Effect of cycloheximide, β -D-xylosides and β -D-galactosides on heparin biosynthesis in mouse mastocytoma

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Heparin biosynthesis has been investigated with mouse mastocytoma *in vitro*. Minced tumour tissue catalysed the incorporation of [^{35} S]sulphate and [^{3} H]glucosamine into heparin and to a smaller extent into chondroitin sulphate. Addition of cycloheximide caused an inhibition (>80%) of incorporation of each labelled precursor into both polysaccharides. Addition of benzyl β -D-xyloside relieved the inhibition of incorporation into chondroitin sulphate and restored it to more than threefold that of the control incubation. The effect of β -D-xyloside on incorporation into heparin was less marked although a consistent small increase of incorporation into this polysaccharide was observed. β -D-Xyloside did, however, cause a marked incorporation of ^{35} S and ^{3} H labels into material of low molecular weight, which appeared to comprise heparin-like fragments. It is proposed that these fragments arise through a breakdown of the usual process of heparin biosynthesis.

Many of the glycosaminoglycans present in mammalian tissues occur in the native state as proteoglycans, macromolecules composed of polysaccharide chains covalently linked to protein (Muir & Hardingham, 1975). Studies on the structure of macromolecular heparin (Horner, 1977; Ögren & Lindahl, 1971; Robinson *et al.*, 1978) have demonstrated that this glycosaminoglycan also exists as a proteoglycan and contains the characteristic linkage oligosaccharide, GlcAp β 1–3Galp β 1–3Galp β 1–4Xylp1–Ser, at the site of covalent attachment of polysaccharide to protein (Lindahl & Rodén, 1972). This linkage also occurs in chondroitin sulphate and dermatan sulphate proteoglycans.

The biosynthesis of chondroitin sulphate proteoglycan in cartilage is markedly inhibited by cycloheximide and other inhibitors of mammalian protein synthesis (Telser *et al.*, 1965). Certain β -D-xylosides and β -D-galactosides relieve this inhibition and cause a stimulation of incorporation of labelled precursors into chondroitin sulphate (Robinson *et al.*, 1975). Addition of these compounds results in the biosynthesis of free chondroitin sulphate chains, not attached to protein, and

these compounds are thought to act as artificial initiators of polysaccharide synthesis.

Since heparin is linked to protein through a linkage oligosaccharide identical with that found in chondroitin sulphate, the effect of cycloheximide and β -D-xyloside on heparin synthesis in mouse mastocytoma tissue was investigated to determine if these compounds would produce similar changes in the rate of synthesis of this glucosaminoglycan.

Materials and methods

Materials

The Furth mast-cell tumour used was maintained in (A/Sn $\times$ Leaden) F $_1$ mice as described by Ögren & Lindahl (1971).

Samples of hyaluronic acid isolated from rooster comb and chondroitin 4-sulphate isolated from bovine nasal septum were a generous gift from Dr. Å. Wasteson. Heparin (stage 14) from pig intestinal mucosa was obtained from Inolex, Pharmaceutical Div., Park Forest South, IL, U.S.A., and had been purified as described by Lindahl *et al.* (1965).

[^{35} S]Sulphate (carrier-free), D-[1- ^{3} H]galactose (13.0 Ci/mmol) and D-[6- ^{3} H]glucosamine hydrochloride (13.4 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethansulphonic acid.

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Crystalline papain was prepared from a crude papain preparation by the method of Kimmel & Smith (1958). Pronase was purchased from Calbiochem, Los Angeles, CA, U.S.A. and chondroitin ABC lyase from Miles-Seravac, Maidenhead, Berks., U.K. Benzyl β -D-xylopyranoside was prepared as described by Robinson *et al.* (1975). Cycloheximide, methyl β -D-xylopyranoside, phenyl β -D-galactopyranoside, phenethyl β -D-galactopyranoside and phenyl β -D-thiogalactopyranoside were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

DEAE-cellulose (DE-52) was purchased from Whatman, Maidstone, Kent, U.K., Dowex 1 (X2; 200–400 mesh; Cl^- form) and Dowex 50 (X8; 200–400 mesh; H^+ form) from Serva Feinbiochemica, Heidelberg, Germany and Sephadex G-25 and G-50 from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Preparation and incubation of the mastocytoma tissue

Tumour-bearing mice were killed by cervical dislocation and the tumours, each about 1.0 g wet weight, were immediately dissected and transferred into ice-cold incubation medium. Tissue from five to 10 mice was then pressed through a stainless-steel mesh screen (no. 18) by careful scraping with a stainless-steel spatula. The minced tissue was collected in fresh medium in an ice-cold centrifuge tube, then suspended in about 50 ml of medium and centrifuged at 60 g for 3 min. The supernatant was discarded and the tissue was resuspended in about 10–20 ml of fresh medium and samples were transferred directly into incubation tubes. A separate sample was removed for dry-weight estimation. Incubations were performed in one of the following media: medium A contained 0.13 M-NaCl, 4.74 mM-KCl, 2.54 mM- CaCl_2 , 1.9 mM- MgCl_2 , 10 mM-D-glucose, 1.0 mM-L-glutamine, 1.19 mM- KH_2PO_4 and 0.02 mg of Phenol Red/ml and was buffered with 25.0 mM-Hepes adjusted to pH 7.4 with NaOH solution; medium B was Ham's F-10 medium (Ham, 1963) supplemented with 25 mM-Hepes adjusted to pH 7.4 with NaOH solution. Each solution was kept saturated with oxygen at atmospheric pressure.

Tissue suspensions (10–30 mg dry wt./ml) were incubated with constant shaking at 37°C under oxygen in tubes or conical flasks sealed with a rubber cap. Incubations were carried out in a total volume of 2.0 or 5.0 ml.

To each incubation was added 0.1 mM- $\text{Na}_2^{35}\text{SO}_4$ (about 100 mCi/mmol), 2.5 $\mu\text{Ci/ml}$ of [$1\text{-}^3\text{H}$]galactose or 10 $\mu\text{Ci/ml}$ of [$6\text{-}^3\text{H}$]glucosamine. Addition of isotope, cycloheximide and β -D-xyloside to the incubation mixture was always in a total volume of <0.1 ml. The reactions were stopped by chilling of the tubes in ice and by addition of 0.2 ml of a

solution of 0.15 M- NaBH_4 in 3.0 M-KOH per ml of incubation mixture.

Isolation of labelled glycosaminoglycans

After alkaline treatment at 4°C overnight, the tube contents were neutralized by addition of 0.05 ml of acetic acid/ml of incubation mixture. Each incubation mixture was then digested with papain at 60°C for 16 h after addition of 0.3 ml of 1.0 M-sodium acetate buffer, pH 6.0, containing 0.05 M-EDTA and 10 mg of cysteine hydrochloride/ml, 0.2 ml of 2.0 M-NaCl and 0.5 ml of papain suspension (30 mg/ml). A small amount of insoluble material remaining after papain digestion was removed by centrifugation, and labelled glycosaminoglycans were then isolated from the supernatant by precipitation with cetylpyridinium chloride or by ion-exchange chromatography on DEAE-cellulose.

Precipitation with cetylpyridinium chloride was performed directly on paper strips by the micro-method of Wasteson *et al.* (1973) or, after the addition to each sample of 0.2 ml of carrier heparin solution (10 mg/ml), by the method described previously (Robinson *et al.*, 1975). Glycosaminoglycans isolated by this method were then assayed for radioactivity and hexuronate content.

Before ion-exchange chromatography on DEAE-cellulose, the polysaccharides in the papain digest were desalted and separated from excess labelled precursor by gel filtration on a column (80 cm $\times$ 2.0 cm diameter) of Sephadex G-25 equilibrated with pyridine/acetic acid buffer. The polysaccharide fraction, which was eluted close to the void volume of the column, was then taken to dryness and dissolved in 10 ml of 0.05 M-LiCl, buffered at pH 6.0 with imidazole/HCl buffer (0.01 M with respect to imidazole).

Ion-exchange chromatography

Labelled glycosaminoglycans were separated on columns (25 cm $\times$ 1.0 cm diameter) of DEAE-cellulose buffered with 0.01 M-imidazole/HCl buffer at pH 6.0. The columns were eluted with a linear concentration gradient of LiCl, the details of which are given in the Figure legends.

Pooled fractions containing labelled peaks from the DEAE-cellulose column were concentrated to a small volume and desalted by gel filtration on a column (80 cm $\times$ 2.0 cm diameter) of Sephadex G-25 in pyridine/acetic acid buffer. The labelled compounds were recovered, free of chloride, close to the void volume of the column and were evaporated to dryness, dissolved in 0.5 ml of water and stored at -20°C .

Chromatography of labelled oligosaccharide fractions was also performed on a column of Dowex 1 (X2; 200–400 mesh; formate form) eluted with a

linear concentration gradient of ammonium formate in formic acid.

Enzymic degradation of glycosaminoglycans

Digestion with chondroitin ABC lyase was performed for 24 h at room temperature in 0.15 ml of 0.07 M-Tris/HCl buffer (pH 7.55) containing 35 μ g of Phenol Red/ml and 1.0 unit of chondroitin ABC lyase/ml. After digestion the samples (50000 c.p.m.) were stored frozen at -20°C until they were applied quantitatively to a column (95 cm $\times$ 1.0 cm diameter) of Sephadex G-50 equilibrated with 1.0 M-NaCl. Fractions (4.0 ml) were collected directly into scintillation vials and each was assayed for radioactivity. Samples of some of the digestion mixtures were also subjected to paper chromatography.

Deaminative degradation of glycosaminoglycans

Degradation with nitrous acid was performed on samples (50000 c.p.m.) by treatment with 0.24 M- NaNO_2 in 1.8 M-acetic acid for 80 min at room temperature (Lindahl *et al.*, 1973). This procedure leads to cleavage of glycosidic bonds of *N*-unsubstituted and *N*-sulphated hexosamine residues, with concomitant release of *N*-sulphate groups as inorganic sulphate. After reaction, the undegraded polysaccharide was separated from inorganic sulphate and small oligosaccharides produced during the degradation, by gel filtration on Sephadex G-50 as described in the preceding section.

Buffer solutions

Stock Hepes/NaOH buffer was prepared 0.25 M with respect to Hepes and was adjusted with saturated NaOH solution until a sample diluted to 0.025 M gave a pH of 7.4. Stock imidazole/HCl buffer was prepared 0.5 M with respect to imidazole and was adjusted with conc. HCl until a sample diluted to 0.01 M gave a pH of 6.0. Pyridine/acetic acid buffer was prepared 0.2 M with respect to pyridine and 0.2 M with respect to acetic acid.

Analytical methods

Hexuronic acid was determined by the method of Bitter & Muir (1962), with glucuronolactone as standard. Hexosamines were detected by reaction with picrylsulphonic acid (Satake *et al.*, 1960). The dry weight of the tissue suspension was estimated gravimetrically after precipitation with 5% (w/v) trichloroacetic acid. The washed precipitate was suspended in 1.0 ml of 1.0 M-ammonia solution, freeze-dried, stored for 24 h under vacuum at room temperature over P_2O_5 , and then weighed.

Samples (about 15×10^3 c.p.m. of ^3H) for hexosamine analysis were hydrolysed at 95°C for 6 h in 6.0 M-HCl and the ^3H -labelled hexosamines were then separated on a column (95 cm $\times$ 1.0 cm

diameter) of Dowex 50 (X8; 200–400 mesh; H^+ form) in 0.3 M-HCl as described by Antonopoulos (1966).

Radioactivity was measured in a Packard model 2450 scintillation counter with Insta-Gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) as scintillant.

Paper chromatography was performed on Whatman no. 3MM paper with isobutyric acid/2.0 M-ammonia solution (5:3, v/v).

Results

Glycosaminoglycan biosynthesis was studied in mouse mastocytoma tissue by incubating minced tissue *in vitro* with $\text{Na}_2^{35}\text{SO}_4$, $[6\text{-}^3\text{H}]\text{glucosamine}$ or $[1\text{-}^3\text{H}]\text{galactose}$. The results in Fig. 1 show that incorporation of label into polysaccharide was proportional to the incubation time for periods up to 4 h.

Table 1 shows the effect of cycloheximide and benzyl β -D-xylopyranoside on the incorporation of $[^{35}\text{S}]\text{sulphate}$ into polysaccharide by the mastocytoma tissue. It is apparent that cycloheximide markedly inhibited the incorporation of ^{35}S and that

Fig. 1. Time course of incorporation of label from $[^{35}\text{S}]\text{sulphate}$ (●—●) and $[1\text{-}^3\text{H}]\text{galactose}$ (○—○) into glycosaminoglycans by mouse mastocytoma tissue. Minced tissue was incubated in 2.0 ml of medium A with $\text{Na}_2^{35}\text{SO}_4$ or with $[1\text{-}^3\text{H}]\text{galactose}$ and the labelled glycosaminoglycans were isolated by precipitation with cetylpyridinium chloride as described in the Materials and methods section.

Table 1. *Effect of cycloheximide and benzyl β -D-xyloside on the incorporation of [^{35} S]sulphate into glycosaminoglycans of mouse mastocytoma tissue*

Minced mastocytoma tissue was incubated in 2.0ml of medium A for 2h with $\text{Na}_2^{35}\text{SO}_4$. Cycloheximide and benzyl β -D-xyloside were added as shown and the incorporation of [^{35} S]sulphate was measured after precipitation of the glycosaminoglycans on to paper strips with cetylpyridinium chloride as described in the Materials and methods section.

Tube no.	Additions (mM)		$10^{-6} \times$ Incorporation of ^{35}S into glycosaminoglycan (c.p.m./flask)
	Cycloheximide	Benzyl β -D-xyloside	
1	—	—	3.25
2	0.5	—	0.33
3	0.5	0.5	1.43
4	0.5	1.25	1.85
5	0.5	2.50	1.83
6	0.5	5.0	2.02
7	0.5	10	1.99
8	0.5	15	1.91
9	0.5	20	1.89
10	0.5	25	1.40

Table 2. *Effects of cycloheximide, β -D-xyloside and β -D-galactoside on [^{35} S]sulphate incorporation into glycosaminoglycans in mouse mastocytoma tissue*

Minced mastocytoma tissue was incubated in 2.0ml of medium A for 2h with $\text{Na}_2^{35}\text{SO}_4$. Additions were made as shown and the incorporation of ^{35}S into glycosaminoglycan was measured after precipitation with cetylpyridinium chloride (with carrier heparin added after the incubation) as described in the Materials and methods section.

Flask no.	Cycloheximide (mM)	Addition (mM)	$10^{-3} \times$ Sp. radioactivity of glycosaminoglycan (c.p.m./mg of hexuronate)
1	—	—	14.4
2	0.5	—	2.09
3	0.5	Benzyl β -D-xyloside (12.5)	9.40
4	0.5	Methyl β -D-xyloside (25)	4.80
5	0.5	Phenyl β -D-galactoside (25)	1.34
6	0.5	Phenethyl β -D-galactoside (25)	2.04
7	0.5	Phenyl β -D-thiogalactoside (25)	1.77

benzyl β -D-xyloside relieved this inhibition but to a limited extent only. At optimum xyloside concentration the incorporation was only 62% of the control value, yet benzyl β -D-xyloside completely relieves cycloheximide inhibition of chondroitin sulphate biosynthesis. In preparations of embryonic chicken cartilage, for instance, 1.0mM-benzyl β -D-xyloside stimulated the incorporation of [^{14}C]-acetate into chondroitin sulphate to three times the control value (Robinson *et al.*, 1975). Table 2 shows that methyl β -D-xylopyranoside also partially relieved the inhibition of glycosaminoglycan synthesis caused by cycloheximide, but β -D-galactosides had no detectable effect.

Previous studies (Helting *et al.*, 1972) on glycosaminoglycan synthesis in mouse mastocytoma have demonstrated that this tissue makes some chondroitin sulphate together with the main glycosaminoglycan product which is heparin. β -D-

Xylopyranosides and β -D-galactopyranosides are thought to function as artificial initiators of chondroitin sulphate synthesis in cartilage (Robinson *et al.*, 1975; Galligani *et al.*, 1975) so the observed effects of benzyl β -D-xyloside on glycosaminoglycan formation in the mastocytoma tissue could be due to a specific activation of chondroitin sulphate biosynthesis alone or to an effect on both chondroitin sulphate and heparin biosynthesis. To clarify this point the labelled glycosaminoglycans formed by the mastocytoma tissue were characterized.

Separation and identification of glycosaminoglycans labelled after incubation of mastocytoma tissue in vitro

Mastocytoma tissue was incubated separately with $\text{Na}_2^{35}\text{SO}_4$ and with [^3H]glucosamine as outlined in the Materials and methods section. Labelled

glycosaminoglycans were isolated after treatment of the tissue suspension with alkaline borohydride to release free chondroitin sulphate chains (Robinson & Hopwood, 1973). After alkaline treatment the entire incubation mixture was digested with papain to complete the dissolution of the tissue. The glycosaminoglycan fraction was desalted and separ-

ated from excess radioactive precursor by gel filtration on Sephadex G-25, as described in the Materials and methods section and was then subjected to ion-exchange chromatography on DEAE-cellulose. Figs. 2 and 3 show typical elution profiles for the labelled glycosaminoglycans.

Fig. 2(a) shows that, in the tissue suspension incubated in the absence of cycloheximide and β -D-xyloside, the main peak of ^{35}S -labelled poly-

Fig. 2. Ion-exchange chromatography on DEAE-cellulose of labelled glycosaminoglycans isolated from mouse mastocytoma tissue incubated *in vitro* with $\text{Na}_2^{35}\text{SO}_4$. The labelled glycosaminoglycans were mixed with carrier hyaluronic acid (2.0 mg), chondroitin sulphate (2.0 mg) and heparin (2.0 mg) and the mixture was applied to a column (25 cm × 1.0 cm diameter) of DEAE-cellulose and eluted with a gradient generated from 250 ml of 0.05 M-LiCl in 0.01 M-imidazole/HCl buffer (pH 6.0) and 250 ml of 2.0 M-LiCl in the same buffer. Fractions (10 ml) were collected and assayed for radioactivity and hexuronate content. Vertical bars marked A, B and C show the elution positions of carrier hyaluronate, chondroitin sulphate and heparin respectively. The horizontal bars indicate how the collected fractions were pooled to give fraction I (6–12) and the main polysaccharide peak, fraction II (13–30). Labelled glycosaminoglycan from tissue incubated (a) in the absence of cycloheximide and β -D-xylosides (flask 1 in Table 3) and (b) with 0.5 mM-cycloheximide and 2.5 mM-benzyl β -D-xyloside (flask 3 in Table 3) was examined.

Fig. 3. Ion-exchange chromatography on DEAE-cellulose of labelled glycosaminoglycans isolated from mouse mastocytoma tissue incubated with [^3H]glucosamine. The experimental conditions are as described in Fig. 2. Labelled glycosaminoglycan from tissue incubated with (a) no additions (flask 4 in Table 3), (b) with 0.5 mM-cycloheximide (flask 5 in Table 3) or (c) with 0.5 mM-cycloheximide and 2.5 mM-benzyl β -D-xyloside (flask 6 in Table 3). Vertical bars A, B and C; elution positions of carriers as in Fig. 2.

saccharide was eluted at the same position as the added heparin carrier although there was a leading shoulder to this peak at the position of elution of the carrier chondroitin sulphate. Fig. 3(a) shows a similar elution profile for polysaccharide labelled under the same conditions with [^3H]glucosamine. In this profile, in addition to the peak and shoulder eluted with heparin and chondroitin sulphate respectively, there was also a large peak of radioactivity eluted before the hyaluronic acid carrier probably consisting of labelled oligosaccharides derived from glycoproteins.

These elution profiles are consistent with the

Fig. 4. Gel chromatography on Sephadex G-50 of labelled glycosaminoglycans after treatment with chondroitin ABC lyase or with nitrous acid

Mouse mastocytoma tissue was incubated with [^{35}S]sulphate and labelled glycosaminoglycans were isolated by ion-exchange chromatography on DEAE-cellulose as outlined in the Materials and methods section. Samples of the polysaccharide were treated (a) with chondroitin ABC lyase or (b) with nitrous acid and applied to a column (95 cm $\times$ 1.0 cm diameter) of Sephadex G-50 equilibrated with 1.0M-NaCl. Fractions were assayed for ^{35}S radioactivity and the extent of degradation of the glycosaminoglycan was equated to the percentage of the radioactivity eluting in the retarded peak of low-molecular-weight material.

reported studies of Helting *et al.* (1972) in that they indicate that the main polysaccharide formed by the mastocytoma tissue is heparin with a much smaller amount of chondroitin sulphate. However, since the separation of these labelled polysaccharides was incomplete and to confirm the identity of these products, the fractions containing the labelled polysaccharide (fraction II in Figs. 2 and 3) were pooled and the glycosaminoglycans were characterized by degradation with chondroitin ABC lyase from *Proteus vulgaris* and with nitrous acid. The degradation products formed by this treatment were separated from undegraded polysaccharide by gel filtration on Sephadex G-50 (Fig. 4). In this manner, the extent of incorporation of label into chondroitin sulphate could be equated to the extent of degradation by chondroitin ABC lyase and the incorporation into heparin to the extent of degradation with nitrous acid (Lindahl *et al.*, 1973).

In addition, sulphated glycosaminoglycans isolated after incubation of mastocytoma tissue with [^3H]glucosamine were also characterized by acid hydrolysis of the polysaccharides and separation of labelled hexosamines by ion-exchange chromatography (Antonopoulos, 1966). In this method the incorporation of ^3H into glycosaminoglycan-linked glucosamine was taken as a measure of heparin synthesis, and incorporation into glycosaminoglycan-linked galactosamine as a measure of chondroitin sulphate synthesis. For these samples the results for the two methods agreed well (Table 3). It is clear from the results that in the absence of cycloheximide or β -xyloside about 33% of the label from [^3H]glucosamine was incorporated into the glycosaminoglycan fraction as chondroitin sulphate (or conceivably as dermatan sulphate if it were formed by this tissue) and about 67% as heparin. The corresponding values for ^{35}S -labelled polysaccharide were 20 and 80% respectively. This difference in the labelling pattern probably reflects the different molar ratios of sulphate to hexosamine in the two polysaccharides; about 1.0 for chondroitin sulphate and 2.5 for heparin.

The results in Table 3 also show that cycloheximide inhibited the incorporation of label from each of the labelled precursors into chondroitin sulphate and into heparin. This effect of cycloheximide on heparin formation suggests that the biosynthesis of this polysaccharide is similar to that of chondroitin sulphate in which the polysaccharide chains are built up on a preformed protein backbone. Inhibition of synthesis of this protein backbone causes the inhibition of biosynthesis of the entire molecule.

The results in Table 3 show that the benzyl β -D-xylopyranoside relieved the inhibition caused by cycloheximide and stimulated incorporation of label from [^{35}S]sulphate and [^3H]glucosamine into each

Table 3. *Effects of cycloheximide and benzyl β -D-xyloside on chondroitin sulphate and heparin biosynthesis in mouse mastocytoma tissue*

Mastocytoma tissue was incubated in 5.0 ml of medium B with [^{35} S]sulphate or [^{3} H]glucosamine for 4 h. The labelled glycosaminoglycans were isolated by gel filtration and ion-exchange chromatography on DEAE-cellulose as shown in Figs. 2 and 3. The incorporation of label into chondroitin sulphate and heparin was estimated by degradation with chondroitin ABC lyase and nitrous acid treatment as described in the Materials and methods section. The incorporation of label from [^{3} H]glucosamine into chondroitin sulphate was also measured by acid hydrolysis of the polysaccharide and ion-exchange separation of labelled galactosamine. Values obtained by this method are shown in parentheses. Incorporation of radioactivity into fraction I refers to the unidentified components that were eluted from DEAE-cellulose before hyaluronate (Figs. 2b and 3c).

$10^{-6} \times$ Total incorporation of radioactivity (c.p.m.)

Flask no.	Labelled precursor	Additions (mM)	Total incorporation of radioactivity (c.p.m.)			
			Total glycosaminoglycans	Chondroitin sulphate	Heparin	Fraction I
1	Na $_2^{35}$ SO $_4$	—	5.32	1.11	4.21	0.705
2	Na $_2^{35}$ SO $_4$	Cycloheximide (0.5)	0.48	0.20	0.28	0.132
3	Na $_2^{35}$ SO $_4$	Cycloheximide (0.5) + benzyl β -D-xyloside (2.5)	7.06	6.32	0.74	4.190
4	[^{3} H]Glucosamine	—	1.21	0.400 (0.410)	0.807	4.14
5	[^{3} H]Glucosamine	Cycloheximide (0.5)	0.08	0.022 (0.024)	0.044	0.80
6	[^{3} H]Glucosamine	Cycloheximide (0.5) + benzyl β -D-xyloside (2.5)	1.47	1.360 (1.36)	0.104	2.92

polysaccharide. However, the effect on chondroitin sulphate was much greater than the rather marginal effect on incorporation into heparin. This result is borne out by the elution profiles shown in Figs. 2(b) and 3(c), which exhibit peaks of radioactivity coincident with the carrier chondroitin sulphate and a small shoulder at the position of the carrier heparin. It is clear that the effect of β -D-xyloside on the biosynthesis of the two polysaccharides was quite different; incorporation into chondroitin sulphate was stimulated well above that in the control incubation (i.e. the incubation without cycloheximide), whereas incorporation into heparin remained below that for the control but was, nevertheless, greater than in the incubation with cycloheximide alone.

When the labelled glycosaminoglycan fraction, which had been isolated from mastocytoma tissue incubated with Na $_2^{35}$ SO $_4$, cycloheximide and benzyl β -D-xylopyranoside, was fractionated on DEAE-cellulose the elution profile shown in Fig. 2(b) was obtained. In addition to the main peak of radioactively labelled polysaccharide, eluted close to the carrier chondroitin sulphate, additional peaks of radioactivity were eluted just ahead of the hyaluronic acid standard. These peaks were not present in the corresponding elution profile of polysaccharide from the control incubation (Fig. 2a) or from tissue incubated with cycloheximide alone. This novel radioactive material (fraction I in Fig. 2b) appears to consist of sulphated compounds, the formation of which was a direct consequence of the addition of β -xyloside.

A similar large peak (fraction I in Fig. 3c) of ^{3} H-labelled material was observed when the polysaccharide fraction, isolated from mastocytoma tissue incubated with [^{3} H]glucosamine, cycloheximide and benzyl β -D-xyloside, was fractionated on DEAE-cellulose in the same manner (Fig. 3c). Although a large peak of radioactivity was eluted in this same position when the polysaccharide fraction from the control incubation was run (Fig. 3a), this peak was almost completely eliminated by addition of cycloheximide (Fig. 3b). It seems likely that the radioactively labelled material eluted in this early peak from the control incubation consisted of oligosaccharides derived from newly formed glycoproteins. The biosynthesis of such glycoproteins would be inhibited by cycloheximide and such oligosaccharides would not be sulphated.

The novel components in fraction I isolated from mastocytoma tissue incubated with cycloheximide and β -D-xyloside did not correspond in elution position to a known polysaccharide component of the mastocytoma tissue and for this reason the radioactivity in these peaks has not been included in the total glycosaminoglycan fraction shown in Table 3. The incorporation into these peaks is, however, shown separately as fraction I in this Table and was clearly large in relation to that of the identified glycosaminoglycans.

Characterization of the components in fraction I

These compounds were eluted close to the non-sulphated glycosaminoglycan hyaluronic acid (Figs. 2b and 3c). The elution profiles shown in Fig.

5 were obtained when samples of fraction I, labelled with [^{35}S]sulphate and [^3H]glucosamine respectively, were eluted separately from DEAE-cellulose with a more shallow concentration gradient of LiCl. The radioactivity in each sample was eluted as a number of overlapping peaks, which were eluted after glucuronic acid but before hyaluronic acid. The two elution profiles were not identical and indicated clearly that each sample contained a number of labelled compounds.

Elution profiles obtained by gel filtration of fraction I components, labelled with [^3H]glucosamine, on Sephadex G-25 and G-50 are shown in Fig. 6. The elution profiles of fraction I components labelled with [^{35}S]sulphate were essentially identical

with these. The labelled compounds were eluted from Sephadex G-25 close to the void volume of the column but they were retarded considerably on Sephadex G-50. These results indicate that the components in these fractions are of relatively low molecular weight, probably within the range 1500–5000. The low molecular weight of these labelled compounds was also confirmed by the observation that most of the radioactivity in fraction I, labelled with [^{35}S]sulphate, moved from the origin during paper chromatography.

The elution profiles of these compounds from Sephadex G-25 and G-50 were not appreciably altered by prior treatment of the material with

Fig. 5. Ion-exchange chromatography on DEAE-cellulose of fraction I

(a) A sample of fraction I labelled with [^3H]glucosamine (Fig. 3c; fractions 5–13) was subjected to ion-exchange chromatography on DEAE-cellulose with a shallow concentration gradient of LiCl generated from 250 ml of 0.01 M-imidazole/HCl buffer (pH 6.0) and 250 ml of 0.8 M-LiCl in the same buffer. Fractions (7.0 ml) were collected and assayed for radioactivity. The bars marked G and A show the elution positions of glucuronic acid and hyaluronic acid standards respectively. (b) A sample of fraction I labelled with [^{35}S]sulphate (Fig. 2b; fractions 6–12) was fractionated in the same way.

Fig. 6. Gel filtration of fraction I labelled with [^3H]glucosamine

A sample of this material was subjected to gel-filtration on: (a) a column (95 cm $\times$ 1.0 cm diameter) of Sephadex G-50 in 1.0 M-NaCl and (b) a column (188 cm $\times$ 1.0 cm) of Sephadex G-25 in 0.2 M-NaCl, as outlined in the Materials and methods section. Eluted fractions were assayed for radioactivity.

Fig. 7. Ion-exchange chromatography on Dowex 1 of a mixture of fraction I labelled separately with [^3H]glucosamine (1.8×10^5 c.p.m.) and with [^{35}S]sulphate (5.75×10^4 c.p.m.)

The mixture was applied to a column (28 cm $\times$ 1.0 cm diameter) of Dowex 1 (X2; 200–400 mesh; formate form) and was eluted with a linear gradient generated from 250 ml of water and 250 ml of 2.0 M-ammonium formate in 2.5 M-formic acid, followed by a linear gradient generated from 250 ml of 2.0 M-ammonium formate in 2.5 M-formic acid and 250 ml of 4.0 M-ammonium formate in 0.5 M-formic acid. The column was eluted at a rate of 30 ml/h and fractions of (5.0 ml) were collected and assayed for ^{35}S (●—●) and ^3H (O—O) radioactivity.

nitrous acid indicating the absence of *N*-sulphate groups (Lindahl *et al.*, 1973).

Fraction I, labelled with [^3H]glucosamine, was subjected to acid hydrolysis in 6.0 M-HCl at 95°C for 4 h and the labelled hexosamines were then separated by ion-exchange chromatography as described in the Materials and methods section. Radioactivity was eluted with glucosamine (65% of the eluted radioactivity) and with galactosamine (35%).

Samples of fraction I labelled with [^3H]glucosamine and with [^3H]sulphate were combined and the mixture was fractionated by ion-exchange chromatography on Dowex 1 (X2) with an ammonium formate gradient (Fig. 7). The elution profiles of ^{35}S and ^3H did not coincide exactly, but one or two of the major peaks clearly corresponded to components labelled with both isotopes. A sample from the main peak (Fig. 7; fractions 78–81) was isolated, subjected to acid hydrolysis and the labelled hexosamines were separated by ion-exchange chromatography. More than 85% of the radioactivity was eluted with the glucosamine peak and the remainder with galactosamine. These results indicate that the main component in fraction I was a compound of low molecular weight containing both glucosamine and sulphate.

It seems likely that such a compound would be an oligosaccharide containing benzyl β -D-xyloside at the reducing terminal position and, in addition, the components of the heparin linkage region together with a limited number of disaccharide units com-

posed of glucuronic acid, *N*-acetylglucosamine and *O*-sulphate groups (Lindahl, 1966).

The small amount of labelled galactosamine recovered from this main peak does not necessarily indicate that the main component also contained galactosamine, since separation between the different peaks was not complete. It may indicate the presence of analogous oligosaccharides related to chondroitin sulphate but such oligosaccharides have not been detected in similar studies on chondroitin sulphate synthesis in embryonic chicken cartilage (Robinson *et al.*, 1975).

Separate experiments in which mastocytoma tissue was incubated with [^{35}S]sulphate and 2.5 mM-benzyl β -D-xylopyranoside in the absence of cycloheximide established that cycloheximide was not essential for the formation of these novel sulphated compounds in fraction I. Ion-exchange chromatography on DEAE-cellulose of the labelled glycosaminoglycan fraction from such incubation mixtures yielded just as much ^{35}S in the early peak as from a similar incubation containing 0.5 mM-cycloheximide.

Discussion

The proteoglycan form of heparin shows structural features that are shared with other proteoglycans, including those of chondroitin sulphate, dermatan sulphate and heparan sulphate (Lindahl & Höök, 1978). The heparin proteoglycan has, however, unique features, apart from the structure of the

polysaccharide chain itself, that distinguish it from other proteoglycans. The polypeptide core, for instance, contains extended sequences of only two amino acids, serine and glycine (Robinson *et al.*, 1978) and unlike chondroitin sulphate and dermatan sulphate proteoglycans, that are secreted into the extracellular matrix of connective tissues, heparin proteoglycan undergoes, in most tissues, intracellular postbiosynthetic modification that involves release and limited depolymerization of free polysaccharide chains by specific endoglycosidases (Ögren & Lindahl, 1975, 1976; Young & Horner, 1979).

Although biosynthesis of the heparin proteoglycan has been studied in some detail with regard to the formation of the polysaccharide chains [see Jacobsson *et al.* (1979) for references] the mechanisms of macromolecular assembly are virtually unknown. In view of the structural similarities it might tacitly be assumed that the proteoglycan of heparin is formed in a manner analogous to that of other glycosaminoglycans, glycosylation of the protein core preceding and initiating the growth of polysaccharide chains (Rodén & Schwartz, 1975). The results of the present study demonstrate clearly that the biosynthesis of heparin resembles that of chondroitin sulphate, at least to the extent that inhibition of protein synthesis by cycloheximide results in inhibition of polysaccharide chain synthesis. Formation of the polypeptide core is thus prerequisite to the formation of heparin chains. On the other hand, the polypeptide core in the heparin proteoglycan is much more heavily substituted than that of any proteoglycan described, and the formation of such a macromolecule may well require mechanisms that deviate from those of the currently accepted model for glycosaminoglycan biosynthesis. For instance, it may be noted that a polyprenol derivative of the disaccharide GlcA-(1→4)-GlcNAc has been identified in various types of cells, suggesting a possible role for this lipid in the biosynthesis of heparin-like polysaccharides (Turco & Heath, 1977). The involvement of a lipid intermediate cannot be excluded solely on the ground of the demonstrated requirement for protein core biosynthesis (see below).

In contrast to the similarity of the effect of cycloheximide on chondroitin sulphate and heparin biosynthesis respectively, the addition of β -D-xyloside gave markedly divergent results. The effects of β -D-xyloside on chondroitin sulphate biosynthesis in the mouse mastocytoma was essentially the same as that observed in cartilage (Robinson *et al.*, 1975). With the mastocytoma preparation addition of 2.5 mM-benzyl β -D-xyloside caused a marked stimulation of incorporation of label from [^{35} S]sulphate and [^{3} H]glucosamine into chondroitin sulphate, which more than completely relieved the inhibition

caused by cycloheximide. The effect of β -D-xyloside on heparin biosynthesis was quite different. Incorporation of [^{35} S]sulphate and [^{35} H]glucosamine into heparin by mastocytoma tissue incubated with β -D-xyloside and cycloheximide was increased over the level incorporated by tissue incubated with cycloheximide alone but fell far short of the level of incorporation in tissue incubated in the absence of cycloheximide or β -D-xyloside. This difference in the effect of β -D-xyloside on the biosynthesis of the two glycosaminoglycans within the same tissue preparation (and probably within the same cells; Helting *et al.*, 1972) must reflect a difference in the mechanism of chain elongation. Free chondroitin sulphate chains formed by cartilage in the presence of cycloheximide and β -D-xyloside are of high molecular weight and do not exhibit a markedly altered pattern of sulphation (Robinson *et al.*, 1975). This indicates that the protein-core moiety is not essential to the formation of the chondroitin sulphate chains subsequent to their initiation. Heparin biosynthesis appears to be different in this regard. The lack of any marked effect of β -D-xyloside on heparin formation could reflect: (a) the inability of the galactosyltransferases involved in the biosynthesis of the heparin-protein linkage oligosaccharide to use benzyl β -D-xyloside as a substrate in place of the natural core-protein substrate or (b) a breakdown in polysaccharide chain formation at some stage subsequent to the synthesis of the linkage oligosaccharide and before the completion of entire polysaccharide chains. The identification of a large quantity of labelled material that appeared to consist of oligosaccharide fragments formed in the presence of β -D-xyloside supports the latter notion. The slight stimulation of incorporation of [^{35} S]sulphate and [^{3} H]glucosamine into heparin on addition of β -D-xyloside also suggests that the first choice is unlikely. However, the lack of any stimulation of glycosaminoglycan synthesis by high concentrations of β -D-galactosides, that are effective initiators of chondroitin sulphate biosynthesis in cartilage (Robinson *et al.*, 1975), does indicate that the galactosyl transferases of mouse mastocytoma are somewhat different from those found in cartilage.

The nature of the biosynthetic block that causes an early breakdown of polysaccharide synthesis and leads to the production of incomplete chain fragments is intriguing. One possibility, alluded to above, implies that the nascent heparin chains are lipid-bound, and are transferred, after completion, on to oligosaccharide acceptors that are attached to the polypeptide core. In the absence (induced by cycloheximide) of appropriate oligosaccharide acceptors polysaccharide synthesis may stop owing to lack of free lipid carrier; the results of xyloside administration could be explained by assuming that the polypeptide-bound oligosaccharides are more

efficient as polysaccharide chain acceptors than are the xyloside-bound analogues. However, alternative possibilities, based on the 'conventional' biosynthesis model may also be considered. Previous studies on the biosynthesis of heparin (summarized by Lindahl *et al.*, 1977) point to a highly organized process in which membrane-bound polymeric intermediates undergo a series of modification reactions (*N*-deacetylation, *N*-sulphation, uronosyl C-5-epimerization and *O*-sulphation) in a sequential, stepwise manner. The overall process must be profoundly influenced by the subcellular organization of the biosynthetic apparatus, including such factors as the spatial arrangement of enzymes in the membranes and the mode of transport of intermediates between, and along, these enzymes. This organization assumes an additional degree of complexity, when the macromolecular properties of the polysaccharide substrate are considered, each intermediate molecule presumably consisting of a large number of polysaccharide chains held in close proximity to each other by attachment to a short polypeptide core. It is tempting to speculate that the polypeptide core has an ordering function that facilitates access, in the appropriate fashion, of the initiated polysaccharide chains to the polymerizing enzymes, and subsequently to the polymer-modifying enzymes. The formation of oligosaccharides containing *O*-sulphate groups could thus reflect a biosynthetic error due to unordered transport of synthesis products between the various enzymes.

In consequence of the above views the biosynthesis of chondroitin sulphate would seem to be less dependent on the ordering influence of the polypeptide core (although some galactosamine-containing oligosaccharides were also formed by the mastocytoma tissue in the presence of β -D-xyloside). This may suggest that the ordering effect of the polypeptide core becomes less important as the distance between the polysaccharide-substituted serine residues increases. Without direct evidence to that effect, it may be relevant to consider the effects of xylosides on the biosynthesis of other glycosaminoglycans. With different types of cultured cells the formation of both dermatan sulphate (Galligani *et al.*, 1975; Bradbeer & Robinson, 1979) and heparin sulphate (Johnston & Keller, 1979; however, see Galligani *et al.*, 1975) was stimulated to a much larger extent than was the formation of heparan in the mastocytoma system. Although the molecular size of the resulting products was somewhat reduced in relation to that of untreated controls it was still clearly within the polysaccharide range, indicating minor interference with the mechanism of polymerization. Proteoglycans of heparan sulphate and dermatan sulphate generally contain much more protein than the heparin proteoglycans studied, and the polypeptide-core

structures are obviously less densely substituted [see Lindahl & Höök (1978) for references]. These findings are thus in good agreement with the notions expressed above. The results relating to heparan sulphate biosynthesis are of special interest, since the carbohydrate structure of heparan sulphate is similar to that of heparin; the major structural difference between the corresponding proteoglycans lies presumably in the polypeptide-core moieties.

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