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β-D-Xylosides and their analogues as artificial initiators of glycosaminoglycan chain synthesis

Aglycone-related variation in their effectiveness *in vitro* and *in ovo*

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A series of aryl and alkyl *O*-β-D-xylosides and their analogues with S, NH or CH_2 in the glycosidic linkage were prepared and examined for their ability to act as artificial chain initiators of chondroitin (dermatan) sulphate synthesis in embryonic chick cartilage, foetal rat skin and 6-week-old-rat aorta under conditions where normal protein-core synthesis was inhibited by cycloheximide. For all these tissues in culture, phenyl *O*-β-D-xyloside and phenyl β-D-thioxyloside were clearly more effective than the corresponding *N*-xyloside and homo-*C*-xyloside. Introduction of a carboxy group to the *para* position of their aglycone yielded derivatives with far lower initiator activity. In a concentration range lower than 0.1 mM, the effectiveness of alkyl β-D-thioxyloside was greatly influenced by the carbon number (*n*) of the alkyl group and was at a maximum at $n = 7$ or 8 for the cartilage, at $n = 5$ for the skin and at $n = 4$ for the aorta. In the β-xyloside-treated cartilages, the average length of newly formed chondroitin sulphate chains reflected the chain-initiator activity of added xyloside, i.e. the higher the initiator activity, the shorter the average chain length. In the skin and aorta, none of the drugs could relieve the inhibition of heparan sulphate synthesis caused by cycloheximide. Fertilized hens' eggs were each injected on day 9 with 9.2 μmol of β-xyloside and the skeletal systems of embryos were examined a week later. The embryos treated with β-xylosides of relatively high initiator activity showed a 30–40% decrease in the overall growth rate of skeletons, whereas those treated with β-xylosides of low initiator activity showed little or no decrease in the growth rate. The results are consistent with the notion that the observed change in skeletal morphology results mainly, if not completely, from β-xyloside-induced synthesis of core-protein-free chondroitin sulphate, and further suggest that a procedure employing a series of β-xyloside homologues with various initiator activities will furnish an easily applied criterion on which to test the specificity of xyloside action on biological processes.

INTRODUCTION

Since the demonstration of the ability of *p*-nitrophenyl β-D-xyloside to act as an artificial chain initiator of chondroitin sulphate biosynthesis (Okayama *et al.*, 1971, 1973), this and other related compounds have become a popular tool for studying the role of chondroitin (dermatan) sulphate proteoglycans in a wide variety of biological processes. However, the precise mechanism of their action on such complex biological processes as tissue morphogenesis and cell differentiation remains to be established. We do not know, for example, how many of the β-xyloside-induced changes in biological processes are caused by the production of core-protein-free glycosaminoglycan with a resultant decrease in proteoglycan deposition and which, if any, are secondary (for example, those caused by the non-specific toxicity of the drugs). One approach to such questions is the quantitative autoradiographic analysis of proteoglycan deposition and processing at the critical zone of β-xyloside-treated and untreated tissues to demonstrate a direct relationship between the extent of inhibition of proteoglycan deposition and the extent of inhibition of the biological process, as has recently been described by Spooner *et al.* (1985) in their studies on branching morphogenesis of embryonic salivary glands.

An interesting property of β-xylosides described by Robinson *et al.* (1975) and Robinson & Robinson (1981) suggests another approach to the questions regarding the specificity of xyloside effects. Those authors found that the nature of the aglycone group of β-xylosides influenced the extent of stimulation of chondroitin sulphate synthesis by intact cartilage in tissue culture, a clue which has led to the present finding that a series of aryl and alkyl β-xylosides can be used in a simple procedure for furnishing a more rigorous criterion on which to test the specificity of β-xyloside action *in vivo*.

With a view towards developing a method applicable to the study of the function of proteoglycans *in vivo*, we have synthesized aryl and alkyl *O*-β-D-xylosides and their analogues with a different atom or grouping in the glycosidic linkage. The present results indicate that the drugs added to tissue-culture systems at appropriately low concentrations stimulate the synthesis of core-protein-free chondroitin (dermatan) sulphate chains in a well-defined aglycone-dependent manner and further suggest that these characteristics of the drugs can be exploited to ascertain whether xyloside-induced changes

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in biological processes result specifically from the perturbation of proteoglycan biosynthesis.

EXPERIMENTAL

Synthesis of xylosides

The following known compounds were prepared by previously described methods: phenyl *O*-β-D-xylopyranoside, m.p. 179 °C, $[\alpha]_D^{20}$ −49.4° (*c* 1 in water) (Montgomery *et al.*, 1942); phenyl β-D-thioxylopyranoside, m.p. 130–135 °C, $[\alpha]_D^{20}$ −67° (*c* 1 in water) (Purves, 1929); *N*-phenyl D-xylopyranosylamine, m.p. 142–148 °C, $[\alpha]_D^{20}$ −90°→−45° (*c* 1 in methanol; note that the final solution is at α,β-equilibrium) (Waygand *et al.*, 1951); *N*-*p*-carboxyphenyl D-xylopyranosylamine (sodium salt), m.p. 147–160 °C, $[\alpha]_D^{20}$ 0 (*c* 1 in water; note that the final solution is at α,β-equilibrium) (Inoue *et al.*, 1950); methyl β-D-thioxylopyranoside, m.p. 167–168 °C, $[\alpha]_D^{20}$ −68° (*c* 1 in water) (Staněk *et al.*, 1965); ethyl β-D-thioxylopyranoside, m.p. 117–118 °C, $[\alpha]_D^{20}$ −68° (*c* 1 in water) (Staněk *et al.*, 1965); propyl β-D-thioxylopyranoside, m.p. 105–107 °C, $[\alpha]_D^{20}$ −65.2° (*c* 1 in water) (Staněk *et al.*, 1965); butyl β-D-thioxylopyranoside, m.p. 110–111 °C, $[\alpha]_D^{20}$ −62.7° (*c* 1 in methanol) (Staněk *et al.*, 1965); pentyl β-D-thioxylopyranoside, m.p. 114–115 °C, $[\alpha]_D^{20}$ −65.8° (*c* 1 in methanol) (Staněk *et al.*, 1965) and hexyl β-D-thioxylopyranoside, m.p. 104–105 °C, $[\alpha]_D^{20}$ −68.5° (*c* 1 in methanol) (Staněk *et al.*, 1965).

C-Benzyl β-D-xylopyranoside (phenyl homo-*C*-β-D-xylopyranoside) was synthesized as follows. *C*-Benzyl-2,3,4-tri-*O*-acetyl α,β-D-xylopyranoside was prepared from 2,3,4-tri-*O*-acetyl α-D-xylosyl chloride (50 g) by reaction with an excess amount of benzylmagnesium chloride in diethyl ether, followed by acetylation with acetic anhydride. The tri-*O*-acetylated β-anomer was isolated by chromatography on a silica-gel column eluted with toluene/ethyl acetate (5:1, v/v) and then deacetylated with LiOH in methanol; yield 6.6 g; m.p. 105–107 °C; $[\alpha]_D^{20}$ −56.3° (*c* 1 in water); i.r. (cm^{-1}) 3430, 1600; ^1H n.m.r. (^2H_2O) δ(p.p.m.) 2.3–4.3 (m, 8H), 7.4 (m, 5H).

p-Carboxyphenyl β-D-*O*-xylopyranoside was synthesized as follows. *p*-Methoxycarbonylphenyl-2,3,4-tri-*O*-acetyl α,β-D-xylopyranoside was prepared from 2,3,4-tri-*O*-acetyl-α-D-xylosyl chloride (59 g) by reaction with methyl *p*-hydroxybenzoate (30 g)/Ag_2O (58 g)/anhydrous $CaSO_4$ (100 g) in 300 ml of acetonitrile, followed by acetylation with acetic anhydride. The tri-*O*-acetylated β-anomer was isolated by silica-gel chromatography and then deacetylated as above: yield 17 g; $[\alpha]_D^{20}$ −34° (*c* 1 in water); ^1H n.m.r. (^2H_2O) δ(p.p.m.) 3.2–4.3 (m, 5H), 5.2 (1H), 7.17, 7.93 [dd, $J = 8.4$, 4H (φH)].

p-Carboxyphenyl β-D-thioxylopyranoside was synthesized as follows. *p*-Carboxyphenyl-2,3,4-tri-*O*-acetyl α,β-D-thioxylopyranoside was prepared from 2,3,4-tri-*O*-acetyl-α-D-xylosyl chloride (30 g) by reaction with *p*-carboxyphenylthiophenol (20 g) in dimethylformamide, followed by acetylation with acetic anhydride. The tri-*O*-acetylated β-anomer was isolated by silica-gel chromatography and then deacetylated as above; yield, 8 g as sodium salt; $[\alpha]_D^{20}$ −49.5° (*c* 1 in water); ^1H n.m.r. (^2H_2O) δ(p.p.m.) 3–4.3 (m, 5H), 4.83 (d, $J = 9.6$, 1H), 7.6, 7.9 [dd, $J = 8.4$, 4H (φ4)].

C-*p*-Carboxybenzyl β-D-xylopyranoside (*p*-carboxyphenyl homo-*C*-β-D-xyloside) was synthesized as follows. *C*-*p*-Bromobenzyl-2,3,4-tri-*O*-acetyl α,β-D-xylopyranoside was prepared from 2,3,4-tri-*O*-acetyl-α-D-xylosyl chloride (11.8 g) by reaction with an excess amount of *p*-bromobenzylmagnesium bromide, followed by acetylation with acetic anhydride. The tri-*O*-acetylated β-anomer was isolated by silica-gel chromatography (see above) and then converted into *C*-*p*-bromobenzyl-2,3,4-tri-*O*-benzoyl β-D-xyloside by deacetylation with LiOH in methanol followed by benzoylation of the hydroxy groups with an excess amount of benzoyl chloride/KOH. The resulting compound was refluxed with a tetrahydrofuran solution of $MgCl_2$ (3.4 g), KI (5.4 g) and metallic potassium (2.5 g), and then treated with dried CO_2 to give *C*-*p*-carboxybenzyl-2,3,4-tri-*O*-benzoyl β-D-xylopyranoside (5.3 g). The compound thus obtained was subjected to catalytic reduction with palladium/carbon (1:9, w/w) (0.9 g): yield 2.8 g as sodium salt; $[\alpha]_D^{20}$ −31.1° (*c* 1 in water); i.r. (cm^{-1}) 1440, 1550, 1600, 3400, ^1H n.m.r. (^2H_2O) δ(p.p.m.) 2.3–4.1 (m, 8H), 7.44, 7.94 [dd, $J = 8.4$, 4H (φH)].

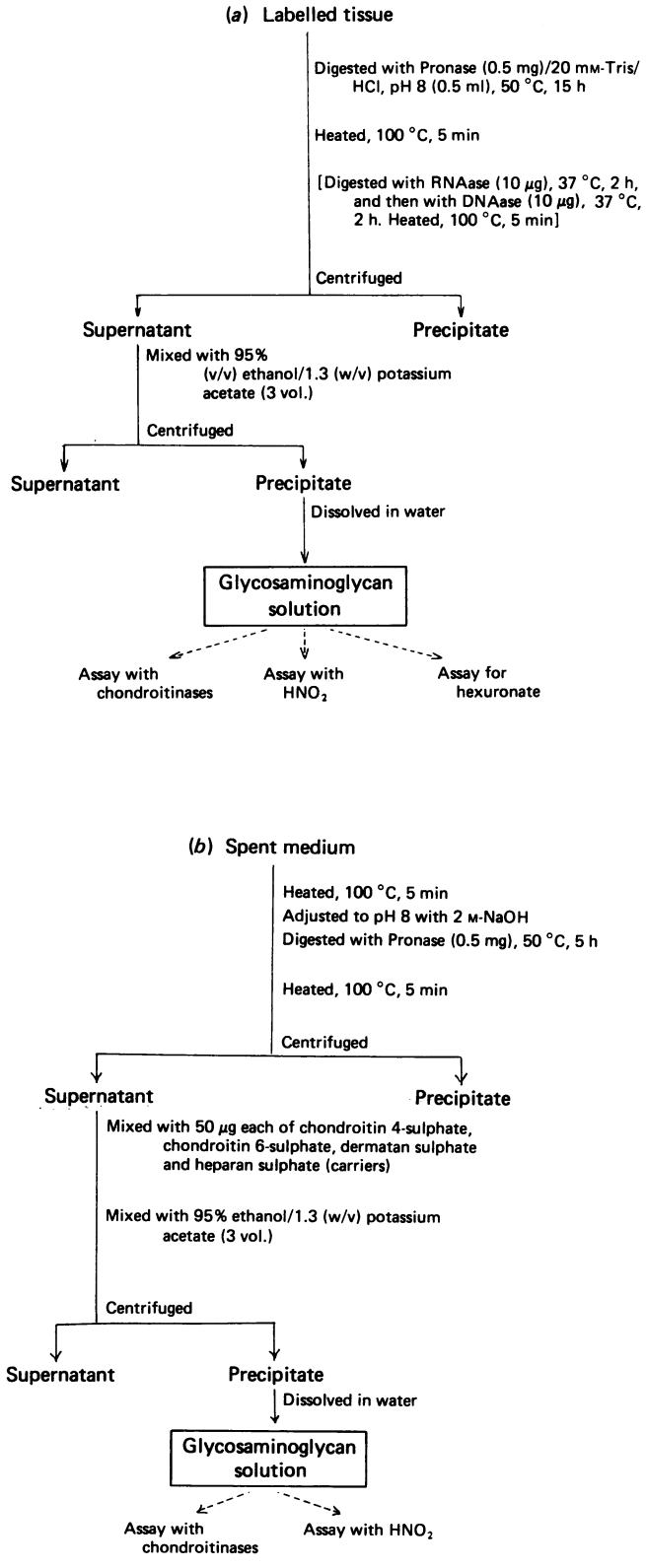
Alkyl β-D-thioxylosides were prepared from 2,3,4-tri-*O*-acetyl β-D-xylopyranosyl mercaptan by reaction with the appropriate alkyl halide by the method of Staněk *et al.* (1965), followed by deacetylation with LiOH in methanol. In this way, the following compounds were prepared: heptyl β-D-thioxyloside, m.p. 106 °C, $[\alpha]_D^{20}$ −68.5° (*c* 1 in methanol); octyl β-D-thioxyloside, m.p. 114 °C, $[\alpha]_D^{20}$ −68.5° (*c* 1 in methanol); nonyl β-D-thioxyloside, m.p. 119.5 °C, $[\alpha]_D^{20}$ −64.8 (*c* 1 in methanol); decyl β-D-thioxyloside, m.p. 121 °C, $[\alpha]_D^{20}$ −62.6° (*c* 1 in methanol) and undecyl β-D-thioxyloside, m.p. 122 °C, $[\alpha]_D^{20}$ −60.6° (*c* 1 in methanol).

C-Alkyl β-D-xylopyranosides (*C*-β-D-xylopyranosyl *n*-alkanes) were prepared as follows. *C*-Alkyl 2,3,4-tri-*O*-acetyl α,β-D-xylopyranoside were prepared from tri-*O*-acetyl α-D-xylopyranosyl chloride by reaction with the appropriate Grignard reagent, followed by acetylation with acetyl anhydride. The β-anomers were isolated by silica-gel chromatography and then deacetylated with LiOH in methanol. In this way the following compounds were prepared: *C*-ethyl β-D-xyloside, m.p. 146.5 °C, $[\alpha]_D^{20}$ −46.4° (*c* 1 in methanol); *C*-propyl β-D-xyloside, m.p. 119–120 °C, $[\alpha]_D^{20}$ −52.6° (*c* 1 in methanol); *C*-butyl β-D-xyloside, m.p. 100–101 °C, $[\alpha]_D^{20}$ −74.1° [*c* 1 in methyl chloride/methanol (5:1, v/v)]; *C*-amyl β-D-xyloside, m.p. 97–98 °C, $[\alpha]_D^{20}$ −44.8° (*c* 1 in methanol); *C*-hexyl β-D-xyloside, m.p. 105–106 °C, $[\alpha]_D^{20}$ −54.5° (*c* 1 in methanol); *C*-heptyl β-D-xyloside, m.p. 110–111 °C, $[\alpha]_D^{20}$ −43.5° (*c* 1 in methanol); *C*-octyl β-D-xyloside, m.p. 95–96 °C, $[\alpha]_D^{20}$ −37.0° (*c* 1 in methanol) and *C*-nonyl β-D-xyloside, m.p. 97–98 °C, $[\alpha]_D^{20}$ −35.0° (*c* 1 in methanol).

All of these xylosides have become available from a commercial supplier (Seikagaku Kogyo Co. Ltd., Nihonbashi-Honcho 2–9, Tokyo 103, Japan).

Other materials

Chondroitinase ABC, chondroitinase AC (Yamagata *et al.*, 1968), chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and heparan sulphate were the products of Seikagaku Kogyo Co. (Tokyo, Japan). Pronase was the product of Kaken Kagaku Co. (Tokyo, Japan) and was kindly donated by K. Takamine of that company. Bovine pancreas ribonuclease I and bovine pancreas deoxyribonuclease I were purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.);



Scheme 1. Method of isolation of ^{35}S-labelled glycosaminoglycans from tissue (*a*) and spent medium (*b*)

The digestion with ribonuclease (RNAase) and deoxyribonuclease (DNAase), the procedure shown in square brackets, was omitted in experiments with cartilage.

tissue-culture materials were from Gibco (Grand Island, NY, U.S.A.); inorganic [^{35}S]sulphate (carrier-free) was from the Japan Radioisotope Association (Tokyo, Japan) and cycloheximide was from Boehringer-Mannheim Yamanouchi (Tokyo, Japan).

Sternal cartilages were dissected from 15-day chick embryos, abdominal skins were from 17-day-old-rat foetuses and abdominal and thoracic aortas were from 6-week-old rats. The isolated tissues were chopped into pieces about 0.5 cm^3. During the dissection and before incubation with xylosides, the tissue segments were kept immersed in Eagle's minimal essential medium at room temperature.

Assay of chain-initiator activities of xyloside analogues

Tissue segments (10 mg of cartilage, 50 mg of skin or 50 mg of aorta) were placed in a culture dish containing a modified Eagle's minimal essential medium (0.5, 1.0 or 1.0 ml for cartilage, skin or aorta respectively) in which the amount of inorganic sulphate was decreased to give 50 μM and which contained 10% (v/v) dialysed foetal-calf serum, 0.3 mM-cycloheximide and 10 mM-Hepes/NaOH buffer, pH 7.4. Appropriate volumes (1 ~ 10 μl) of xyloside solutions were added to the flasks to give the desired final concentrations. To ensure complete solubilization of β-xylosides, the stock solutions of β-xylosides were prepared in dimethyl sulphoxide/water, in which the amount of dimethyl sulphoxide was adjusted to give a final concentration of 0.1% (v/v) after addition to the incubation medium (control experiments indicated that the added dimethyl sulphoxide had no effect on the rate of [^{35}S]sulphate incorporation into glycosaminoglycans under the assay conditions described below). The flasks plus tissue were preincubated at 37 °C for 45 min. The tissue segments were then transferred to 0.5 ml (for cartilage) or 1.0 ml (for skin or aorta) of fresh medium containing all the above reagents and 1 μCi (for cartilage), 50 μCi (for skin) or 4 μCi (for aorta) of inorganic [^{35}S]sulphate, and incubation was continued for 2 h (cartilage or aorta) or 5 h (skin).

For separate determination of media- and tissue-associated [^{35}S]glycosaminoglycans, media were removed and the tissue segments were washed twice with Earle's solution. The media and washed tissues were separately subjected to the isolation procedures shown in Scheme 1. The contents of chondroitin [^{35}S]sulphate and dermatan [^{35}S]sulphate in the isolated glycosaminoglycan samples were determined by a modification of the methods using chondroitinases (Saito *et al.* 1968). Two reaction mixtures and a blank mixture were prepared. The mixtures contained 100 μl, including glycosaminoglycan sample (50–80 μl) and the following enzyme and buffer solutions: tube 1 (control), 2 μl each of 1 M-Tris/HCl, pH 8.0, and 1 M-sodium acetate; tube 2, 2 μl each of 1 M-Tris/HCl, pH 8.0, 1 M-sodium acetate and chondroitinase ABC stock solution (20 units/ml); tube 3, 2 μl each of 1 M-Tris/HCl, pH 7.2, 1 M-sodium acetate and chondroitinase AC stock solution (20 units/ml). After incubation at 37 °C for 2 h, aliquots of the solutions were applied on Toyo No. 50 filter paper and chromatographed in butyric acid/0.5 M-NH_3 (5:3, v/v) at room temperature for 48 h to separate radioactive inorganic sulphate and oligosaccharides from undigested ^{35}S-labelled materials remaining at the origin. The areas at the origin where the samples were originally spotted were cut out and counted for radioactivity in a liquid-

scintillation spectrometer. The amounts (c.p.m.) of ^{35}S-labelled glycosaminoglycans are given by:

$$\text{Chondroitin}[^{35}S]\text{sulphate} + \text{dermatan}[^{35}S]\text{sulphate} = CPM_1 - CPM_2$$
$$\text{Chondroitin}[^{35}S]\text{sulphate} = CPM_1 - CPM_3$$
$$\text{Dermatan}[^{35}S]\text{sulphate} = CPM_3 - CPM_2$$

where CPM_n is the radioactivity (c.p.m.) measured at the origin of the chromatogram of tube n.

As shown below, some of the skin and aorta samples contained considerable amounts of chondroitinase ABC-resistant radioactive glycosaminoglycans. Most, if not all, of these compounds were susceptible to degradation with HNO_3 (results not shown), indicating that they are [^{35}S]heparin or heparan [^{35}S]sulphate, or both. For estimation of the amounts of ^{35}S-labelled heparin/heparan sulphate, aliquots of the sample solutions (Scheme 1) were directly subjected to HNO_2 treatment (Lindahl *et al.*, 1973) as follows. To 500 μl of the sample solutions from medium (but not to the sample solutions from tissue) were added 50 μg each of chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate and heparan sulphate as carriers. Aliquots of the solutions were treated at room temperature with 60 μl each of 18% (w/v) $NaNO_2$ and acetic acid for 80 min. Controls were without $NaNO_2$/acetic acid. Undegraded glycosaminoglycans were recovered by precipitation with ethanol, redissolved in a small volume of water and chromatographed on filter paper as described above. The amount (c.p.m.) of [^{35}S]heparin/heparan [^{35}S]sulphate is given by the difference between the radioactivity of the control and that of the HNO_2-treated sample (determined at the origin of the chromatogram).

In relation to the sources of error of the assay method, the following should be noted. (1) The SO_4^{2-} concentration in medium (50 μM) is higher than the critical SO_4^{2-} concentration for synthesis of chondroitin sulphate with the normal extent of sulphation (30 μM in a modified Eagle's medium) reported in our previous paper (Sobue *et al.*, 1978). When glycosaminoglycans were isolated from cartilage, skin or aorta specimens that had been incubated with $^{35}SO_4^{2-}$ at SO_4^{2-} concentrations ranging from 50 μM to 100 μM, electrophoresis on cellulose acetate strips in pyridine/acetic acid/water (1:9:115, by vol.) showed that the distribution of radioactivity was nearly centred under the Alcian Blue-stainable bands of unlabelled (endogenous) chondroitin sulphate, dermatan sulphate and/or heparan sulphate, regardless of whether or not β-xyloside was included in the medium. Therefore there were no obvious complications (e.g. synthesis of undersulphated chains) arising from the effect of environmental SO_4^{2-} concentration on the extent of sulphation of glycosaminoglycans. (2) In the presence of 0.3 mM-cycloheximide, $^{35}SO_4^{2-}$ incorporation into the total glycosaminoglycans of cartilage, skin or aorta was inhibited by 95±2%, indicating that, under the given incubation conditions, the rate of core-protein-dependent synthesis of sulphated glycosaminoglycan chains was only 5±2% of that in control tissues. (3) When measurements were made of hexuronate-positive material in the tissues (which represents the bulk of endogenous proteoglycans), no indication was obtained that the total amount of hexuronate was altered by incubation of the tissues at different β-xyloside concentrations. (4) Under the given conditions for paper chromatography, a sulphated tetrasaccharide with a non-reducing terminal unsaturated hexuronic acid residue and an internal L-iduronic acid residue moved down the paper, whereas higher oligosaccharides remained at the origin. In no case, however, did proportions of ^{35}S-labelled tetrasaccharide exceed 2% of the total ^{35}S applied, as ascertained by radioactivity scanning of the chromatographic strips. Therefore the difference between CPM_3 and CPM_2 may actually represent the extent of incorporation of ^{35}S into dermatan sulphate.

The results, taken together, indicate that the specific radioactivity of ^{35}S incorporated into the glycosaminoglycan fractions can be taken as a measure of newly synthesized glycosaminoglycan chains.

Gel chromatography

Samples of ^{35}S-labelled glycosaminoglycans (about 20000 c.p.m.) were dissolved in 0.1 ml of water and applied to a column (1 cm × 45 cm) of Bio-Gel A-1.5m that had been equilibrated with 0.2 M-NaCl. The column was eluted with the same solution at the rate of 3 ml/h at room temperature. Fractions (0.5 ml) were collected and assayed for radioactivity.

Treatment of chick embryos *in ovo* with xylosides

Fertilized eggs were obtained from inbred White Leghorn hens. β-Xylosides [9.2 μmol in 0.2 ml of phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 2.9 g of Na_2HPO_4, $12H_2O$ and 0.2 g of KH_2PO_4/litre, pH 7.2)] were injected on day 9 in the egg white through a hole in the shell. Control eggs were injected with phosphate-buffered saline only. The eggs were maintained in a humidified incubator at 38 °C for 1 week. For measurement of the length of skeletons, embryos were fixed in 80% (v/v) ethanol for 2 days and then defatted by placing in acetone for 1 day. Skeletons of the embryos were stained by the KOH/Alizarin Red S technique (Staples & Schnell, 1964)

RESULTS

Effect of aryl *O*-, *S*-, *C*- and *N*-xylosides on glycosaminoglycan synthesis in cartilage, skin and aorta

When cartilages of 15-day-old chick embryos were incubated with [^{35}S]sulphate in culture medium without cycloheximide, the specific radioactivity (c.p.m./μmol of hexuronate) of the isolated glycosaminoglycan fraction increased linearly with time (1.5×10^5 c.p.m./h) for at least 2 h. At the end of incubation, about 98% of the labelled glycosaminoglycan remained unreleased from the tissues, while the remainder was found in the medium. Of the total ^{35}S in the tissue-associated glycosaminoglycan, about 99% was sensitive to chondroitinase AC and, therefore, must represent chondroitin [^{35}S]sulphate. The addition of 0.3 mM-cycloheximide decreased the incorporation of $^{35}SO_4^{2-}$ into chondroitin sulphate to 4.8% of control, indicating that core-protein-dependent synthesis of chondroitin sulphate (i.e. the usual proteoglycan biosynthesis) was greatly inhibited.

Phenyl *O*-β-D-xyloside and its analogues with modifications in the atom in the glycosidic linkages were tested for their ability to relieve the inhibition of chondroitin sulphate synthesis caused by cycloheximide. Previous studies (Kato *et al.* 1978) showed that, although the core-protein-free chondroitin [^{35}S]sulphate chains formed in the presence of β-xyloside were rapidly released into culture medium, some protein-free chondroitin [^{35}S]sulphates were retained in the tissue unless chase incubation had been performed. In the present assay, therefore, the extents of incorporation of $^{35}SO_4^{2-}$ into chondroitinase AC-digestible material in medium and tissue fractions were separately measured as in Scheme 1, and the sum of medium and tissue materials was used as representative of newly synthesized chondroitin sulphate chains. Fig. 1 shows the relationship between the concentration of added xylosides and the specific radioactivity of chondroitin sulphate chains expressed as a percentage of the specific radioactivity of core-protein-bound chondroitin sulphates from control tissue incubated without cycloheximide (3.0×10^5 c.p.m./μmol of hexuronate). The *O*- and *S*-xylosides were clearly more effective than the corresponding *C*- and *N*-xyloside analogues over a concentration range lower than 0.1 mM. In this low-concentration range the rate of chondroitin sulphate synthesis increased linearly as the xyloside concentration was increased. The curves for *O*- and *S*-xylosides reached a plateau at a xyloside concentration of 0.1 mM, whereas the effectiveness of *C*- and *N*-xylosides continued to increase beyond this concentration. Thus, at a xyloside concentration of 1.0 mM, the extent of linkage-atom-dependent variation of effectiveness was much smaller than that at xyloside concentrations lower than 0.1 mM.

Analysis with HNO_2 of the whole labelled material in medium plus tissue fractions indicated that [^{35}S]heparin/heparan [^{35}S]sulphate was produced only at extremely low levels in cartilages, if at all, regardless of whether or not β-xylosides were included in the medium.

Fig. 2 shows that introduction of a carboxy group to the *para* position of the aglycone yields, in each case, a derivative with much lower initiator activity.

Although triplicate assays of the specific radioactivities of chondroitin [^{35}S]sulphate produced showed a coefficient of variation ranging from 5 to 8%, the patterns of aglycone-dependent variation in the effectiveness (Figs. 1 and 2) were highly reproducible. To see whether this pattern of changes is common among proteochondroitin sulphate-synthesizing systems of different tissues or is specific to the cartilage, the chain-initiator ability of xyloside analogues was studied with 17-day-rat foetus skin and 6-week-rat aorta.

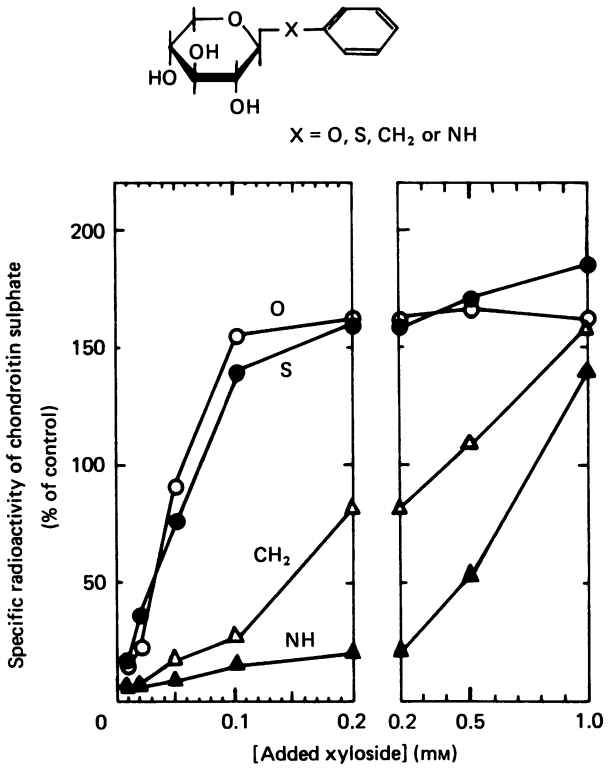


Fig. 1. Abilities of phenyl *O*-β-D-xyloside and its analogues with S, CH_2 or NH in the glycosidic linkage to relieve the cycloheximide-induced inhibition of chondroitin sulphate synthesis by cartilage

Chick-embryo cartilage was incubated for 2 h in medium containing cycloheximide and $^{35}SO_4^{2-}$ as described in the Experimental section. Phenyl *O*-β-D-xyloside (○), phenyl β-D-thioxyloside (●), phenyl homo-*C*-β-D-xyloside (△) and phenylamine *N*-α,β-D-xyloside (▲) were added as shown in the Figure. The general structural formula for the compounds is shown at the top of the Figure. The specific radioactivity of the chondroitin sulphate in medium plus tissue is expressed as a percentage of that isolated from medium plus tissue incubated without cycloheximide and xyloside (3.0×10^5 c.p.m./μmol of hexuronate). Note the different scales for the horizontal axes.

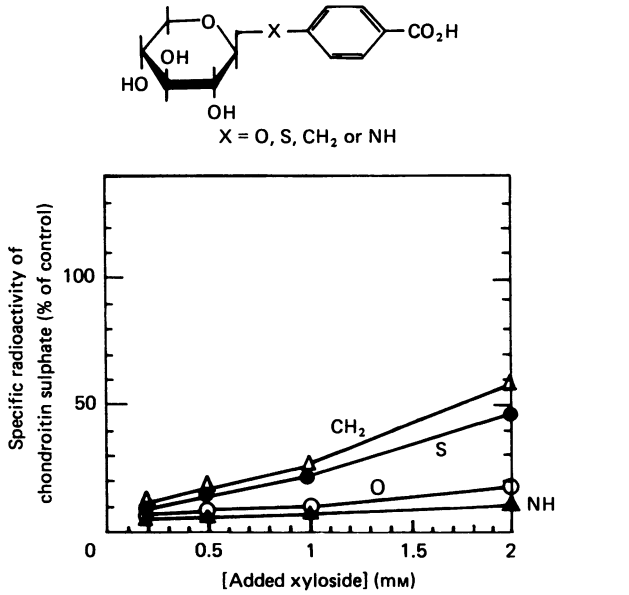


Fig. 2. Abilities of *p*-carboxyphenyl *O*-β-D-xyloside and its analogues with S, CH_2 or NH in the glycosidic linkage to relieve the cycloheximide-induced inhibition of chondroitin sulphate synthesis by cartilage

Chick-embryo cartilage was incubated for 2 h in medium containing cycloheximide and $^{35}SO_4^{2-}$ as described in the Experimental section. *p*-Carboxyphenyl β-D-*O*-xyloside (○), *p*-carboxyphenyl β-D-thioxyloside (●), *p*-carboxyphenyl homo-*C*-β-D-xyloside (△) and *p*-carboxyphenylamine *N*-α,β-D-xyloside (▲) were added as shown in the Figure. The general structural formula for the compounds is shown at the top of the Figure. For an explanation for the values on the ordinate scale, see the legend to Fig. 1.

When tissue specimens were incubated with [^{35}S]sulphate in culture medium without cycloheximide, the specific radioactivity (c.p.m./μmol of hexuronate) of the isolated glycosaminoglycan fraction increased linearly with time (1×10^6 c.p.m./h for skin and 3.5×10^4 c.p.m./h for aorta) for at least 5 h (skin) or 2 h (aorta). At the end of incubation, about 98% of the labelled glycosaminoglycans remained unreleased from the tissues, while the remainder was found in the medium. Of the total ^{35}S in the tissue-associated glycosaminoglycan, about 35, 16 and 48% (skin) or 35, 36 and 27% (aorta) were accounted for by chondroitin sulphate, dermatan sulphate and heparan sulphate respectively. The addition of 0.3 mM-cycloheximide decreased the total incorporation of ^{35}S into glycosaminoglycans to 5 ± 2% of control. Fig. 3 shows the abilities of various aryl xylosides to relieve the inhibition with cycloheximide. In this figure the rate of synthesis of chondroitinase ABC-sensitive material in the presence of 0.05 mM- or 0.1 mM-xyloside is expressed as a percentage of the specific radioactivity of chondroitinase ABC-sensitive product from control tissue incubated without cycloheximide (5.35×10^6 c.p.m./μmol of hexuronate for skin and 7.2×10^4 c.p.m./μmol of hexuronate for aorta). As the histograms show, the skin and aorta do not differ greatly from the cartilage in their specificity to aglycone modifications either in the glycosidic linkage or at the *para* position of the benzene ring.

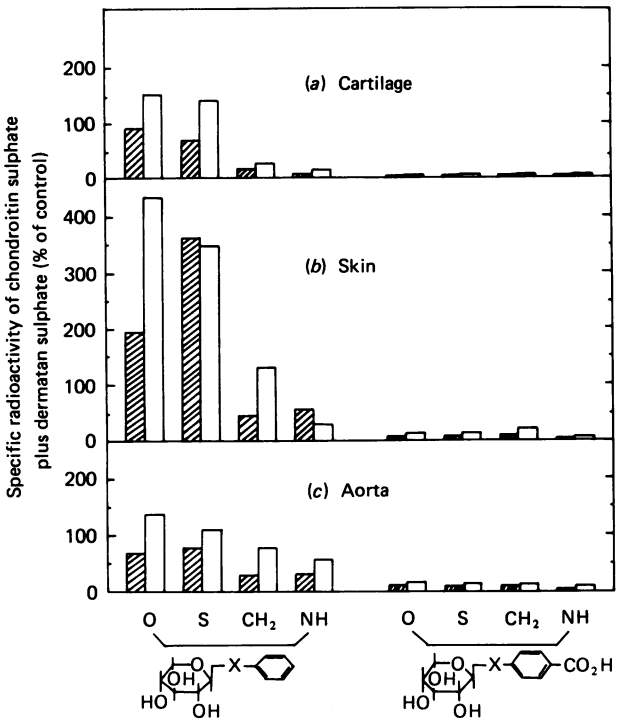


Fig. 3. Effect of phenyl and *p*-carboxyphenyl *β*-D-*O*-xyloside and their analogues on the incorporation of $^{35}SO_4^{2-}$ into chondroitin sulphate plus dermatan sulphate in the presence of cycloheximide

Chick-embryo cartilage (*a*), rat embryo skin (*b*) and rat aorta (*c*) were incubated for 2 h, 5 h and 2 h respectively in medium containing cycloheximide and $^{35}SO_4^{2-}$ as described in the Experimental section. The compounds shown at the bottom of the Figure were added to give a final concentration of 0.05 mM (hatched bars) or 0.1 mM (open bars). The specific radioactivity of chondroitin sulphate plus dermatan sulphate in medium plus tissue is expressed as a percentage of that isolated from medium plus tissue incubated without cycloheximide and xyloside [3.0×10^5 c.p.m. (cartilage), 5.35×10^6 c.p.m. (skin) or 7.2×10^4 c.p.m. (aorta)/μmol of hexuronate].

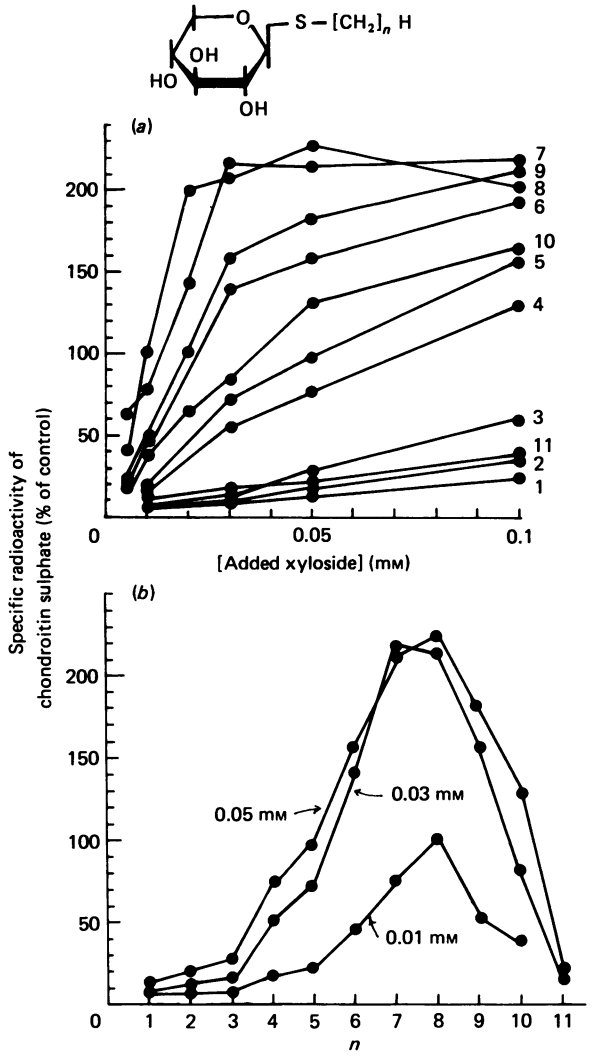


Fig. 4. Effect of the carbon number of the aglycone moiety on the extent of stimulation of chondroitin sulphate synthesis (cartilage) by alkyl *β*-D-thioxyloside

Chick-embryo cartilage was incubated for 2 h in medium containing cycloheximide and $^{35}SO_4^{2-}$ as described in the Experimentatal section. (*a*) Alkyl *β*-D-thioxylosides with various carbon numbers (*n*, shown on the extreme right) of the aglycone (see the structural formula at the top of the Figure) were added as shown in the Figure. (*b*) The data obtained at xyloside concentrations of 0.01 mM, 0.03 mM and 0.05 mM are plotted against *n* to show the mode of variation of the effectiveness with *n*. For explanation of the values of the ordinate, see the legend to Fig. 1.

Since both mammalian skin and aorta synthesize proteodermatan sulphate and proteoheparan sulphate in addition to proteochondroitin sulphate (Habuchi *et al.*, 1986; Oegema *et al.* 1979), the chondroitinase ABC-sensitive radiolabel may actually represent the sum of chondroitin [^{35}S]sulphate and dermatan [^{35}S]sulphate. To obtain further information on the chemical nature of glycosaminoglycans produced under the influence of

these xyloside analogues, enzyme digestions and HNO_2 treatment were performed as described in the Experimental section. Thus individual glycosaminoglycans which appeared only after phenyl *O* (or *S*)-β-D-xyloside treatment in the presence of cycloheximide were shown to be predominantly chondroitin sulphate (46±4% in skin and 66±8% in aorta) and dermatan sulphate (50±4% in skin and 34±6% in aorta). No stimulation of synthesis of HNO_2-sensitive material was observed with these xylosides. In control tissues incubated without cycloheximide, however, about 48% (skin) and 27% (aorta) of the total ^{35}S-labelled glycosaminoglycans synthesized on core proteins were sensitive to HNO_2, suggesting that, in the presence of cycloheximide, the added β-xylosides supported polymerization of chondroitin sulphate and dermatan sulphate, but not of heparan sulphate and heparin.

It is noteworthy that the added β-xylosides caused a tissue-specific change in dermatan [^{35}S]sulphate/ chondroitin [^{35}S]sulphate ratio; in controls the radioactivity ratios were about 0.43 (skin) and 1.0 (aorta), and after treatment with the β-xylosides the ratio in skin increased 2.6-fold, whereas the ratio in aorta decreased 2-fold.

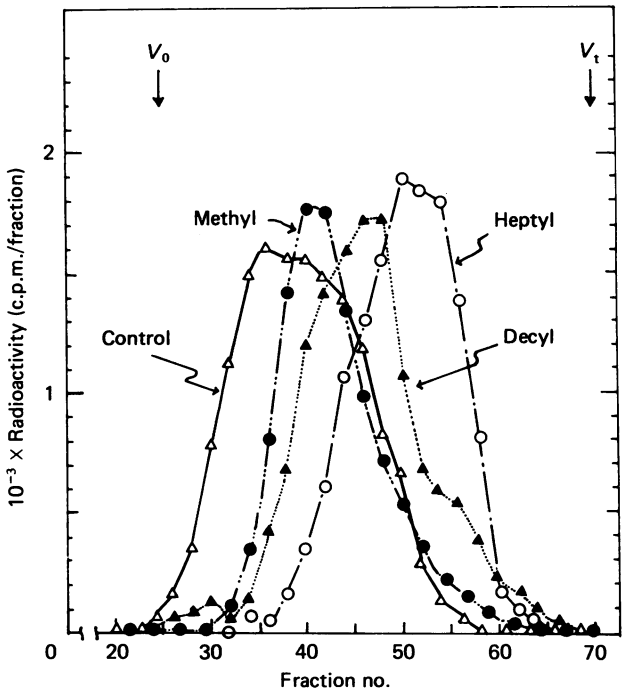


Fig. 5. Elution profiles from Bio-Gel A-1.5m of labelled chondroitin sulphate extracted from cartilage plus medium by Pronase digestion

Chick-embryo cartilage was incubated for 2 h in medium containing cycloheximide and $^{35}SO_4^{2-}$ in the presence of β-D-thioxyloside. Labelled chondroitin sulphate isolated from medium plus tissue by Pronase treatment was chromatographed on a Bio-Gel A-1.5m column as described in the Experimental section. Elution profiles for labelled chondroitin sulphate formed in the presence of 0.1 mM-methyl β-D-thioxyloside (●), 0.1 mM-heptyl β-D-thioxyloside (○) or 0.1 mM-decyl β-D-thioxyloside (▲) are shown, together with the profile of chondroitin sulphate formed in the absence of both cycloheximide and β-thioxyloside (△). The arrows indicate the void volume (V_0) and the effective total volume of the column (V_t).

Effect of alkyl thioxyloside homologues on chondroitin sulphate synthesis

The results shown in Fig. 4(*a*) illustrate differences between a number of alkyl thioxylosides that were tested for their chain-initiator activity in the cartilage. Over a concentration range lower than 0.03 mM their effectiveness increased with the increase of carbon number (*n*) of the alkyl group and was at a maximum at $n = 7$ or 8 (Fig. 4*b*). The maximum extent of stimulation by the homologues of $n = 7$ and 8 was reached at far lower concentrations (0.03 mM ~ 0.05 mM) than by phenyl β-thioxyloside (0.1 mM; see Fig. 1).

The sizes of the chondroitin [^{35}S]sulphate chains formed in the presence of 0.1 mM-methyl β-thioxyloside, heptyl β-thioxyloside and decyl β-thioxyloside were compared by Bio-Gel A-1.5 m chromatography (Fig. 5). It is clear that the chain size of chondroitin sulphate altered in response to changes in the effectiveness of alkyl β-thioxyloside as chain initiator, i.e. the greater the

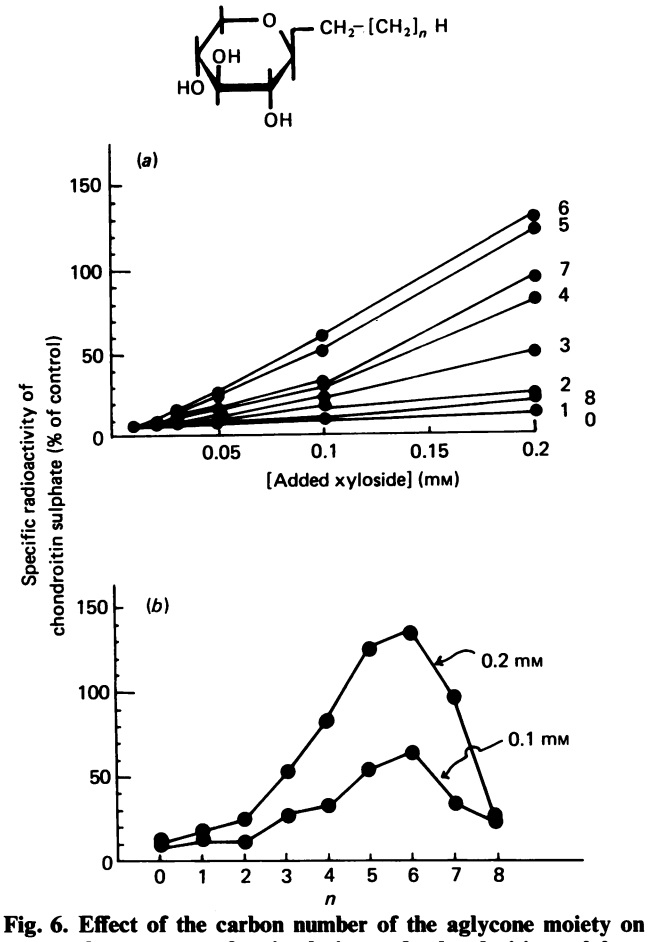


Fig. 6. Effect of the carbon number of the aglycone moiety on the extent of stimulation of chondroitin sulphate synthesis (cartilage) by alkyl β-D-*C*-xylosides

(*a*) The experimental conditions were the same as in Fig. 4, except that the *C*-xyloside analogues indicated by the general formula at the top of the Figure were employed (*n* values shown on the extreme right). (*b*) The data obtained at xyloside concentrations of 0.1 mM and 0.2 mM are plotted against the carbon number (*n*) of the aglycone. For explanation of the values on the ordinate, see the legend to Fig. 1.

effectiveness of chain initiator, the smaller the average size of initiator-stimulated chains. Consistent with this view, an increase of the concentration of methyl β-thioxyloside to 1 mM resulted in the production of chondroitin [^{35}S]sulphate chains with an average molecular size comparable with that of the chains synthesized on 0.1 mM-heptyl β-D-thioxyloside. The inverse relationship between synthetic rate and molecular size holds for phenyl β-xyloside. Thus a progressive increase of phenyl β-D-xyloside concentration caused a progressive decrease in the average molecular size of newly synthesized chondroitin sulphate chains, so that, at 0.1 mM-phenyl β-xyloside, their average chain size was larger ($K_{av.}$ 0.45) than that of the chains synthesized on 0.1 mM-heptyl β-thioxyloside ($K_{av.}$ 0.57), whereas at 1.0 mM-phenyl β-xyloside the average chain size was considerably smaller ($K_{av.}$ 0.67).

Compared with these alkyl thioxylosides, the *C*-xyloside analogues had very small effects on chondroitin sulphate biosynthesis (Fig. 6*a*) with a maximum effectiveness at $n = 5$ or 6 (Fig. 6*b*).

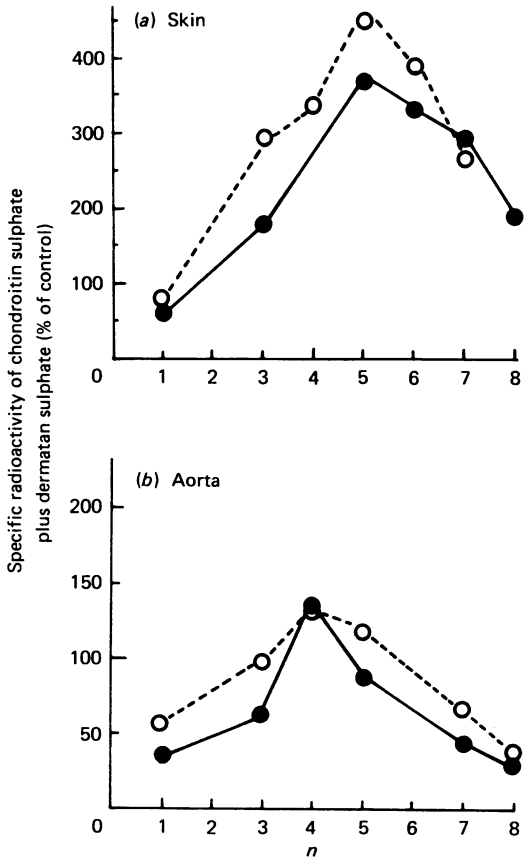


Fig. 7. Variation in skin and aorta of the effectiveness of alkyl β-D-thioxylosides with the carbon number of the aglycone

Rat-embryo skin (*a*) and rat aorta (*b*) were incubated for 5 h and 2 h respectively in medium containing cycloheximide and $^{35}SO_4^{2-}$ as described in the Experimental section. Alkyl β-D-thioxylosides with various carbon numbers (*n*) of the aglycone (for the general structural formula, see Fig. 4) were added to give a final concentration of 0.05 mM (●) or 0.1 mM (○). For explanation of the values on the ordinate, see the legend to Fig. 3. Note that different scales are used on the ordinate.

Fig. 7 shows the effect of carbon number of the alkyl group of alkyl β-thioxyloside on the extent of stimulation of chondroitin sulphate/dermatan sulphate synthesis in rat skin and aorta. The curves for skin (Fig. 7*a*) and aorta (Fig. 7*b*) are different from the curve for cartilage (Fig. 4) and maximum effectiveness was now obtained at $n = 5$ and $n = 4$ respectively.

Changes in size of skeletons of chick embryos treated *in ovo* with β-xylosides

Gibson *et al.* (1978) described a teratological syndrome of chick embryo produced by injection of 4-methylumbelliferyl β-D-xyloside or *p*-nitrophenyl β-D-xyloside

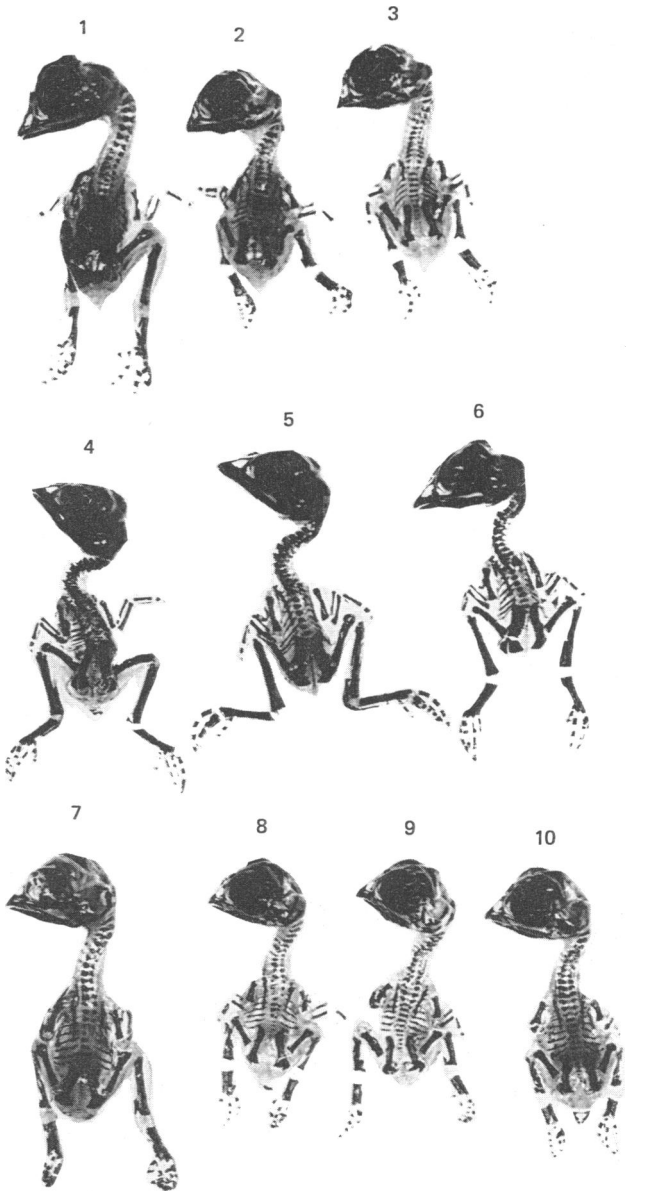


Fig. 8. Skeletons of chick embryos that had received β-D-xyloside on day 9 and were killed on day 16

The drugs injected were: 1, phosphate-buffered saline (control); 2, phenyl *O*-β-D-xyloside; 3, phenyl β-D-thioxyloside; 4, phenyl homo-*C*-β-D-xyloside; 5, *p*-carboxyphenyl β-D-thioxyloside; 6, methyl β-D-thioxyloside; 7, ethyl β-D-thioxyloside; 8, butyl β-D-thioxyloside; 9, pentyl β-D-thioxyloside; 10, hexyl β-D-thioxyloside.

into the amniotic sac of fertilized egg on day 9 (the 'stage 35' described by Hamburger & Hamilton, 1951). The morphological changes induced by the drugs included a decrease in the overall growth rate of cartilage and bone. The major chemical change in β-xyloside-treated embryos was a marked undersulphation and a decrease in the average chain length of chondroitin sulphate, resulting partly from a decrease in chain length of the chondroitin sulphates linked to core protein but chiefly from the presence of large numbers of shortened chains of core-protein-free (xyloside-linked) chondroitin sulphates (Gibson *et al.* 1979). The results suggest that the observed change in morphology is closely related to the ability of β-xyloside to stimulate synthesis of core-protein-free chondroitin sulphate. If this is so, xylosides with different chain-initiator activities should have different inhibiting effects on the growth of cartilage and bone *in ovo*.

To test this possibility, we investigated the effects of various aryl and alkyl xylosides on the growth *in ovo* of chick embryo cartilage and bone. Drugs (9.2 μmol) were administerd to 9-day-old embryos by injection into the albumen on day 9. If injected drugs diffuse uniformly throughout the egg, their concentration in the vicinity of the embryo would be 0.1 ~ 0.2 mM.

Fig. 8 shows the skeletons of embryos that had received drug on day 9 and were killed on day 16. Control embryos had received phosphate-buffered saline only. In embryos treated with drugs of relatively high initiator activities the overall growth of skeletons was greatly retarded in comparison with the growth of skeletons in controls or in embryos treated with drugs of low initiator activities: phenyl *O*-β-D-xyloside, phenyl β-D-thioxyloside and alkyl β-D-thioxyloside homologues of $n = 4 \sim 6$ had most prominent effects. Table 1 summarizes the effects of various β-D-xylosides on the growth of the femur, tibiofibula and second metatarsus. Growth of each bone was inhibited, relative to controls, by some 60–70% when treated with phenyl *O*-β-D-xyloside, phenyl β-D-thioxyloside or alkyl β-D-thioxylosides of $n = 4 \sim 6$, whereas no measurable change in size of the bones was found in embryos treated with phenyl homo-*C*-β-D-xyloside, *p*-carboxyphenyl β-D-thioxyloside or alkyl β-D-thioxylosides of $n = 1$ and 2.

DISCUSSION

p-Nitrophenyl β-D-xyloside and 4-methylumbelliferyl β-D-xyloside were the first xylosides described in artificial chain initiators of chondroitin sulphate synthesis (Okayama *et al.*, 1971, 1973; Schwartz *et al.*, 1974; Fukunaga *et al.*, 1975), and much of early methodology was based on work with these two compounds. In the meantime, Robinson *et al.* (1975) prepared methyl, ethyl, butyl, octyl, phenyl and benzyl *O*-β-D-xylosides and phenyl β-D-thioxyloside, and pointed out that the nature of the aglycone group of the β-xylosides influenced the extent of stimulation of chondroitin sulphate synthesis and the concentration of β-xyloside necessary to produce the maximum effect. However, the role of the aglycone or non-carboxyhydrate moiety in determining the specificity and effectiveness of β-xylosides apparently is largely unappreciated in both the old and recent literature describing effects of *p*-nitrophenyl β-D-xyloside or 4-methylumbelliferyl β-D-xyloside on complex biological processes. Our results demonstrate clearly that the initiator activity of xyloside varies in a well-defined aglycone-dependent and tissue-specific manner. Thus, over a concentration range lower than 0.1 mM, the effectiveness of alkyl β-D-thioxyloside is markedly influenced by the carbon number (n) of the alkyl group and is at a maximum at $n = 7$ or 8 for the cartilage, at $n = 5$ for the skin and at $n = 4$ for the aorta. All of the drugs can relieve the inhibition of both chondroitin sulphate and dermatan sulphate synthesis, but not the inhibition of heparan sulphate synthesis caused by cycloheximide. Not only the size or shape of aglycone, but also the atom or grouping in the glycosidic linkage, is shown to have a marked effect on the effectiveness of xylosides; for all the three tissues, phenyl *O*-β-D-xyloside and phenyl β-D-thioxyloside are clearly more effective than the corresponding homo-*C*-β-D-xyloside and *N*-D-xyloside at α,β-equilibrium, provided that the initial concentration of added xyloside is lower than 0.1 mM.

Table 1. Length of skeletons in embryos treated with β-xylosides

Results are given as means ± S.E.M. for the numbers of embryos shown in parenthesis. Abbreviation: IA, initiator activity expressed as:

$$\frac{\text{Radioactivity (c.p.m.) of chondroitin sulphate synthesized on 0.1 mM-β-xyloside}}{\text{Radioactivity (c.p.m.) of chondroitin sulphate synthesized on core protein}} \times 100$$

Drug administered to embryos	IA	Length (mm)		
		Femur	Tibiofibula	Second metatarsus
None	0	8.3 ± 0.2 (6)	11.7 ± 0.2 (6)	8.2 ± 0.3 (6)
Phenyl *O*-β-xyloside	150	6.0 ± 0.2 (4)	7.8 ± 0.5 (4)	5.1 ± 0.1 (4)
Phenyl β-thioxyloside	140	6.2 ± 0.2 (3)	8.8 ± 0.6 (3)	6.0 ± 0.2 (3)
Phenyl homo-*C*-β-xyloside	25	8.4 ± 0.2 (3)	12.3 ± 0.5 (3)	8.4 ± 0.4 (3)
p-Carboxyphenyl β-xyloside	5	9.2 ± 0.2 (3)	12.6 ± 0.2 (3)	9.5 ± 0 (3)
Methyl β-thioxyloside	25	8.3 ± 0.2 (3)	11.4 ± 0.3 (3)	8.3 ± 0.3 (3)
Ethyl β-thioxyloside	40	7.8 ± 0.2 (5)	11.3 ± 0.3 (5)	7.9 ± 0.4 (5)
Butyl β-thioxyloside	125	5.7 ± 0.2 (5)	7.8 ± 0.6 (5)	4.9 ± 0.1 (5)
Pentyl β-thioxyloside	150	5.1 ± 0.02 (4)	7.3 ± 0.3 (4)	5.0 ± 0 (4)
Hexyl β-thioxyloside	190	5.7 ± 0.2 (3)	7.2 ± 0.2 (3)	5.0 ± 0 (3)

The fall on either side of the optimum of size of alkyl group (Figs. 4, 6 and 7) could conceivably be due to a decreased rate of transport of the xyloside to the site of chondroitin sulphate synthesis in an intact tissue. It seems likely that a critical proportion of the hydrophilic and hydrophobic moieties is required for the transport of the xyloside molecules through cell membranes. Robinson & Robinson (1981) compared the rates of galactose transfer from UDP-galactose to D-xylose, methyl β-D-xyloside, ethyl β-D-xyloside, benzyl β-D-xyloside, phenyl β-D-thioxyloside and several other xylosides, catalysed by a cell-free microsomal preparation from embryonic-chick cartilage. Although the aglycone group was shown to have some effect on galactosyltransferase activity, as evidenced by difference $V_{max.}$ and K_m values, the marked effect of aglycone group on initiator activities observed with intact cartilage was not apparent, indicating that the galactosyltransferase activity in chondrocytes is not a point of regulation of the aglycone effect.

We have shown that the extent of aglycone-dependent variation of the xyloside effectiveness is gradually decreased with increasing xyloside concentration in the medium. At a xyloside concentration of 0.1 mM, for example, the effectiveness of phenyl *O*-β-D-xyloside in cartilage is about six times that of phenyl homo-*C*-β-D-xyloside, whereas at 1.0 mM their effectivenesses are almost the same (Fig. 1). This concentration effect is in keeping with the notion that differences in the rate of membrane transport of xyloside are an important factor in the aglycone-related changes in effectiveness. Although the precise mechanism of the aglycone effect is not yet known, we may suggest that more attention be directed at the effects of the nature of the aglycone and xyloside concentration, especially when a complex biological system consisting of different types of cell or tissues is examined for its response to xylosides. It should be noted in this respect that various types of cell (e.g. fibroblasts and chondrocytes) exposed to high concentrations (> 1 mM) of *p*-nitrophenyl β-D-xyloside or 4-methylumbelliferyl β-D-xyloside show increased vacuolization or decreased proliferative activity, or both, probably caused by non-specific cell toxicity of the drugs (see Johnston & Keller, 1979; Lohmander *et al.*, 1979*a*; Robinson & Gospodarowicz, 1984, among others). Thus, without appropriate control experiments, it is difficult to interpret such events as the abnormal embryogenesis caused by treatment with 4 mM-*p*-nitrophenyl β-D-xyloside (Akasaka *et al.*, 1980) as solely resulting from the perturbation of proteoglycan synthesis.

Since heparan sulphate and heparin are linked to core protein in a manner identical with that by which chondroitin sulphate is linked (Rodén, 1980), it might be assumed that β-xylosides can stimulate heparan sulphate (heparin) synthesis in a manner analogous to that by which chondroitin sulphate synthesis is stimulated. In the present study, the test for this possibility with skin and aorta demonstrated that cycloheximide inhibits almost completely heparan sulphate synthesis, consistent with the notion that formation of the protein core is prerequisite to the formation of heparan sulphate chains. Nonetheless, the cycloheximide inhibition of heparan sulphate synthesis was not overcome at all by any of the xylosides we have described. This agrees with previously published information in which neither *p*-nitrophenyl β-D-xyloside nor 4-methylumbelliferyl β-D-xyloside stimulates the synthesis of heparan sulphate or heparin in rat glial cells, mouse neuroblastoma cells (Galligani *et al.*, 1975), rat serosal mast cells (Stevens & Austen, 1982) and haemopoietically active mouse bone-marrow cells (Spooncer *et al.*, 1983) in culture. The lack of β-xyloside stimulation of heparan sulphate synthesis may reflect an inability of the galactosyltransferase involved in the synthesis of heparan sulphate–protein linkage oligosaccharide to use β-xyloside as a substrate in place of the natural core-protein precursor. Alternatively, the intracellular compartment for heparan sulphate synthesis may be different from that for chondroitin sulphate synthesis in being resistant to the entry of xylosides.

Injecting 5 mg of *p*-nitrophenyl β-D-xyloside in 0.3 ml of saline or 10 mg of 4-methylumbelliferyl β-D-xyloside in 0.1 ml of corn oil into the amniotic sac of a fertilized egg on day 9, Gibson *et al.* (1978) showed that the drugs caused morphological abnormalies of the embryo 1 week later. The xyloside-induced abnormalities include an overall decrease in skeleton size, a change expected from the known effects of β-xyloside on cartilage proteoglycan synthesis, i.e. stimulation of free chondroitin sulphate chain synthesis with a resultant production of proteoglycan with shortened, undersulphated, chondroitin sulphate chains (Gibson *et al.*, 1977). Since, however, both of the xylosides have been shown to inhibit the synthesis of DNA (Gibson *et al.*, 1979), collagen and non-collagenous proteins (Lohmander *et al.*, 1979*b*) in both cartilage and chondroyctes in culture, the possibility that the observed change in morphology might result from such nonspecific effects cannot completely be excluded. Our examination of the morphogenesis of skeletons of embryos treated with a variety of β-xylosides with diverse aglycone structures indicates a positive correlation between the abilities of the drugs to initiate core-protein-free chondroitin sulphate synthesis in cartilage and their abilities to inhibit the growth of skeletons. The results strengthen the possibility that the β-xyloside inhibition of skeletal growth stems from enhanced production of free chondroitin sulphate, which may cause decreased deposition of proteoglycans in the extracellular matrix of growing cartilage. Although the results with each of the drugs, taken individually, could be explained on a basis other than the perturbation of cartilage proteoglycan, the combined results with the variety of xyloside homologues constitute strong evidence for the direct effect of xylosides on the growth rate of skeletons through perturbation of proteoglycan synthesis. The probability of obtaining such a combination of consistent results by chance is exceedingly small. Delineating the precise mechanisms that underlie the observed correlation should contribute to our understanding of the function of proteoglycans in histogenetic processes.

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