isolated from the

Fig. 2. Effect of acetate concentration and L-glutamine concentration on the incorporation of $[^{14}\text{C}]$ acetate into chondroitin sulphate

(a) Cartilage was incubated for 2h in medium A containing 1.0mM-L-glutamine and sodium $[^{14}\text{C}]$ acetate (10mCi/mmol). Results from three separate experiments are combined. Each point is a mean value of at least two determinations at each acetate concentration. The bars indicate $\pm$ s.d. calculated from a number (4-6) of values obtained at each acetate concentration. (b) Cartilage was incubated in medium B containing 1.0mM-sodium $[^3\text{H}]$ acetate (10mCi/mmol) as outlined under 'Methods'. Individual values obtained at different concentrations of added L-glutamine are shown.

entire tissue sample after incubation, was used as a measure of the rate of biosynthesis of the polysaccharide. The specific radioactivity was independent of the mass of tissue used within the range 25-125mg of dry tissue (5-25 bones) per 5.0ml of incubation medium.

The rate of incorporation of labelled acetate is dependent, however, on the incubation medium used (Table 1) and on the concentration of acetate (Fig. 2a) and of L-glutamine (Fig. 2b) added to the medium. In most of the experiments described below, the incubation medium used was a modified Eagle's medium buffered with 25mM-Hepes and saturated with O_2 as outlined under 'Methods'.

Effect of D-xylitol on chondroitin sulphate synthesis *in vitro*

The results shown in Table 2 indicate that the incorporation of labelled acetate into chondroitin sulphate *in vitro* is inhibited by added cycloheximide

Table 2. *Effect of xylose, puromycin, cycloheximide and glutamine on [³H]acetate incorporation into chondroitin sulphate*

Embryonic cartilage was incubated for 2h in medium C containing 1.0mM-sodium [³H]acetate (10mCi/mmol) and labelled chondroitin sulphate was isolated as outlined under 'Methods'. Additions to the incubation medium were made as shown in the Table before a 30min pre-incubation in the absence of [³H]acetate.

Additions to the incubation medium	10 ⁻³ × Specific radioactivity of chondroitin sulphate (c.p.m./mg of hexuronate)
None	10.3
1.0mM-Glutamine	27.1
0.35mM-Cycloheximide	1.85
0.35mM-Cycloheximide + 1.0mM-glutamine	1.83
0.35mM-Cycloheximide + 25mM-xylose	17.6
0.35mM-Cycloheximide+25mM-xylose+1.0mM-glutamine	12.0
0.10mM-Puromycin	2.72
0.10mM-Puromycin + 25mM-xylose	12.90

Table 3. *Effect of puromycin and xylose on the incorporation of [¹⁴C]serine into protein*

Embryonic cartilage was incubated for 2h in medium A containing 1.0mM-L-glutamine and 0.054mM-[¹⁴C]serine (7.42mCi/mmol) and protein was isolated as outlined under 'Methods'. The zero-time reaction, which was stopped by addition of trichloroacetic acid before the addition of [¹⁴C]serine, was carried out to detect adsorption of label on to the precipitated protein.

Additions to the incubation medium	10 ⁻³ × Specific radioactivity of isolated protein (c.p.m./mg)
None (zero time)	0.18
None	42.0
0.01 mM-Puromycin	26.1
0.02 mM-Puromycin	17.2
0.04 mM-Puromycin	11.7
0.10 mM-Puromycin	4.10
0.21 mM-Puromycin	1.55
0.10 mM-Puromycin + 25 mM-xylose	3.20
0.10 mM-Puromycin + 50 mM-xylose	3.14

and puromycin and is stimulated by added L-glutamine. The addition of 25mM-D-xylose to the incubation medium completely relieves the inhibitory action of cycloheximide and of puromycin in the absence of L-glutamine and partially abolishes the inhibition in the presence of L-glutamine. Added

Fig. 3. *Effect of β -D-xylosides on the incorporation of [³H]acetate into chondroitin sulphate*

Embryonic chicken cartilage was incubated for 2h in medium C containing 1.0mM-L-glutamine, 1.0mM-sodium [³H]acetate (10mCi/mmol) and 0.35mM-cycloheximide as outlined under 'Methods'. Methyl α -xyloside ($\bullet$), methyl β -xyloside ($\circ$), ethyl β -xyloside ($\blacksquare$), *n*-butyl β -xyloside ($\square$) and *n*-octyl β -xyloside ($\blacktriangle$) were added as shown in the Figure. The specific radioactivity of the chondroitin sulphate is expressed as a percentage of that isolated from tissue incubated without cycloheximide (3.52×10^5 d.p.m./mg of hexuronate).

D-xylose, however, has no appreciable effect on the inhibition of incorporation of [¹⁴C]serine into protein caused by puromycin (Table 3).

The effect of D-xylose is a relatively specific one, since other pentoses and a number of other monosaccharides fail to relieve the inhibition caused by cycloheximide (Table 4). A number of β -D-xylosides, however, are very active and completely abolish the inhibitory action of cycloheximide at low concentrations, even in the presence of L-glutamine. α -D-Xylosides, however, have no such action. The effect of the concentration of β -D-xyloside on the incorporation is shown in Fig. 3. It is apparent that the structure of the aglycone portion of the molecule determines to a considerable extent the amount of incorporation of acetate and also the concentration of xyloside required to produce 50% of the maximum effect.

The results in Table 4 also show that although D-galactose has no effect on the acetate incorporation in the presence of cycloheximide, even at high concentration, several β -D-galactosides can also relieve the inhibition caused by cycloheximide. The effect of β -D-galactoside concentration is shown in Fig. 4; methyl α -D-galactoside has no such action.

Table 4. *Effect of sugars and glycosides on the incorporation of [³H]acetate into chondroitin sulphate in the presence of puromycin and cycloheximide in vitro*

Embryonic cartilage was incubated for 2h in medium C containing 1.0mM-L-glutamine and 1.0mM-sodium [³H]acetate (10mCi/mmol) and chondroitin sulphate was isolated as outlined under 'Methods'. Additions to the incubation medium were made as shown in the Table before a 30min preincubation in the absence of [³H]acetate.

Expt. no.	Additions to the incubation medium (mM)			Incorporation of [³ H]acetate into chondroitin sulphate	
	Puromycin	Cycloheximide	Other	(10 ⁻³ × c.p.m./mg of hexuronate)	(% of control)
1	—	—	—	36.6	100
	0.2	—	—	9.3	25
	0.2	—	D-Xylose (20)	39.3	107
	0.2	—	D-Galactose (20)	9.1	25
	0.2	—	N-Acetylgalactosamine (20)	6.4	18
2	—	—	—	63.6	100
	0.1	—	—	1.73	2.7
	0.1	—	D-Xylose (10)	12.9	20
	0.1	—	D-Lyxose (10)	2.37	3.7
	0.1	—	L-Arabinose (10)	1.85	2.9
	0.1	—	D-Ribose (10)	2.23	3.5
	0.1	—	D-Galactose (10)	2.06	3.2
	0.1	—	Lactose (10)	2.20	3.5
	0.1	—	N-Acetylchondrosine (10)	1.97	3.1
	0.1	—	p-Nitrophenyl β-glucuronide (10)	1.74	2.7
3	—	—	—	47.5	100
	—	0.35	—	2.53	5.3
	—	0.35	D-Xylose (20)	11.7	25
	—	0.35	Methyl β-D-xyloside (1.0)	46.5	98
	—	0.35	Methyl α-D-xyloside (1.0)	2.47	5.2
4	—	—	—	86.0	100
	—	0.35	—	4.65	5.4
	—	0.35	Ethyl β-D-xyloside (1.0)	112	130
	—	0.35	Butyl β-D-xyloside (1.0)	239	278
	—	0.35	Octyl β-D-xyloside (1.0)	263	305
	—	0.35	Benzyl β-D-xyloside (1.0)	260	302
	—	0.35	Phenyl β-D-xyloside (1.0)	181	210
	—	0.35	Phenyl β-D-thioxyloside (1.0)	146	170
	—	0.35	Benzyl α-D-xyloside (1.0)	5.25	6.1
5	—	—	—	67.0	100
	—	0.35	—	1.61	2.4
	—	0.35	Methyl α-D-galactoside (20)	1.67	2.5
	—	0.35	Phenyl galactoside (20)	74.5	111
	—	0.35	Phenethyl β-D-galactoside (20)	59.6	89
	—	0.35	p-Nitrophenyl β-D-galactoside (20)	34.8	52
	—	0.35	Methyl β-D-thiogalactoside (10)	1.54	2.3
	—	0.35	p-Nitrophenyl-N-acetyl β-D-galactosaminide (10)	1.41	2.1
	—	0.35	p-Nitrophenyl-N-acetyl β-D-glucosaminide (10)	0.5	0.7
	—	0.35	p-Nitrophenyl β-D-glucuronide (10)	1.81	2.7
	—	0.35	Methyl β-D-xyloside (10)	76.5	114

Structure of chondroitin sulphate formed in the presence of puromycin and xylose

The labelled material formed by embryonic chicken cartilage incubated with [¹⁴C]acetate in the presence of D-xylose (or β-D-xyloside) and cycloheximide has been identified as chondroitin sulphate by ion-

exchange chromatography on ECTEOLA-cellulose and by digestion with chondroitin sulphate ABC-lyase from *Proteus vulgaris* (Robinson & Hopwood, 1973). After digestion with this enzyme, more than 95% of the radioactivity was recovered and characterized as labelled disaccharide. The distri-

bution of radioactivity in the three disaccharides produced (10% in 3-O- β -gluc-4-enuronosyl *N*-acetylgalactosamine, 59% in the corresponding 4-O-sulphate ester and 31% in the 6-O-sulphate ester) was determined by paper chromatography as described by Robinson & Dorfman (1969). The distribution was not markedly different from that obtained with chondroitin sulphate isolated from a control incubation (20, 49 and 31% respectively). This result shows that the degree of sulphation of the chondroitin sulphate and the ratio of chondroitin 4-sulphate to chondroitin 6-sulphate formed in the presence of β -D-xyloside and cycloheximide is not very different from the values found for the normal glycosaminoglycan of the tissue (Robinson, 1969).

After incubation of embryonic chicken cartilage with [14 C]acetate, labelled proteoglycan was isolated by extraction with 5.0M-LiCl as outlined under 'Methods'. The extracted polysaccharide was separated from [14 C]acetate by gel chromatography on Bio-Gel P4 and was then subjected to gel filtration on a column of Sephadex G-200 equilibrated with 5.0M-LiCl in imidazole-HCl buffer, pH 7.2. The elution profile of this column is shown in Fig. 5(a). All of the hexuronate-positive material and all of the 14 C label was eluted immediately after the void volume of the column. The elution profile of the same fraction isolated from cartilage incubated with [14 C]acetate in the presence of 0.18mM-puromycin and 50mM-D-xylose is shown in Fig. 5(b). The hexuronate-positive material was eluted at the same position as the peak in Fig. 5(a), but most of the radioactivity was retarded by the gel and was eluted as a separate broad peak. This result indicates that the chondroitin sulphate formed in the presence of puromycin and D-xylose differs considerably in molecular size from the proteoglycan formed in the control incubation. The elution profile shown in

Fig. 5(c) is that of the corresponding fraction from cartilage incubated with 50mM-D-xylose in the absence of puromycin. The same retarded peak of radioactivity is observed, but in this incubation, a considerable incorporation of radioactivity into the main proteoglycan peak has occurred. Essentially similar results have been obtained with cycloheximide plus 1.0mM-methyl β -D-xyloside.

The size of the chondroitin sulphate chains formed during incubation *in vitro* was determined after extraction from the tissue with 0.5M-KOH containing 0.02M-NaBH₄. This procedure is known to cause specific breakdown of proteoglycan and to release single polysaccharide chains free of any attached

Fig. 4. Effect of β -galactosides on the incorporation of [3 H]acetate into chondroitin sulphate

Experimental conditions were as shown in Fig. 3: ●, methyl α -galactopyranoside; ■, phenethyl β -galactopyranoside.

Fig. 5. Gel filtration of labelled polysaccharide

Cartilage was incubated for 4h in medium B with 1.18mM-sodium [3 H]acetate (34.4mCi/mmol). Polysaccharide was extracted with 5.0M-LiCl and was separated from [3 H]acetate and applied to a column (37cm $\times$ 2.8cm diam.) of Sephadex G-200 as outlined under 'Methods'. Each fraction (6.0ml) was assayed for radioactivity (○) and hexuronate content (●). (a) Cartilage incubated with no additions, (b) cartilage incubated with 0.1mM-puromycin and 50mM-D-xylose, (c) cartilage incubated with 50mM-D-xylose.

Fig. 6. Elution profiles from Sephadex G-200 of labelled chondroitin sulphate extracted from embryonic chicken cartilage with 0.1M-NaOH

Cartilage was incubated for 2 h in medium C with 1.0mM-sodium [^3H]acetate (10mCi/mmol). Labelled chondroitin sulphate, isolated as outlined under 'Methods', was applied to a column (90cm $\times$ 2.2cm diam.) of Sephadex G-200 equilibrated with imidazole-HCl buffer, pH7.2 (I 0.05 mol/l) containing 0.005M-NaF and 0.1% sodium dodecyl sulphate. Elution profiles for radioactively labelled chondroitin sulphate formed in the presence of added 0.35mM-cycloheximide and 1.0mM-methyl β -D-xyloside (□), 0.35mM-cycloheximide and 1.0mM-*n*-octyl β -D-xyloside (Δ) and that formed in the absence of cycloheximide and β -xyloside (○) are shown in the Figure. The elution profile of hexuronate (●) is also shown and was essentially the same in each experiment. The arrows indicate the void volume and the effective total volume of the column.

peptide (Robinson & Hopwood, 1973). The chondroitin sulphate was isolated by precipitation with cetylpyridinium chloride and applied to a column of Sephadex G-200 as outlined under 'Methods'. This column had previously been calibrated with chondroitin sulphate of known molecular weight (Hopwood & Robinson, 1973), so that a relationship between the molecular weight of chondroitin sulphate and the elution volume was known. The elution profile of hexuronate and ^3H -labelled chondroitin sulphate formed in the presence of 0.35mM-cycloheximide and 1.0mM-methyl β -D-xyloside is shown in Fig. 6. Table 5 shows the molecular weights calculated for chondroitin sulphate corresponding to the position of the peak of the radioactivity profile obtained with some of the β -D-xylosides and with *p*-nitrophenyl β -D-galactoside. It is clear that the molecular size of the chondroitin sulphate chains formed in the presence of these compounds is smaller than that of the chondroitin sulphate chains already formed in the tissue or of those synthesized in the absence of added β -D-xyloside and cycloheximide. The molecular-weight distribution of the chains formed in the

presence of these compounds also seems to be somewhat broader than that of the control incubation. Nevertheless these results clearly indicate that chondroitin sulphate chains of large size are formed under conditions where protein synthesis has been almost completely inhibited.

Discussion

Sodium [^3H]acetate and sodium [^{14}C]acetate are convenient substrates for measuring the rate of chondroitin sulphate biosynthesis in intact cartilage preparations *in vitro*. The labelled product has been identified as chondroitin sulphate by digestion with chondroitin sulphate ABC-lyase (Robinson & Dorfman, 1969); the acetate is incorporated almost exclusively into *N*-acetyl residues in polysaccharide (Schiller *et al.*, 1955). Acetate incorporation is linear with time for 5–6 h (Fig. 1), and is dependent on the concentration of labelled acetate and on the incubation medium used (Fig. 2a, Table 1).

L-Glutamine stimulates the incorporation of [^3H]acetate (Fig. 2b) and of [^{35}S]sulphate and [^{14}C]glucose (Rodén, 1956). L-Glutamine is required for the formation of glucosamine 6-phosphate (Ghosh *et al.*, 1960) so the stimulation probably reflects increased flux through the pathway to UDP-*N*-acetylhexosamine and chondroitin sulphate. However, stimulation could also arise from an increase in the specific radioactivity of intracellular UDP-*N*-acetylglucosamine. The latter possibility seems unlikely since the incorporation of [^{35}S]sulphate, which involves a quite different set of reactions, is also stimulated by L-glutamine (Rodén, 1956).

Chondroitin sulphate biosynthesis occurs by the addition of alternating residues of *N*-acetylglucosamine and glucuronic acid from the corresponding UDP-sugars on to the non-reducing end of the polysaccharide chain (Telser *et al.*, 1966; Rodén, 1970). The process of polysaccharide-chain synthesis is initiated by transfer of xylose from UDP-xylose to serine in protein (Stoolmiller *et al.*, 1972; Baker *et al.*, 1972). Puromycin and cycloheximide which inhibit chondroitin sulphate biosynthesis in intact cartilage (Telser *et al.*, 1965; Cole & Lowther, 1969) are thought to act by inhibiting synthesis of protein which acts as an acceptor for xylose. This inhibition is abolished by the addition of D-xylose, β -D-xylosides or β -D-galactosides (Table 4). D-Xylose, added in an amount sufficient to relieve the inhibition of polysaccharide synthesis, has no effect on the inhibition of [^{14}C]serine incorporation into protein; a direct effect of xylose on protein synthesis is therefore unlikely, and a specific effect of xylose on the synthesis of core protein seems improbable. Levitt & Dorfman (1973) have shown that D-xylose relieves the inhibition of glycosaminoglycan synthesis caused by 5-bromodeoxyuridine, which suggests that D-

Table 5. *Effect of β -D-xylosides and β -D-galactosides on chondroitin sulphate chain size*

Embryonic cartilage was incubated as shown in Table 4 together with 0.35mM-cycloheximide and the other compounds shown below. The chondroitin sulphate from each incubation was isolated, subjected to gel filtration on Sephadex G-200 and a molecular-weight value for chondroitin sulphate corresponding to the radioactive peak was determined as described under 'Methods'. The control contained no added cycloheximide.

Chondroitin sulphate initiator	Molecular weight at peak maximum
Control	36500
Xylose (25mM)	32000
Methyl β -D-xyloside (1.0mM)	23000
Ethyl β -D-xyloside (1.0mM)	19000
Butyl β -D-xyloside (1.0mM)	18000
Octyl β -D-xyloside (1.0mM)	17000
p-Nitrophenyl β -D-galactoside (10mM)	35000

xylose does not specifically modify the action of puromycin or of cycloheximide.

D-Xylose, methyl β -D-xyloside and certain β -D-galactosides function as acceptors for galactose transfer from UDP-galactose in cell-free systems (Helting & Rodén, 1969a,b). The results presented here indicate that such compounds also function as initiators of chondroitin sulphate-chain synthesis in intact tissue as shown by Okayama & Lowther (1973) with 4-methylumbelliferyl β -D-xyloside.

Labelled polysaccharide formed in the presence of D-xylose and puromycin was eluted from Sephadex G-200 (Fig. 5) at a position quite separate from that of proteoglycan formed in a control incubation. This result is consistent with the suggestion of Okayama *et al.* (1973) that chondroitin sulphate formed in the presence of these compounds occurs not as proteoglycan but as free polysaccharide chains.

D-Xylose acts as an initiator of chondroitin sulphate synthesis only to a limited extent and at relatively high concentration. In the presence of L-glutamine and cycloheximide, β -D-xylosides such as benzyl β -D-xyloside, produce a marked stimulation of incorporation, above that of a control (Table 4), but D-xylose does not (Table 2). The aglycone moiety of the β -D-xyloside influences the degree of stimulation observed and the concentration of xyloside necessary to produce a maximum effect (Fig. 3); non-polar aglycone residues such as *n*-butyl, *n*-octyl, phenyl and benzyl groups clearly are best. This effect might result from differences in the rate of transport of xylosides through cell membranes or from differences in the affinity of the xylosides with the enzymes involved in the initiation of chondroitin sulphate chains. It is noteworthy that these enzymes

are located in subcellular fractions derived from the endoplasmic reticulum and the golgi apparatus and are probably closely associated with sub-cellular membranes (Rodén, 1970). Phenyl β -D-thioxyloside is almost as active as phenyl β -D-xyloside, which suggests that the enzymes do not distinguish sulphur from oxygen in the glycosidic linkage.

Although chondroitin sulphate formed in the presence of cycloheximide and β -D-xyloside is smaller than that synthesized in a control incubation, it is clear (Table 5) that quite large polysaccharide chains are made. There is no evidence for an accumulation of short-chain polysaccharide. Richmond *et al.* (1973) have shown that chondroitin sulphate primers are rapidly elongated to chains of molecular weight 25000–35000 with little or no formation of intermediate-size material. Chain elongation therefore appears to be a highly integrated process. The use of β -D-xyloside enables chain elongation to be studied in intact tissue as an event separate from the synthesis of protein and proteoglycan. The marked stimulation of acetate incorporation suggests that the synthesis of protein may be a rate-controlling step in proteoglycan biosynthesis.

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