Control of chondroitin sulphate biosynthesis

β -D-Xylopyranosides as substrates for UDP-galactose: D-xylose transferase from embryonic-chicken cartilage

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Embryonic-chicken epiphyseal cartilage was incubated *in vitro* with a variety of β -xylosides and the amount of [3 H]acetate incorporation into chondroitin sulphate was determined under conditions when normal protein core production was inhibited by cycloheximide. The ability of the different β -xylosides to relieve the cycloheximide-mediated inhibition of chondroitin sulphate synthesis was influenced by the nature of the aglycan group of the xyloside. β -Xylosides with apolar and uncharged aglycan groups were most effective and produced a severalfold stimulation of chondroitin sulphate biosynthesis. β -Xylosides with charged aglycan groups were less effective initiators of chondroitin sulphate synthesis. The rate of galactose transfer from UDP-galactose to each of the β -xylosides, catalysed by a cell-free microsomal preparation from embryonic cartilage, was measured. This study showed that the nature of the aglycan group of the β -xyloside was a factor determining the capacity of the xyloside to act as an acceptor for galactosyltransferase I, the enzyme that catalyses the first galactose transfer reaction of chondroitin sulphate synthesis. The aglycan group of the xyloside also appeared to influence other steps leading to chondroitin sulphate chain initiation *in vitro*.

D-Xylose and β -D-xylosides function as chain initiators of chondroitin sulphate synthesis in intact chondrocytes (Brett & Robinson, 1971; Okayama *et al.*, 1973; Galligani *et al.*, 1975). The usual initiator in this pathway is thought to be the polypeptide, which constitutes the protein core of cartilage proteoglycan and is substituted with xylose at specific serine residues in the course of proteoglycan biosynthesis. The use of fluorescent 4-methylumbelliferyl β -D-xyloside (Fukunaga *et al.*, 1975) and of [3 H]benzyl β -D-xyloside (Sandy *et al.*, 1980) has demonstrated incorporation of the aglycan groups into the reducing end of newly synthesized chondroitin sulphate, which is released as a free polysaccharide chain not attached to protein. β -Xylosides have been used to determine a tissue's capacity to synthesize chondroitin sulphate under conditions when normal proteoglycan production is limited (Okayama *et al.*, 1973; Levitt & Dorfman, 1973; Stearns & Goetinck, 1979; Sandy *et al.*, 1980).

Robinson *et al.* (1975) found that the nature of the aglycan group of the β -D-xylosides influenced the degree of stimulation of chondroitin sulphate synthesis and the concentration of β -xyloside necessary to produce the maximum effect. β -D-Xylosides with hydrophobic aglycan groups were most effective.

The influence of the aglycan group on chondroitin sulphate biosynthesis could arise in the following ways. (a) The aglycan group could determine the rate of transport of the β -xyloside to the site of chondroitin sulphate biosynthesis. (b) The aglycan group could determine the final concentration of the β -xyloside at the site of synthesis by affecting its concentration within intracellular compartments or its rate of breakdown by intracellular glycosidases. (c) The aglycan group could determine the affinity of the β -xyloside for one of more of the several glycosyltransferase enzymes that constitute the pathway of polysaccharide chain synthesis.

The present study was undertaken to investigate whether the effectiveness of β -xylosides as substrates for galactosyltransferase I, the first enzyme to act on β -xylosides in the pathway of chondroitin sulphate chain initiation (Helting & Rodén, 1969), was determined by the aglycan moiety of the β -D-xylosides.

A preliminary communication of these results has been published (Robinson & Robinson, 1979).

Experimental

Materials

Embryonic chickens were obtained from incu-

bated fertile eggs and their femurs and tibias were dissected as described previously (Robinson *et al.*, 1975). Eagle's basal medium 10X concentrate was purchased from the Commonwealth Serum Laboratories (Melbourne, Australia).

β -Galactosidase from *Escherichia coli* was purchased from Boehringer-Mannheim G.m.b.H. (Mannheim, Germany). 4-Methylumbelliferyl β -D-xyloside, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Mes (4-morpholine-ethanesulphonic acid), thiosalicylic acid, 2-mercaptopyridine, D-xylose, NaBH₄, methyl β -D-xyloside, UDP-galactose, Nonidet P40 detergent and Dowex 50 (X8; H⁺ form; 100–200 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex gel-filtration materials were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Bio-Gel materials from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were of analytical grade.

Sodium [³H]acetate, UDP-[U-¹⁴C]galactose and UDP-[6-³H]galactose were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). [³H]Benzyl β -D-xyloside was prepared from 1.0 g of the unlabelled compound by catalytic exchange in solution with tritium gas carried out by The Radiochemical Centre. A sample of the labelled product (sp. radioactivity >1.2 Ci/mmol) was mixed with unlabelled benzyl β -D-xyloside and recrystallized to constant specific radioactivity (125 Ci/mol). Acid hydrolysis of the labelled product followed by paper chromatography demonstrated that <0.2% of the label resided in the xylose moiety and >70% could be recovered in benzyl alcohol isolated as its crystalline 3,5-dinitrobenzoate.

Synthesis of β -xylosides

The synthesis of ethyl β -D-xyloside, benzyl β -D-xyloside and phenyl β -D-thioxyloside has been described previously (Robinson *et al.*, 1975). Tri-*O*-acetyl- α -D-xylopyranosyl chloride was also condensed with a variety of thiol compounds by the method described for the preparation of phenyl β -D-thioxyloside. In this way, β -D-thioxylosides were prepared from the following compounds: thioglycolic acid, thiosalicylic acid, 2-mercaptopyridine and 2-mercaptoethylamine. Thiosalicylic acid β -D-xyloside (2.86 g) was dissolved in 30 ml of anhydrous methanol and mixed with excess diazomethane (freshly distilled from a mixture of 6.0 g of *p*-tolylsulphonylmethylnitrosamide, 1.0 g of KOH, 75 ml of diethyl ether and 10 ml of 95% ethanol) in 60 ml of diethyl ether. The mixture was left at room temperature for 1 h and then acidified with 0.5 ml of acetic acid. White crystals of the methyl ester of thiosalicylic acid β -D-thioxyloside (2.5 g) were obtained after storage at -20°C overnight.

Imidazole *N*- β -D-xylopyranoside was prepared

from tri-*O*-acetylxylosyl chloride and chloromercuri-imidazole by the method of Bauer (1962). Each of these β -xylosides was isolated as a crystalline solid and behaved as a single compound when analysed by paper chromatography in solvents A, B, C and D. Each of the compounds had the calculated composition when assayed for xylose content and when elemental analysis was carried out.

Methods

Chondroitin sulphate synthesis in intact cartilage. Embryonic-chicken bones (10–15/flask) were incubated with [³H]acetate in 5.0 ml of Eagle's basal medium supplemented with 1.0 mM-L-glutamine and 25 mM-Hepes/NaOH buffer, pH 7.4, as described by Robinson *et al.* (1975). Appropriate volumes (0.01–0.25 ml) of xyloside solutions were added to the flasks to give the desired final concentrations, together with 0.02 ml of 50 mM-cycloheximide. The flasks were pre-incubated for 20 min before addition of [³H]acetate. After a further 2 h incubation, the reactions were stopped by chilling each flask in ice and the addition of 1.0 ml of 0.15 M-NaBH₄ in 3.0 M-KOH. The flasks were kept at 4°C for 2 h and then excess borohydride was decomposed by the addition of 0.05 ml of acetone to each flask. After 30 min at room temperature, the incubation mixtures were neutralized with acetic acid, and labelled chondroitin sulphate was isolated by precipitation with cetylpyridinium chloride from a papain digest of the entire flask contents as described previously (Robinson *et al.*, 1975). The potassium salt of the chondroitin sulphate was finally prepared by dissolving the precipitate, obtained with cetylpyridinium chloride, in 1.0 ml of 4.0 M-potassium acetate and adding 5.0 ml of ethanol. The dry precipitate was dissolved in 5.0 ml of water and assayed for radioactivity and hexuronate content.

Preparation of the microsomal enzyme. Femurs and tibias were dissected from 50–60 chick embryos (14 days old), cleaned of soft tissue and homogenized in freshly prepared ice-cold 'enzyme' buffer that contained 62.5 mM-Mes, 0.5 M-KCl, 15 mM-MgCl₂, 1.25 mM-EDTA and was adjusted to pH 5.5 with NaOH. In some experiments a pH of 6.0 was used. The initial homogenization of the bones was carried out with a motor-driven loose-fitting glass homogenizer and the suspension obtained from this procedure was then homogenized again by hand with a tight-fitting Tenbroeck tissue grinder (Kontes Glass Co., Vineland, NJ, U.S.A.). The homogenate was then 'sonicated' (25 × 1 s pulses at medium intensity) and then centrifuged at 10 000 rev./min for 15 min (MSE 18; 8 × 50 ml rotor). The pellet was discarded and the supernatant was centrifuged at 40 000 rev./min for 1 h (MSE Superspeed 75; 10 × 10 ml rotor). Resuspension of the microsomal pellet in 'enzyme' buffer, pH 5.5, at a concentration of

about 1.0 mg of protein/ml, provided the particulate enzyme preparation used in the initial experiments.

In some experiments the enzyme was solubilized by a procedure based on the method described by Schwartz & Rodén (1975). The pellet fraction obtained after centrifugation at 40000 rev./min was homogenized in a fine-glass homogenizer in a small volume of 'enzyme' buffer at pH 6.0, to which 0.5% (w/v) Nonidet P40 detergent had been added. The homogenate was then frozen and thawed three times, and finally centrifuged at 40000 rev./min. The clear supernatant fraction, which contained approx. 80% of the enzymic activity, was used as 'solubilized' enzyme.

Assay of galactosyltransferase activity. The microsomal enzyme preparation (10–50 μ g of protein) was routinely incubated with 1.25 mM-UDP-[3 H]galactose (sp. radioactivity 1.98 Ci/mol) or UDP-[14 C]galactose (sp. radioactivity 4.93 Ci/mol), 10 mM-MnCl₂, 12 mM-MgCl₂, 1.0 mM-EDTA, 0.4 M-KCl and added β -xylosides (0–50 mM) in 50 μ l of Mes/NaOH buffer, pH 5.5 (0.05 M with respect to Mes). In experiments with solubilized enzyme Mes/NaOH buffer, pH 6.0, was used. The effect of Nonidet P40 detergent on the particulate enzyme was determined by adding 0.4% Nonidet to the assay mixture.

Tubes were incubated at 37°C for 4 h. If the reaction products were to be isolated by paper chromatography, the reaction was stopped by the addition of 0.05 ml of propan-2-ol to each tube and by chilling the mixture rapidly in ice. The reaction mixtures were then stored at –20°C until they were applied to sheets of chromatography paper. When pyridine 2- β -thioxyloside or 2-aminoethyl β -thioxyloside was used as substrate, the reaction was stopped by addition of 0.5 ml of 6% trichloroacetic acid solution. The precipitated protein was then removed by centrifugation and the labelled products were isolated from the supernatant solution by adsorption on Dowex 50.

Isolation of the labelled products of galactose transfer. Paper chromatography. After centrifugation to remove insoluble material, the supernatant solutions were applied to Whatman no. 3MM paper and the chromatogram was developed by descending chromatography for 8 h in solvent B or for 18 h in solvent C. The positions of the radioactively labelled disaccharides were detected on the paper by exposure of the chromatogram to X-ray film for 1–2 weeks. The radioactive products were then cut out and assayed for radioactivity.

Ion-exchange chromatography. The supernatant solutions were applied to columns (2.5 cm $\times$ 0.7 cm diam.) of Dowex 50 (X8; H⁺ form; 100–200 mesh). The columns were washed with 10 $\times$ 1 ml of water and the product was then eluted with 5 $\times$ 1 ml of

2M-NH₃ solution directly into scintillation counting vials. After evaporation to dryness at 60°C overnight, the samples were dissolved in 2 ml of water and assayed for radioactivity.

The reproducibility of these two procedures for isolation of the products of the cell-free incubations was determined with pyridine β -thioxyloside as substrate. Reaction tubes containing 1, 2, 5 and 10 mM concentrations of the xyloside were set up in duplicate and the standard incubation procedure was followed. The product from one set of tubes was isolated by paper chromatography in solvent C, and the product from the duplicate set was isolated by ion-exchange chromatography on Dowex 50.

The extent of [14 C]galactose incorporation measured by the two methods agreed within 10% at each xyloside concentration.

Characterization of the product of galactose transfer to [3 H]benzyl β -D-xyloside. [3 H]Benzyl β -D-xyloside (sp. radioactivity 125 Ci/mol; 0.61 μ mol), UDP-[14 C]galactose (sp. radioactivity 4.93 Ci/mol; 0.091 μ mol) and the microsomal preparation (0.275 mg of protein) were incubated at pH 5.5 under the standard conditions of assay for 24 h at 37°C in a final volume of 0.1 ml. The reaction was stopped by addition of 0.1 ml of propan-2-ol. The labelled product was isolated by paper chromatography in solvent B. The product was detected by autoradiography, eluted from the dry paper with water, evaporated to dryness and dissolved in 5.0 ml of 0.1 M-boric acid/NaOH buffer, pH 7.0. This solution was applied to a column (87 cm $\times$ 2 cm diam.) of Dowex 1 (X4; borate form; 200–400 mesh) equilibrated with the same buffer. The column was eluted with a linear gradient formed from 250 ml of 0.1 M-boric acid/NaOH buffer, pH 7.0, and 250 ml of 0.2 M-Na₂SO₄ in 0.4 M-boric acid/NaOH buffer, pH 7.0, and elution then continued with the latter buffer. Fractions (10 ml) were collected and assayed for radioactivity. The main peak of 14 C label was pooled and desalted by filtering the solution through short columns of Dowex 50 (H⁺ form) and Dowex 1 (acetate form). It was then evaporated to dryness from methanol several times to remove residual boric acid.

The dry sample was redissolved in 0.5 ml of water and a 0.1 ml sample was removed and subjected to β -galactosidase digestion with 0.05 units (2.5 μ g) of enzyme in 0.01 M-Tris/HCl buffer, pH 7.2, for 4 h at room temperature. The digest was analysed by paper chromatography in solvent C. Another digested sample was applied to a second column of Dowex 1 (borate form) together with 10 μ mol of galactose and 10 μ mol of benzyl β -D-xyloside. The column was eluted with the same gradient procedure as described above.

Analytical methods. Hexuronate was determined by the method of Bitter & Muir (1962). Xylose

content of β -xylosides was determined by using the phenol/ H_2SO_4 method of Dubois *et al.* (1956) with D-xylose as standard.

Paper chromatography was carried out by descending development on Whatman 3MM paper in the following solvents: A, propan-2-ol/water (4:1, v/v); B, butan-1-ol/pyridine/water (10:3:3, by vol.); C, ethyl acetate/acetic acid/water (3:1:1, by vol.); D, butan-1-ol saturated with 2.0M- NH_3 solution.

Sugars and glycosides were detected on the paper chromatograms with alkaline silver nitrate reagent.

Autoradiography of paper chromatograms was carried out with Kodirex RP-54 X-ray film at exposure times of 1–2 weeks.

Results and discussion

Chondroitin sulphate biosynthesis was determined by measuring the incorporation of [^3H]-acetate into sulphated polysaccharide during a 2 h incubation of embryonic-chicken cartilage *in vitro*. The results shown in Fig. 1 and Table 1 illustrate differences between a number of β -xylosides and β -thioxylosides that were tested for their ability to relieve the inhibition of chondroitin sulphate biosynthesis caused by cycloheximide. Xylosides with non-polar aglycan groups such as benzyl β -D-xyloside were clearly more effective at relieving the inhibition of chondroitin sulphate synthesis than ethyl β -D-xyloside or methyl β -D-xyloside, and much more effective than D-xylose, which produced relatively poor restoration of chondroitin sulphate synthesis. Charged groups on the aglycan moiety were found to lower the effectiveness of the xyloside since thiosalicylic acid β -thioxyloside stimulated chondroitin sulphate biosynthesis to a much smaller extent than phenyl β -thioxyloside. Similarly, 2-aminoethyl β -thioxyloside and thioglycolic acid β -thioxyloside were less effective than ethyl β -D-xyloside. It seems likely that these differences were caused predominantly by the charge on the aglycan group, since modification of the carboxylic acid group in thiosalicylic acid β -thioxyloside by methylation with diazomethane yielded a derivative that stimulated chondroitin sulphate most effectively (Table 1). In contrast, pyridine β -thioxyloside is an effective initiator of chondroitin sulphate synthesis compared with 2-aminoethyl β -thioxyloside. This may indicate that the pyridine ring is not charged at the intracellular site of chondroitin sulphate chain initiation. It is noteworthy that imidazole β -N-xyloside was also a good initiator of chondroitin sulphate biosynthesis.

Since β -thioxylosides and β -N-xylosides appear to be almost as effective as β -O-xylosides in initiating chondroitin sulphate biosynthesis in intact cartilage, it seems unlikely that the aglycan group influences

Fig. 1. Effect of β -D-xylosides on the incorporation of [^3H]acetate into chondroitin sulphate

Embryonic-chicken cartilage was incubated for 2 h with 0.2 mM-cycloheximide and 2.0 mM-sodium [^3H]acetate (sp. radioactivity 1 Ci/mol). Benzyl β -D-xyloside (●), pyridine β -D-thioxyloside (▲), phenyl β -D-thioxyloside (○), ethyl β -D-xyloside (■) and thiosalicylic acid β -D-thioxyloside (□) were added as shown in the Figure. The specific radioactivity of chondroitin sulphate is expressed as a percentage of that isolated from tissue incubated without cycloheximide (4.5×10^4 d.p.m./mg of hexuronate).

the rate of breakdown of the xylosides by intracellular β -xylosidase. Such breakdown could limit the concentration of β -xylosides at the intracellular site of chondroitin sulphate biosynthesis, but it is unlikely that β -xylosidase would exhibit activity with thioxylosides and N-xylosides as well as O-xylosides.

β -D-Xylosides as substrates for galactosyltransferase

A cell-free preparation of galactosyltransferase was prepared from embryonic-chicken cartilage by the methods described by Schwartz & Rodén (1975). The enzyme, which is firmly associated with the microsomal fraction that sedimented at 40000 rev./min, was in some experiments 'solubilized' by treatment with 0.4% Nonidet P40 and 0.4 M-KCl to eliminate the possibility of an interaction between the aglycan groups of the β -xylosides and the intact membranes of the microsomal

Table 1. *Effect of β -D-xylosides on [^3H]acetate incorporation into chondroitin sulphate in embryonic-chicken cartilage in vitro*

Embryonic-chicken cartilage was incubated under standard conditions with [^3H]acetate, 0.2 mM-cycloheximide and the β -D-xylosides shown in the Table. In the absence of cycloheximide and β -xyloside, [^3H]acetate incorporation was equivalent to 10.2 nmol/h per mg of tissue hexuronate. Cycloheximide inhibited the incorporation by more than 90% to less than 1.0 nmol/h per mg of tissue hexuronate. The first column shows the limiting rate of incorporation obtained with the respective β -xylosides (see Fig. 1) expressed as a percentage of the rate obtained in the absence of β -xyloside and cycloheximide. For those β -xylosides, such as 2-aminoethyl *S*-thioxyloside, which did not reach a limiting value, the highest incorporation rate obtained is given together with the corresponding xyloside concentration in parentheses. The second column lists the concentration of β -xyloside required to achieve 50% of the limiting rate of incorporation.

Substrate	Effect on chondroitin sulphate biosynthesis	
	Limiting rate of incorporation of [^3H]acetate into chondroitin sulphate (% of control)	Substrate concentration required to yield 50% of the limiting rate of incorporation (mM)
D-Xylose	35	7.5
Methyl β -D-xyloside	160	0.7
Ethyl β -D-xyloside	175	0.7
Butyl β -D-xyloside	290	0.2
Benzyl β -D-xyloside	290	0.05
4-Methylumbelliferyl β -D-xyloside	154	0.02
Phenyl β -D-thioxyloside	180	0.2
Pyridine 2- β -D-thioxyloside	210	0.1
2-Aminoethyl β -D-thioxyloside	50 (10 mM)	>4.0
Thioglycollic acid β -D-thioxyloside	162 (10 mM)	>3.0
Thiosalicylic acid β -D-thioxyloside	34 (10 mM)	>4.0
Thiosalicylic acid β -D-thioxyloside (methyl ester)	265	0.1
Imidazole <i>N</i> - β -D-xyloside	232	1.5

fraction. Galactose transfer from UDP-[^{14}C]-galactose to β -xylosides was measured after separating the labelled products by paper chromatography as outlined under 'Methods'. Table 2 shows the relative R_F values observed for the several reactants and products in the solvents used. The products of [^{14}C]-galactose transfer to [^3H]benzyl xyloside and to pyridine 2- β -thioxyloside were characterized in detail.

[^3H]Benzyl β -D-xyloside was incubated with UDP-[^{14}C]galactose and the microsomal preparation for 24 h at 37°C as outlined under 'Methods'. The product was separated by preparative paper chromatography and detected by autoradiography. It was then eluted from the paper with water and purified further by ion-exchange chromatography on Dowex 1 (X4) in borate buffer. A major peak containing ^{14}C and ^3H was eluted from the column (Fig. 2). The molar ratio of galactose/benzyl xyloside in this peak was calculated from the ratio of $^{14}\text{C}/^3\text{H}$ and the specific radioactivities of the initial labelled substrates. A value of 0.95 was obtained. The radioactive product was desalted and then digested with β -galactosidase. Paper chromatography of the digest mixture and repeated ion-exchange chromatography on Dowex 1 in borate buffer, with added standards, confirmed the release of [^{14}C]galactose and [^3H]benzyl xyloside as the only detectable products of the digestion. These

Fig. 2. *Elution from Dowex 1 (X4) of the reaction product of [^{14}C]galactose transfer to [^3H]benzyl β -D-xyloside*

[^3H]Benzyl β -D-xyloside (sp. radioactivity 125 Ci/mol; 0.61 μmol) and UDP-[^{14}C]galactose (sp. radioactivity 4.93 Ci/mol; 0.091 μmol) were incubated with the microsomal enzyme preparation. Product was isolated by preparative paper chromatography and by ion-exchange chromatography on a column of Dowex 1 in borate buffer. ^3H radioactivity (—) and ^{14}C radioactivity (---) were measured.

Table 2. *Paper chromatography of β -xylosides and products of galactosyltransferase*

β -D-Xylosides and labelled products obtained by incubation of β -D-xylosides with UDP-[14 C]galactose and galactosyltransferase (see under 'Methods') were subjected to descending paper chromatography on Whatman 3MM paper in the following solvents: A, propan-2-ol/water (4:1, v/v); B, butan-1-ol/pyridine/water (10:3:3, by vol.); C, ethyl acetate/acetic acid/water (3:1:1, by vol.); D, butan-1-ol saturated with 2.0M-NH₃ solution. The R_F values listed in parentheses are those of the labelled products formed from the corresponding xylosides as a result of galactose transfer. The R_F value in square brackets under pyridine thioxyloside is that of a minor component formed at high UDP-[14 C]galactose concentration and is presumed to be the pyridine 2-thioglycoside of the trisaccharide galactosylgalactosylxylose.

Compound	R_F values			
	Solvent A	Solvent B	Solvent C	Solvent D
D-Xylose	0.45	0.23	0.21 (0.05)	0.135
Methyl β -D-xyloside	0.63	0.50	0.41	0.30
Ethyl β -D-xyloside	0.70	—	0.53 (0.14)	—
Benzyl β -D-xyloside	0.81	0.72 (0.26)	0.75 (0.23)	0.67
Lactose	0.14	0.03	0.044	0.013
Phenyl β -D-thioxyloside	0.80	0.75	0.78 (0.29)	0.70
Pyridine 2- β -D-thioxyloside	0.69 (0.45) [0.23]	0.58 (0.23)	0.58 (0.19)	0.51 (0.12)
Thiosalicylic acid β -D-thioxyloside	0.21	0.09	0.59 (0.19)	0.11
Thiosalicylic acid β -D-thioxyloside (methyl ester)	0.79	—	0.76 (0.22)	0.63
2-Aminoethyl β -D-thioxyloside	0.27	—	0.10	—
Imidazole <i>N</i> - β -D-xyloside	0.56	—	0.26	0.79

results clearly demonstrated the formation of a disaccharide glycoside composed of galactose bonded by a β -linkage to benzyl xyloside, presumably by a β 1 $\rightarrow$ 4 linkage (Helting & Rodén, 1969).

Essentially similar results were obtained with pyridine 2- β -thioxyloside. The labelled product was isolated by paper chromatography in solvent A and produced a radioactive spot that coincided exactly with a spot of u.v. absorption. Treatment with β -galactosidase liberated free [14 C]galactose and an unlabelled u.v.-absorbing component that moved to the same position as pyridine 2- β -thioxyloside on paper chromatography in solvent A.

Different β -D-xylosides were tested as substrates for galactosyltransferase activity in a cell-free preparation from chicken cartilage. Initial experiments performed with pyridine 2- β -thioxyloside and benzyl β -xyloside established that the transfer of [14 C]galactose was directly proportional to incubation time for 8–10 h and to the amount of enzyme added within the range 40–1000 μ g/ml (final concentration). Addition of Nonidet P40 detergent caused a 4–5-fold increase in the galactosyltransferase activity ($V_{\max}$) with benzyl β -D-xyloside and with pyridine 2- β -D-thioxyloside as substrate. The effect of Nonidet P40 on the apparent K_m value for

these substrates was less marked; in one experiment with benzyl β -D-xyloside it produced an increase from 5.3 mm to 14 mm and with pyridine 2- β -D-thioxyloside a change from 13 mm to 20 mm. Addition of KCl, which facilitates solubilization of the galactosyltransferase (Schwartz & Rodén, 1975), had no detectable effect on activity when added in concentrations ranging from 0.03 M to 1.0 M.

The effect of UDP-galactose concentration was consistent with the findings of Helting & Rodén (1969). A final UDP-galactose concentration of 1.25 mM was selected for routine experiments. The pH optimum was 5.6, but the activity did not change markedly over the entire pH range of 5.5–7.0. In subsequent experiments standard incubation conditions were used at pH 5.5 or at pH 6.0.

The effect of the different β -xyloside aglycan groups was studied in two ways. In the first group of experiments, each of the β -xylosides studied was incubated at pH 5.5 with UDP-[14 C]galactose and the microsomal fraction in the absence of Nonidet P40. The products were separated by paper chromatography in solvents B and C and the locations of the radioactive disaccharide glycosides on the paper were detected by autoradiography. The products were then cut out and assayed for radioactivity.

The effect of β -D-xyloside concentration on the galactosyltransferase activity in a single microsomal preparation was analysed by double reciprocal plots and the following results were obtained. D-Xylose gave an apparent K_m value of 22 mM and a V_{max} of 2.9 nmol of galactose transferred/h per mg of protein. The corresponding values for other β -xylosides were: methyl β -D-xyloside, 42 mM and 3.9 nmol/h per mg; ethyl β -D-xyloside, 13 mM and 3.6 nmol/h per mg; benzyl β -D-xyloside, 6.5 mM and 4.3 nmol/h per mg; phenyl β -D-thioxyloside, 5.1 mM and 5.2 nmol/h per mg; thiosalicylic acid β -D-thioxyloside, 9.5 mM and 5.25 nmol/h per mg; the methyl ester of thiosalicylic acid β -D-thioxyloside, 10.3 mM and 5.2 nmol/h per mg.

The second group of experiments was carried out with pyridine 2- β -thioxyloside and UDP-[3 H]galactose as substrates for the solubilized enzyme assayed in the presence of 0.4% Nonidet P40. The effect of added D-xylose and uncharged β -xylosides as competitive inhibitors of galactose transfer to pyridine 2- β -thioxyloside was investigated. The labelled disaccharide thioglycoside of 2-mercaptopyridine could be separated readily from the uncharged products. The kinetic data obtained in these experiments were analysed by double-reciprocal plots and the results were consistent with linear competitive inhibition (Fig. 3). Values of K_i were determined for each of the competitive β -D-xyloside substrates after replotting the slopes from the double-reciprocal plots against inhibitor concentration (Cleland, 1963).

This method produced the following apparent K_i (slope) values: D-xylose, >100 mM; methyl β -D-xyloside, 30 mM; benzyl β -D-xyloside, 10.5 mM; 4-methylumbelliferyl β -D-xyloside, >30 mM; phenyl β -D-thioxyloside, 8.9 mM; thiosalicylic acid β -D-thioxyloside, 8.2 mM; methyl ester of thiosalicylic acid β -D-thioxyloside, 8.9 mM; methyl α -D-xyloside, >100 mM; phenyl β -D-galactoside, >100 mM. The apparent K_m values measured for pyridine 2- β -D-thioxyloside and imidazole *N*- β -D-xyloside with the solubilized enzyme in the presence of Nonidet P40 were 9.3 mM and 44 mM respectively.

Although the aglycan group has some effect on galactosyltransferase activity as evidenced by the different V_{max} values, K_m values and K_i values listed above, the marked effect of charged and non-polar substituents observed with intact cartilage (Table 1) is not apparent. The concentration of β -xyloside necessary to elicit half maximum activity is much greater with the cell-free preparations than with intact cartilage. These results suggest that affinity for the active site of galactosyltransferase is not a major factor in determining the relative effectiveness of β -xylosides in initiating chondroitin sulphate chain synthesis in chondrocytes.

The results also show that methyl α -D-xyloside and phenyl β -D-galactoside do not inhibit transfer of

Fig. 3. Effect of β -D-xyloside concentration on galactose transfer to pyridine 2- β -D-thioxyloside

Galactosyltransferase from embryonic-chicken cartilage was incubated with UDP-[3 H]galactose and pyridine 2- β -D-thioxyloside. The effect of pyridine 2- β -D-thioxyloside concentration is shown in the absence (●) and presence (O) of 10 mM-thiosalicylic acid β -D-thioxyloside. The points shown for pyridine 2- β -D-thioxyloside alone are mean values and the vertical bars indicate $\pm$ S.D. calculated from 10 separate estimations. The maximum velocity of the enzyme determined from this plot was 48 nmol/h per mg of protein and the K_m value for pyridine 2- β -D-thioxyloside was 9.26 mM. The values obtained in the presence of 10 mM-thiosalicylic acid β -D-thioxyloside were 55 nmol/h per mg of protein and 23.8 mM respectively.

galactose to pyridine 2- β -thioxyloside by the chicken cartilage galactosyltransferase preparation. Methyl α -D-xyloside is not a substrate for galactose transfer and does not stimulate chondroitin sulphate biosynthesis in intact cartilage (Robinson *et al.*, 1975). Phenyl β -D-galactoside is a substrate for galactose transfer catalysed by the chicken cartilage preparation (Robinson & Robinson, 1980) and does relieve the inhibition of chondroitin sulphate biosynthesis caused by cycloheximide in intact cartilage (Robinson *et al.*, 1975), although only at high concentrations. Because phenyl β -D-galactoside does not compete with pyridine 2- β -thioxyloside in the cell-free assay, it seems probable that the galactoside initiates chondroitin sulphate chain synthesis at a completely different glycosyltransferase reaction, possibly by serving as a substrate for galactosyltransferase II or for glucuronosyltransferase I (Rodén *et al.*, 1972; Brandt *et al.*, 1975).

If differences between the β -D-xylosides in initiating chondroitin sulphate chain synthesis cannot be

explained by their different affinities for the active site of galactosyltransferase I, it remains likely that the aglycan group of the xyloside influences other events such as: (a) transport of the xyloside to the site of chondroitin sulphate biosynthesis, (b) the effective concentration of the xyloside at the intracellular site of the glycosyltransferases or (c) the rate with which the oligosaccharide products arising from glycosyltransferase activity diffuse away from the site of chondroitin sulphate synthesis.

The synthesis of an entire chondroitin sulphate chain, initiated by β -xyloside, appears to require at least five glycosyltransferases and two sulphotransferase enzymes (Rodén & Schwartz, 1975). It seems possible therefore that the aglycan residue of the β -xyloside could exert its effect by altering the affinity of the nascent chondroitin sulphate chain for any one of these enzymes. Since the size and the extent of sulphation of chondroitin sulphate chains in a variety of tissues or cells and initiated by a number of different β -xylosides (Galligani *et al.*, 1975; Robinson *et al.*, 1975; Lohmander *et al.*, 1979) are not markedly different from those of chains on the normal proteoglycan core, the later stages of polysaccharide chain extension and sulphation are not profoundly influenced by the aglycan grouping.

Separate experiments have been unable to detect any low-molecular-weight fragments such as galactosyl xyloside or galactosylgalactosyl xyloside, which might arise if β -D-xyloside was a poor substrate for the second galactosyltransferase or the first glucuronosyltransferase respectively. The absence of such fragments suggests that it is the first galactosyltransferase (galactosyltransferase I) or an earlier event that is the critical step in xyloside-initiated chondroitin sulphate biosynthesis.

The high K_m values (5–40 mM) found for the β -D-xylosides with the cartilage galactosyltransferase suggests that this enzyme may function in the chondrocyte well below its maximum capacity. This notion is supported by the considerable stimulation of chondroitin sulphate chain synthesis produced by added β -xyloside in the presence or absence of cycloheximide. It is possible to calculate from the measured rate of chondroitin sulphate biosynthesis in embryonic-chicken cartilage *in vitro* (Robinson, 1969; Robinson *et al.*, 1975) a rate of galactose transfer to xylosyl-core protein of about 20 nmol/h per g dry wt. of tissue. The measured galactosyltransferase activity with 20 mM-pyridine 2- β -thioxyloside as substrate, in cell-free homogenates of the same tissue was equivalent to 2.0 μ mol/h per g dry wt. of tissue, a value at least 100-fold greater.

These considerations all support the notion that the galactosyltransferase I activity in chondrocytes is not a point of regulation of chondroitin sulphate biosynthesis. Nevertheless, the possibility of allo-

steric or other forms of regulation cannot be excluded. Indeed, galactosyltransferase I is closely associated with the membranes of the endoplasmic reticulum of the chondrocytes and Nonidet P40 is known to prevent a specific interaction between xylosyltransferase and galactosyltransferase I (Rodén & Schwartz, 1975). This type of interaction together with protein-lipid interactions may play a part in the regulation of chondroitin sulphate biosynthesis *in vivo*.

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