The Mode of Action of 4-Methylumbelliferyl β -D-Xyloside on the Synthesis of Chondroitin Sulphate in Embryonic-Chicken Sternum

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1. Embryonic-chicken sterna, incubated in medium containing 0.1 mM 4-methylumbelliferyl β -D-xyloside (4-methylcoumarin 7- β -D-xyloside), synthesize proteochondroitin sulphate that is significantly undersulphated and shorter than usual [Gibson, Segen & Audhya (1977) *Biochem. J.* **162**, 217–233].
2. Neither the β -D-galactoside nor the β -D-glucuronide of 4-methylumbelliferone, nor 4-methylumbelliferone itself, produced the effects. The only metabolites of 4-methylumbelliferone that were detected in cartilages exposed to 4-methylumbelliferyl β -D-xyloside were unchanged xyloside and chondroitin sulphate covalently attached to 4-methylumbelliferone.
3. Gel filtration of salt extracts of sterna incubated in medium containing the xyloside showed that there were two pools of chondroitin sulphate in the tissue. One pool was identified, on the basis of its elution pattern and the linear kinetics of incorporation of sulphate into it, as proteochondroitin sulphate. Incorporation into the other pool, whose properties suggested that it was methylumbelliferyl-chondroitin sulphate, indicated that it underwent partial turnover. The molecular weight of this chondroitin sulphate was about 19000, and it appeared to be about 70% sulphated.
4. When sterna were incubated in medium containing the xyloside, there was a very large incorporation of sulphate and glucose into glycosaminoglycans that were released into the incubation medium. This contrasts with incubations of sterna in the absence of the xyloside, in which less than 5% of the sulphate incorporated could be recovered from the medium. The glycosaminoglycan released into the medium was 4-methylumbelliferyl-chondroitin sulphate, whose average molecular weight was 7000–8000 and degree of sulphation more than 95%.
5. Incorporation of sulphate into proteochondroitin sulphate was stimulated more than 3-fold by addition of 20% (v/v) human serum and 10 nM L-3,3',5-tri-iodothyronine. Incorporation into methylumbelliferyl-chondroitin sulphate, in either the tissue or the medium, was not significantly altered.
6. The decrease in chain length and degree of sulphation of proteochondroitin sulphate is explained in terms of competition between peptide-linked primers and methylumbelliferone-containing primers at the intracellular sites of polysaccharide-chain elongation and sulphation. The implications of the results for the mechanism of stimulation of proteoglycan synthesis by serum factors are discussed.

The ability of embryonic cartilage or differentiated chondrocytes to use β -D-xylosides as primers for chondroitin sulphate synthesis is well documented (Okayama *et al.*, 1973; Schwartz *et al.*, 1974; Fukunaga *et al.*, 1975; Robinson *et al.*, 1975; Galligani *et al.*, 1975; Schwartz *et al.*, 1976). In the presence of inhibitors of protein synthesis, formation of the natural proteochondroitin sulphate by these cells is blocked; but, if a β -xyloside is present as well, chondroitin sulphate synthesis is efficiently promoted. By appropriate choice of the aglycone moiety, β -xylosides have been found that appear to prime the synthesis of chondroitin sulphate more efficiently than does the natural peptidyl-xyloside (Robinson *et al.*, 1975). The chondroitin sulphate formed in such incubations is

known to be covalently linked to the aglycone in at least one case, namely that of 4-methylumbelliferyl β -D-xyloside (Fukunaga *et al.*, 1975).

Gibson *et al.* (1977) presented evidence that embryonic-chicken sternum, incubated *in vitro* in medium containing low concentrations of 4-methylumbelliferyl β -D-xyloside, synthesizes proteochondroitin sulphate with shortened, undersulphated, side chains. At low concentrations of the β -xyloside (0.1 mM), there appeared to be little decrease in the synthesis of the major proteoglycan fraction as measured by the incorporation of serine into this fraction; however, the combination of a 25% decrease in chain length and a 30% deficit in sulphation produced a decrease of 50–70% in the total amount of sulphate incorporated into glycosamino-

glycans. Stimulation of proteoglycan synthesis by serum factors, including L-3,3',5-tri-iodothyronine (Audhya & Gibson, 1974), was comparable with the stimulation observed in the absence of xyloside (Audhya *et al.*, 1976). In the present paper, we report experiments designed to elucidate the mechanism by which the xyloside affects the synthesis of chondroitin sulphate. It is possible that 4-methylumbelliferyl β -D-xyloside itself, or a metabolite, might directly inhibit one or more of the enzymes involved in the elongation and sulphation of chondroitin chains. Alternatively, the presence of the xyloside might prime the initiation of many new chondroitin chains; this would cause a fall in the average length and degree of sulphation of completed chains, if the rates of chain elongation and sulphation did not rise in proportion. The results presented here are not easily reconciled with the first mechanism, but offer strong support for a modified version of the second one. Further, they show that chick-embryo sternum, incubated in medium containing 0.1 mM-4-methylumbelliferyl β -D-xyloside, synthesizes at least two classes of chondroitin sulphate, which differ greatly in chain length and degree of sulphation.

Experimental

Materials

4-Methylumbelliferyl β -D-galactopyranoside, 4-methylumbelliferyl β -D-glucuronide and 4-methylumbelliferone were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sources of all other materials are as described by Gibson *et al.* (1977).

Methods

Incorporation of radioactive precursors into glycosaminoglycans and proteoglycans. Sterna from 12-day chick embryos were incubated individually in 1 ml of medium as described in detail previously (Audhya *et al.*, 1976; Gibson *et al.*, 1977). After preincubation for 30 min, radioactive precursors were added as indicated in the Tables and Figures, and incubation was continued for periods up to 6 h. Incorporation into glycosaminoglycans was determined after digestion of the sterna with papain and precipitation with Alcian Blue (Audhya & Gibson, 1974). In some experiments the wet weights of the sterna were determined before digestion.

When incorporation into glycosaminoglycans released into the medium was to be measured, chondroitin sulphate A+C (100 μ g/ml) was added to the incubation medium at the start of the incubation. At the end of the incubation, portions (0.2–0.4 ml) of the medium were precipitated by addition of Alcian Blue as described for the isola-

tion of glycosaminoglycans from the sterna, except that the precipitate was washed three times with 95% (v/v) ethanol rather than twice.

Crude preparations of glycosaminoglycans were obtained by precipitation of protein with trichloroacetic acid followed by exhaustive dialysis and freeze-drying (Audhya *et al.*, 1976). Proteoglycans were extracted with 0.5 M-LaCl₃ or with 4 M-guanidinium chloride as described (Gibson *et al.*, 1977).

Gel filtration. Preparations of glycosaminoglycans were chromatographed either on a calibrated column (0.9 cm $\times$ 101 cm) of Sepharose 6B, with 0.2 M-NaCl as eluent [see Gibson *et al.* (1977) for details], or on a column (0.9 cm $\times$ 96 cm) of Sephadex G-50 which was developed with the same solvent. The latter column was operated at a flow rate of 2 ml/h; the effluent was separated into fractions (approx. 0.75 ml). Extracts containing proteoglycan were chromatographed either on the column of Sepharose 6B referred to or on a smaller column (0.6 cm $\times$ 98 cm) which was not calibrated. This column was operated at a flow rate of 1 ml/h, and fractions (approx. 0.4 ml) were collected. Fraction sizes were determined gravimetrically in all cases.

Analysis of glycosaminoglycans. Digestion with chondroitin ABC lyase (EC 4.2.2.4) was as described by Gibson *et al.* (1977), except that the enzyme concentration was increased to 0.8–1.0 unit/ml (a unit of activity is defined as 1 μ mol/min). A single batch of enzyme was used in the present work: its activity was checked by incubating 0.02 unit with 0.8 mg of chondroitin sulphate A+C at 37°C in a volume of 1 ml, and following the reaction by the increase in A_{232} (Yamagata *et al.*, 1968). Digestion was complete in 1 h, and had reached 50% completion within 20 min.

Chromatography. Paper chromatography of the products of digestion by chondroitin lyase was as described by Gibson *et al.* (1977), except that the solvent was always freshly made up and the chromatograms were developed for either 40 or 70 h. For measurement of 4-methylumbelliferone fluorescence and radioactivity, the paper was cut into 1 cm strips, which were placed in tubes containing 1 ml of 0.1 M-HCl and heated at 100°C for 15–20 min. The contents of the tubes were adjusted to pH 10–10.5 by addition of 0.3 ml of 1 M-glycine/NaOH, pH 10.3, and 0.1 ml of 1 M-NaOH, and the volume was made up to 2 ml. The paper strips were removed and the fluorescence of the solutions was determined; portions (1.0 ml) of the solutions were then removed for determination of radioactivity.

T.l.c. was performed on cellulose plates, with butan-1-ol/acetic acid/2 M-NH₃ (2:3:1, by vol.) as solvent. The plates were cut into 0.5 cm strips, which were scraped into tubes containing 1 ml of 0.1 M-HCl. Fluorescence and radioactivity were determined as described above.

Analytical methods. Samples containing 4-methylumbelliferone in covalent linkage were hydrolysed in 0.1M-HCl at 100°C for 15 min, neutralized by addition of 1M-NaOH and adjusted to pH 10–10.5 by addition of 1M-glycine/NaOH, pH 10.3, to a final concentration of 0.15M. Standards of 4-methylumbelliferone, and blanks, were made up in the same way. Methylumbelliferone was then determined fluorimetrically with an Aminco-Bowman spectrofluorimeter, by using excitation and emission wavelengths of 364 and 448 nm (Leaback & Walker, 1961). Free methylumbelliferone was determined similarly, but without initial acid hydrolysis.

Radioactivity was determined as described by Gibson *et al.* (1977). In most experiments, incorporations of radioactive isotopes were converted into pmol or nmol by comparison with appropriate standards counted under identical conditions. All other analytical methods have been described (Audhya *et al.*, 1976; Gibson *et al.*, 1977).

Results

When sterna are incubated in the presence of 0.1 mM-4-methylumbelliferyl β -xyloside, incorporation of sulphate into Alcian Blue-precipitable glycosaminoglycans is decreased by 60–75% compared with control incubations (Gibson *et al.*, 1977; see Table 1). In double-labelling experiments with [^3H]glucose and [^{35}S]sulphate, the $^3\text{H}:^{35}\text{S}$ molar ratio in the glycosaminoglycans increases by 40–50%, as a result of a 30% decrease in the degree of sulphation of newly synthesized chondroitin sulphate. Similar effects are observed when 0.25 mM-*p*-nitrophenyl β -xyloside is substituted for 4-methylumbelliferyl β -xyloside. When other glycosides of 4-methylumbelliferone, or 4-methylumbelliferone itself, were added to the incubation medium at concentrations of 0.1 mM or

higher, there was either no change or a small increase in the amount of sulphate incorporated into glycosaminoglycans of cartilage, but in no case was there any significant decrease (Table 1). In the incubations in which 4-methylumbelliferyl β -D-glucuronide was added to the medium, a small increase in the $^3\text{H}:^{35}\text{S}$ ratio implied that the chondroitin sulphate was undersulphated, but not to the same extent as chondroitin sulphate synthesized in the presence of the xyloside. Neither the β -galactoside nor 4-methylumbelliferone itself appeared to affect the $^3\text{H}:^{35}\text{S}$ ratio. Table 1 also shows that at the end of the incubation period the sterna contained 4-methylumbelliferone in all cases, suggesting that the failure of compounds other than the β -xyloside to decrease sulphation did not result from an inability to penetrate the tissue.

When sterna are incubated in synthetic medium alone, incorporation of sulphate proceeds at a constant rate for at least 6 h (Audhya & Gibson, 1974). If the incubation medium contains serum, and especially if thyroid hormones are also added, there is a short lag, lasting less than 2 h, before incorporation again becomes linear with time (Audhya & Gibson, 1975; cf. Fig. 1). However, addition of 0.1 mM-4-methylumbelliferyl β -xyloside to the incubation medium changed the kinetics of incorporation. Sterna were incubated in medium containing the xyloside for 30 min; [^{35}S]sulphate was then added, and incorporation into glycosaminoglycans was followed as a function of time (Fig. 1). There was an initial rapid incorporation lasting for approx. 30 min, followed by a phase of about 1.5 h during which the rate of incorporation fell to a low value, after which it continued to increase slowly until the experiment was terminated. Very similar kinetics were seen when the experiment was repeated in the presence of 20% human serum and 10 nM-tri-iodothyronine, except

Table 1. Incorporation of sulphate and methylumbelliferone into chick-embryo sterna in the presence of derivatives of methylumbelliferone

Sterna were incubated in medium containing [6- ^3H]glucose (3.3 mCi/mmol) and [^{35}S]sulphate (0.5 mCi/mmol) for 6 h. After digestion with papain, portions (0.1 ml) were removed for determination of incorporation into glycosaminoglycans, or for fluorimetric analysis of 4-methylumbelliferone, as described under 'Methods'. Incorporation into glycosaminoglycans and uptake of methylumbelliferone are expressed as mean values $\pm$ S.E.M. for groups of five sterna, after conversion into pmol/ μg of chondroitin sulphate for each sternum. Apparent $^3\text{H}:^{35}\text{S}$ molar ratios are expressed as mean values $\pm$ S.D. for the same groups.

Addition to the incubation medium (mM)	Incorporation into glycosaminoglycans (pmol/ μg of chondroitin sulphate)			Uptake of 4-methylumbelliferone (pmol/ μg of chondroitin sulphate)
	[^3H]Glucose	[^{35}S]Sulphate	Molar ratio $^3\text{H}:^{35}\text{S}$	
None	73.7 $\pm$ 2.9	74.9 $\pm$ 2.9	0.99 $\pm$ 0.07	—
4-Methylumbelliferyl β -xyloside (0.1)	25.9 $\pm$ 1.4	18.9 $\pm$ 0.7	1.38 $\pm$ 0.09	1.56 $\pm$ 0.19
4-Methylumbelliferyl β -galactoside (0.1)	86.2 $\pm$ 5.8	79.2 $\pm$ 4.2	1.08 $\pm$ 0.08	0.71 $\pm$ 0.16
4-Methylumbelliferyl β -galactoside (1.25)	78.3 $\pm$ 3.6	76.3 $\pm$ 2.1	1.03 $\pm$ 0.07	17.8 $\pm$ 1.4
4-Methylumbelliferyl β -glucuronide (0.1)	117.3 $\pm$ 7.5	99.1 $\pm$ 4.5	1.18 $\pm$ 0.06	0.42 $\pm$ 0.11
4-Methylumbelliferone (0.1)	92.1 $\pm$ 1.3	87.7 $\pm$ 1.2	1.05 $\pm$ 0.03	1.03 $\pm$ 0.16

Fig. 1. Time course of incorporation of sulphate into sternal glycosaminoglycans

Sterna were incubated in medium containing [^{35}S]-sulphate (15mCi/mmol) for the times indicated, and incorporation into glycosaminoglycans of cartilage was determined as described under 'Methods'. Results are expressed as mean values $\pm$ S.E.M. for groups of five sterna. Incubations were performed in media to which were added: ●, nothing; ○, 20% (v/v) human serum and 10nM-tri-iodothyronine; ▲, 0.1mM-4-methylumbelliferyl β -xyloside; △, 0.1mM-4-methylumbelliferyl β -xyloside, 20% (v/v) human serum and 10nM-tri-iodothyronine.

that the final rate of incorporation was nearly equal to that in control incubations (Fig. 1).

Another change in the kinetics of incorporation became apparent in pulse-chase experiments. Sulphate incorporated into glycosaminoglycans of sterna in control incubations is not lost during a subsequent chase of up to 4h, and the same is true if the pulse and chase are carried out in medium containing 20% human serum and 10nM-tri-iodothyronine (Audhya *et al.*, 1976). When experiments of this type were performed with 0.1mM-4-methylumbelliferyl β -xyloside added to the incubation medium, there was a significant decrease in the specific radioactivity of glycosaminoglycans of cartilage during chases of up to 4h (Table 2). The decrease occurred within the first 2h of the chase with no subsequent loss of label during the remainder of the chase (Table 2, Expt. 2), and the same decrease was observed whether the chase was performed in medium containing 0.1mM-4-methylumbelliferyl β -xyloside or in medium lacking xyloside (Table 2, Expt. 3). When the chase was

performed in medium containing no xyloside, approx. 80% of the 4-methylumbelliferone that had been taken up by the cartilage during the pulse was lost from the tissue and could be recovered in the medium. Addition of 20% human serum and 10nM-tri-iodothyronine to the incubation medium did not prevent the loss of radioactivity during the chase (Table 2, Expt. 1).

One possibility suggested by the time course of incorporation in Fig. 1 is that 4-methylumbelliferyl β -xyloside is metabolized in the cartilages, and one of its metabolites actually produces the observed effects on chondroitin sulphate synthesis. To test these possibilities, we investigated the uptake of 4-methylumbelliferyl β -xyloside and its subsequent disposition by the sterna. When sterna were incubated in medium containing the xyloside at a concentration of 0.1mM, 4-methylumbelliferone was detected in papain digests after 30min, and reached a peak concentration of about 30pmol/mg wet wt. (range 23–38pmol/mg) after 1h. The concentration in the digests then remained constant or declined slightly during the next 3h. If the incubation medium contained 20% (v/v) human serum and 10nM-tri-iodothyronine, the concentration of 4-methylumbelliferone in the digests was generally about 25% lower. After deproteinization and exhaustive dialysis, 60–70% of the total methylumbelliferone in these digests was recovered in the non-diffusible material. Direct measurement of fluorescence, without initial acid hydrolysis, indicated that no free 4-methylumbelliferone was present in any of the digests. Also, analysis of non-dialysed digests by t.l.c. revealed only two fluorogenic spots; one of these co-migrated with 4-methylumbelliferyl β -xyloside and the other remained at the origin. The results indicated that approximately two-thirds of the 4-methylumbelliferone taken up by the sterna was incorporated into a macromolecular compound and the rest remained in the form of the β -xyloside.

The macromolecular compound containing methylumbelliferone was identified as chondroitin sulphate by subjecting crude preparations of glycosaminoglycans from sterna that had been incubated in medium containing [^{35}S]sulphate and 0.1mM-4-methylumbelliferyl β -xyloside to digestion by chondroitin lyase, followed by paper chromatography and analysis of fluorescence and radioactivity as described under 'Methods'. When the glycosaminoglycans were chromatographed without exposure to chondroitin lyase, all the radioactivity remained at the origin of the chromatogram (Fig. 2a). A significant, but relatively constant, background fluorescence was observed along the length of the chromatogram, but there was only one sharp peak of fluorescence, which coincided exactly with the peak of radioactivity at the origin (Fig. 2a). If the glycosaminoglycans were digested with chondroitin lyase before chromato-

Table 2. *Incorporation of sulphate and methylumbelliferone into chick-embryo sterna in pulse-chase experiments*

Sterna were incubated in medium containing 0.1 mM-4-methylumbelliferyl β-xyloside and [³⁵S]sulphate (2.5 mCi/mmol in Expts. 1 and 2; 20 mCi/mmol in Expt. 3). They were then washed with 5 ml of 0.15 M-NaCl and transferred to medium containing no radioactive isotopes for the period indicated as 'chase'. In Expts. 1 and 2, the composition of the medium during the chase was the same as during the pulse. In Expt. 3, the medium during the chase was either unsupplemented (Expt. 3c) or contained 0.1 mM-4-methylumbelliferyl β-xyloside (Expt. 3b). Portions (0.1 ml) of the papain digests of the sterna were removed for determination of radioactivity in glycosaminoglycans as outlined in the legend to Table 1. In Expt. 3, additional portions (0.1 ml) were removed from the digests, and where indicated from the incubation medium, for fluorimetric analysis of 4-methylumbelliferone. Results are expressed as mean values ± S.E.M. for groups of five sterna, after conversion into pmol/μg of chondroitin sulphate for each sternum.

Expt. no.	Additions to the incubation medium during the pulse	Length of pulse (h)	Length of chase (h)	Incorporation into glycosaminoglycans (pmol/μg of chondroitin sulphate)	4-Methylumbelliferone remaining in cartilage (pmol/μg of chondroitin sulphate)	4-Methylumbelliferone released into chase medium (pmol/μg of chondroitin sulphate)
1	—	2	—	11.6 ± 0.6	—	—
		2	3	7.4 ± 0.2	—	—
	20% (v/v) Human serum	2	—	22.1 ± 1.0	—	—
	+10 nM-tri-iodothyronine	2	3	15.8 ± 0.9	—	—
2	—	1	—	6.0 ± 2.4	—	—
		1	2	2.3 ± 0.1	—	—
		1	4	2.1 ± 0.1	—	—
	—	3	—	12.8 ± 0.4	—	—
		3	2	6.7 ± 0.4	—	—
		3	4	6.9 ± 0.3	—	—
3 (a)	—	1	0	6.0 ± 0.4	3.7 ± 0.1	—
(b)	—	1	2	2.3 ± 0.1	3.4 ± 0.1	—
		1	4	2.1 ± 0.1	3.5 ± 0.2	—
(c)	—	1	2	2.4 ± 0.2	0.6 ± 0.0	3.0 ± 0.1
		1	4	2.1 ± 0.1	0.5 ± 0.0	3.4 ± 0.2

graphy, approx. 80% of the radioactivity was distributed equally between the two peaks, whose migration corresponded to that of Δ-di-4S* and Δ-di-6S (Fig. 2b). Only one significant peak of fluorescence was observed, about 5 cm from the origin of the chromatogram (Fig. 2b). From the *R_F* values reported by Fukunaga *et al.* (1975), we estimate that the position of this peak corresponds to the position that would be expected for the linkage-region tetrasaccharide that is released when chondroitin sulphate covalently linked to methylumbelliferone is digested by chondroitin lyase. Also, after subtracting the background fluorescence due to the paper, the total fluorescence of this peak accounted for more than 95% of the fluorescence applied to the paper. Identical results were obtained with glycosaminoglycans from sterna that had been incubated in medium containing 0.1 mM-4-methylumbelliferyl β-xyloside, 20% (v/v) human serum and 10 nM-tri-iodothyronine (Figs. 2c and 2d).

Further evidence for the presence of chondroitin sulphate covalently linked to 4-methylumbelliferone

* Abbreviations: Δ-di-0S, 3-*O*-β-(gluc-4-eneuronosyl)-*N*-acetylgalactosamine; Δ-di-4S, 3-*O*-β-(gluc-4-eneuronosyl)-4-*O*-sulpho-*N*-acetylgalactosamine; Δ-di-6S, 3-*O*-β-(gluc-4-eneuronosyl)-6-*O* sulpho *N*-acetylgalactosamine; Δ*-GlcA, gluc-4-eneuronic acid; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; Xyl, xylose.

was obtained by extracting proteoglycans from sterna with 0.5 M-LaCl₃ and analysing the extracts by chromatography on an agarose column (Fig. 3). When an extract from sterna that had been incubated with [³⁵S]sulphate in the absence of 4-methylumbelliferyl β-xyloside was examined in this way, all the radioactivity was eluted in the void volume of the column (Fig. 3a), in keeping with the fact that the label was in proteochondroitin sulphate (Audhya *et al.*, 1976). However, when an extract from sterna that had been incubated in the presence of 0.1 mM-4-methylumbelliferyl β-xyloside was chromatographed, only 65% of the radioactivity was eluted in the void volume; the remainder was eluted in a broad peak with *K_{av}* values between 0.31 and 0.83 (Fig. 3b). There was also a broad peak of fluorescence that eluted between *K_{av}* values of 0.30 and 0.85 and corresponded reasonably well to the minor peak of radioactivity (Fig. 3b).

The observation that sterna incubated in medium containing 0.1 mM-4-methylumbelliferyl β-xyloside synthesize two forms of chondroitin sulphate might provide an explanation for the anomalous kinetics of incorporation shown in Fig. 1. Sterna were therefore incubated with [³⁵S]sulphate for various periods in medium containing the xyloside, and proteoglycans and glycosaminoglycans were extracted with 4M-guanidinium chloride and fractionated on

Fig. 2. *Chondroitin lyase digestion of glycosaminoglycans from sterna*

Groups of five sterna were incubated individually in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside and [35 S]sulphate (10 mCi/mmol) for 5 h. The papain digests of the sterna from each group were combined and used to prepare glycosaminoglycans, as outlined under 'Methods'. One-half of each preparation was digested with chondroitin lyase before spotting on paper; the other half was spotted without digestion. After development with butan-1-ol/acetic acid/1 M-NH₃ (2:3:1, by vol.) for 40 h, the chromatogram was cut into 1 cm slices, which were assayed for radioactivity ($\bullet$) and fluorescence due to methylumbelliferone ($\circ$), expressed in arbitrary units, as described under 'Methods'. (a) and (b) Glycosaminoglycans from sterna incubated in medium with no further addition: (a) not digested; (b) after digestion with chondroitin lyase. (c) and (d) Glycosaminoglycans from sterna incubated in medium to which 20% (v/v) human serum and 10 nM-tri-iodothyronine were added: (c) not digested; (d) after digestion with chondroitin lyase.

a column of Sepharose 6B (Fig. 4). In each case the distribution of radioactivity showed a sharp peak eluted in the void volume of the column, followed by a broad peak that was eluted over most of the included volume. The fraction of the total radioactivity that was eluted in the void volume increased steadily during the course of the incubation, and after a chase in medium free of radioactive isotope this fraction accounted for nearly all the radioactivity. When extracts of sterna that had been incubated in medium containing 20% (v/v) human serum and 10 nM-tri-iodothyronine, in addition to the xyloside, were fractionated in the same way, a larger fraction of the radioactivity was eluted in the void volume. By combining the data in Fig. 4 with the specific radioactivities of the original extracts of the sterna, the time course of incorporation into each of these fractions was determined (Fig. 5). In sterna incu-

bated in medium containing [35 S]sulphate and xyloside but no serum or tri-iodothyronine, the incorporation into the fraction eluted in the void volume of the column was linear with time (Fig. 5b). Also, when sterna were incubated with [35 S]sulphate for 3 h and then chased for 2 h, there was no loss of radioactivity from this fraction. Finally, when the incubations were carried out in medium containing serum and tri-iodothyronine as well as xyloside, incorporation into this fraction became linear with time after a short lag period, and continued at a much more rapid rate than in the incubations without serum or tri-iodothyronine (Fig. 5b). These kinetics of incorporation are identical with the kinetics of incorporation into proteochondroitin sulphate in incubations in which no xylosides are added to the medium (Audhya & Gibson, 1974, 1975; Audhya *et al.*, 1976). However, incorporation into the included

Fig. 3. Gel filtration of salt extracts of *sterna*. *Sterna* were incubated in medium containing [^{35}S]sulphate (1.0mCi/mmol) for 6h. Groups of five *sterna* were combined and extracted with 0.5M-L aCl_3 , pH 5.8 (0.3ml/sternum), for 16h. Proteoglycans and glycosaminoglycans were precipitated by addition of water, the precipitate was dissolved in 0.5ml of 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, and portions (0.4ml) were chromatographed on a calibrated column of Sepharose 6B, as described under 'Methods'. Fractions from the column were assayed for methylumbelliferone ($\circ$) and radioactivity ($\bullet$). (a) Extract of *sterna* incubated in medium with no addition; (b) extract of *sterna* incubated in medium to which 0.1mM-4-methylumbelliferyl β -xyloside was added. V_0 , Void volume; V_t , total volume of the column.

fraction followed a very different course (Fig. 5c). The specific radioactivity of this fraction increased rapidly within the first hour after addition of [^{35}S]sulphate; thereafter it rose very slowly during the remainder of the incubation. Further, there was very little difference in the rate of incorporation into this fraction in incubations in which the medium did or did not contain serum and tri-iodothyronine. Finally, more than half of the radioactivity that was incorporated during a 3h incubation was lost during a subsequent 2h chase.

These results indicate that the newly synthesized chondroitin sulphate in the first column fraction, which is probably linked to protein, is stable, whereas the chondroitin sulphate in the second fraction, which is probably linked to 4-methyl-

Fig. 4. Distribution of radioactivity in salt extracts of *sterna* incubated for various times in medium containing 4-methylumbelliferyl β -xyloside

Sterna were incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside and [^{35}S]sulphate (5.0mCi/mmol). Groups of ten *sterna* were combined and extracted with 3ml of 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, for 16h at 4°C. The extracts were dialysed against several changes of water and freeze-dried; the residues were dissolved as far as possible in 0.3–0.5ml of water and clarified by centrifugation for 20min at 10000g. Portions (0.2ml) were chromatographed on a column (0.6cm $\times$ 98cm) of Sepharose 6B, as outlined under 'Methods', and the fractions were analysed for radioactivity. (a), (b) and (c) Extracts of *sterna* incubated in medium containing [^{35}S]sulphate: (a) for 1h; (b) for 3h; (c) for 5h. (d) Extract of *sterna* incubated in medium containing [^{35}S]sulphate for 3h, then transferred to medium lacking radioactive isotopes for a further 2h. The number over the initial peak in each panel is the percentage of the total radioactivity that was associated with the void volume (all K_{av} values less than 0.2); this was determined by tracing each of the distributions and weighing the tracings. V_0 , Void volume; V_t , total volume of the column.

umbelliferone, undergoes a rapid turnover. Two obvious possible explanations for the turnover rate are (1) that this chondroitin sulphate undergoes

Fig. 5. Time course of incorporation into glycosaminoglycan fractions from sterna

The guanidinium extracts of the sterna incubated as described in the legend to Fig. 4, and similar extracts of sterna that had been incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside, 20% (v/v) human serum, 10 nM-tri-iodothyronine and [35 S]sulphate, were assayed for radioactivity and uronic acid. Total specific radioactivities were calculated from these results and are shown in (a). Specific radioactivities of the glycosaminoglycan fraction eluted in the void volume (b) and of the included fraction (c) were calculated from the total specific radioactivities, and the data in Fig. 4, and from the results of similar analyses of the other extracts. ●, Sterna incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside; ○, sterna incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside, 20% (v/v) human serum and 10 nM-tri-iodothyronine; ▲, sterna incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside and [35 S]sulphate for 3 h, then transferred to the same medium lacking radioactive isotopes for 2 h.

hydrolytic cleavage, and (2) that it leaks out rapidly from the tissue into the medium. To test the second possibility, sterna were incubated for various periods in medium containing chondroitin sulphate (100 μ g/ml), with or without the addition of 0.1 mM-4-methylumbelliferyl β -xyloside. At the end of the incubation period, the sterna were weighed and digested with papain; portions of the digests, and also of the original incubation medium, were then precipitated with Alcian Blue to determine the extent of incorporation into glycosaminoglycans (see under 'Methods'). After a 5 h incubation in medium lacking xyloside, the amount of 35 S in glycosaminoglycans in the medium was 5% of the amount found in the glycosaminoglycans from the tissue (Table 3). When the incubation was performed in medium lacking xyloside to which 20% (v/v) human serum and 10 nM-tri-iodothyronine had been added, twice as much 35 S was incorporated, but the glycosaminoglycans in the medium still contained less than 5% 35 S of that found in the tissue glycosaminoglycans. By contrast, in all incubations in which 0.1 mM-4-methylumbelliferyl β -xyloside was present there was a large incorporation of [35 S]sulphate into Alcian Blue-precipitable glycosaminoglycans in the medium (Table 3). The rate of incorporation into the glycosaminoglycans in the medium was almost unaffected by the presence or absence of serum and tri-iodothyronine in the incubation medium. When sterna were incubated in medium containing [35 S]sulphate and 0.1 mM-4-methylumbelliferyl β -xyloside for 3 h, and then chased for 2 h in medium lacking radioactive isotope, the amount of 35 S recovered in the glycosaminoglycans in the medium was essentially equal to that lost from the tissue glycosaminoglycans. [The amount of sulphate incorporated into glycosaminoglycans in the tissue in Table 3 can be related to the amount

Table 3. Incorporation of sulphate into glycosaminoglycans in the tissue and medium

Sterna were incubated in medium containing chondroitin sulphate A + C (100 μ g/ml) and [35 S]sulphate (10 mCi/mmol), for the period indicated under 'pulse'. In some cases, the sterna were washed with 5 ml of 0.15 M-NaCl and transferred to medium of the same composition but without radioactive isotopes for the period indicated under 'chase'. Portions (0.4 ml) of the medium were precipitated with Alcian Blue as described under 'Methods'. The sterna were weighed, digested with papain, and incorporation into glycosaminoglycans was determined after precipitation with Alcian Blue. Results are expressed as mean values $\pm$ S.E.M. for groups of five sterna.

Additions to the incubation medium			Length of pulse (h)	Length of chase (h)	Incorporation into glycosaminoglycans (nmol/mg wet wt.)	
Human serum (% v/v)	Tri-iodothyronine (nM)	4-Methylumbelliferyl β -xyloside (mM)			Tissue	Medium
—	—	—	5	—	1.01 $\pm$ 0.02	0.05 $\pm$ 0.00
20	10	—	5	—	2.08 $\pm$ 0.09	0.10 $\pm$ 0.01
—	—	0.1	1	—	0.11 $\pm$ 0.00	0.35 $\pm$ 0.01
—	—	0.1	3	—	0.20 $\pm$ 0.01	1.11 $\pm$ 0.02
—	—	0.1	5	—	0.25 $\pm$ 0.01	1.41 $\pm$ 0.03
—	—	0.1	3	2	0.11 $\pm$ 0.00	0.07 $\pm$ 0.00
20	10	0.1	1	—	0.14 $\pm$ 0.01	0.39 $\pm$ 0.02
20	10	0.1	3	—	0.41 $\pm$ 0.02	1.20 $\pm$ 0.06
20	10	0.1	5	—	0.75 $\pm$ 0.02	1.53 $\pm$ 0.08
20	10	0.1	3	2	0.32 $\pm$ 0.02	0.06 $\pm$ 0.00

Fig. 6. Gel filtration of glycosaminoglycans in the medium on Sephadex G-50

Sterna were incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside, chondroitin sulphate A+C (100 μ g/ml), [6- 3 H]glucose (7.5 mCi/mmol) and [35 S]sulphate (1.2 mCi/mmol) for 6h. The medium from five incubations was combined and used for the preparation of glycosaminoglycans, as outlined under 'Methods'. The preparation was dissolved in 0.6 ml of water; 0.3 ml of the solution was chromatographed on a column of Sephadex G-50 and the fractions were assayed for methylumbelliferone (Δ) and 3 H ($\circ$) and 35 S ($\bullet$) radioactivity as described under 'Methods'. V_0 , Void volume; V_t , total volume of the column.

incorporated in other experiments by dividing by 11.5, which is the average concentration, in μ g/mg wet wt., of chondroitin sulphate in this tissue (Audhya *et al.*, 1976). When this is done, the incorporation is very similar to that observed in other experiments.]

To investigate the size and composition of the glycosaminoglycans released into the medium, sterna were incubated for 6h in medium containing [6- 3 H]glucose, [35 S]sulphate and 0.1 mM-4-methylumbelliferyl β -xyloside, as outlined in the legend to Fig. 6; the medium was then deproteinized with 5% (w/v) trichloroacetic acid, dialysed and freeze-dried. The residue was dissolved in water and chromatographed on a column of Sephadex G-50 equilibrated with 0.2M-NaCl. All the radioactivity and fluorescence were eluted in a single broad peak centred at a K_{av} value of 0.48 (Fig. 6). The correspondence between the peaks of radioactivity and fluorescence suggests that essentially all the glycosaminoglycans contained 4-methylumbelliferone as aglycone. A semi-logarithmic plot of the apparent molar ratio, 35 S:4-methylumbelliferone, of each of the fractions in Fig. 6 against the K_{av} value of the same fraction yielded a straight-line relationship (Fig. 7), indicating that the column had indeed separated the fractions on the basis of their molecular size. Fig. 7 also includes data from a similar column fractionation of glycosaminoglycans isolated from the medium of an incu-

Fig. 7. 35 S:methylumbelliferone ratio and K_{av} value of glycosaminoglycans in the medium

The ratio of 35 S:methylumbelliferone was calculated from the data in Fig. 6 and plotted against K_{av} values ($\bullet$). Ratios were also calculated from the elution pattern on Sephadex G-50 of a preparation of glycosaminoglycans, isolated from the medium of 6h incubations of sterna in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside, chondroitin sulphate (100 μ g/ml), 20% (v/v) human serum, 10 nM-tri-iodothyronine, [6- 3 H]glucose (7.0 mCi/mmol) and [35 S]sulphate (1.1 mCi/mmol); these ratios are included in the Figure ($\circ$).

bation mixture containing 0.1 mM-4-methylumbelliferyl β -xyloside, 20% (v/v) human serum and 10 nM-tri-iodothyronine; the data from the two preparations of glycosaminoglycan lie on the same line.

The apparent molar ratio of 3 H: 35 S of each fraction from the elution pattern in Fig. 6 was between 0.9 and 1.1 at all K_{av} values; this suggested that the material was probably chondroitin sulphate having a high degree of sulphation. Confirmation was obtained when this and other preparations of glycosaminoglycans were digested with chondroitin lyase and analysed by paper chromatography (Fig. 8). The undigested glycosaminoglycan separated into two peaks, one of which was inorganic sulphate that had not been completely removed during the preparation of the sample (Fig. 8a). The other peak, which contained all the 3 H and the 4-methylumbelliferone as well as the remaining 35 S, stayed at the origin of the chromatogram. The chromatogram of the digested material showed a distribution of radioactivity typical of highly sulphated chondroitin sulphate, together with a peak of inorganic sulphate and another peak close to the origin, which contained

Fig. 8. Digestion of glycosaminoglycans of the medium with chondroitin lyase

Sterna were incubated in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside, [6-³H]glucose (12 mCi/mmol) and [³⁵S]sulphate (1.8 mCi/mmol) for 6 h. Glycosaminoglycans were prepared from the combined medium of six such incubations and dissolved in 0.4 ml of water. A portion (0.1 ml) was digested with chondroitin lyase and spotted on paper; another portion (0.075 ml) was spotted without digestion. The chromatogram was developed for 71 h, and cut into 1 cm slices which were assayed for methylumbelliferone (Δ) and ³H ($\circ$) and ³⁵S ($\bullet$) radioactivity as described under 'Methods'. (a) Not digested; (b) after digestion with chondroitin lyase. Peaks C, D, E and F were identified as Δ -di-6S, inorganic sulphate, Δ -di-4S and Δ -di-0S respectively by comparison with appropriate standards. The possible nature of peak A is discussed in the text.

virtually all the 4-methylumbelliferone together with some ³H and ³⁵S (Fig. 8b). The amounts of radioactivity associated with the peaks of inorganic sulphate in Figs. 8(a) and 8(b) agreed within 5%, showing that no inorganic sulphate was released during the enzymic digestion. The fact that all the 4-methylumbelliferone in the digest was associated with a single peak that had moved slightly from the origin indicates that it had all originally been covalently attached to chondroitin sulphate.

Some characteristics of the chromatographic peaks from these digests are listed in Table 4. The apparent molar ratio ³H:³⁵S in the peaks of Δ -di-4S and Δ -di-6S was close to 0.75 in all cases. Theoretically, the ratio should be 1.0, since 1 mol of ³H from [6-³H]glucose would be expected to be incorporated into each chondrosine residue; however, as discussed elsewhere (Audhya *et al.*, 1976), the observed ratio is likely to be lower than 1.0 as a result of various metabolic transformations that must be undergone by glucose before it can be incorporated into chondroitin sulphate. In the other major ³H-labelled peak (peak A in Fig. 8 and Table 4), which contained the 4-methylumbelliferone and remained close to the origin of the chromatogram, the apparent molar ³H:³⁵S ratio was approx. 2.3, or 3 times the ratio observed in Δ -di-4S and Δ -di-6S. This suggests that peak A contained 3 mol of ³H per mol of ³⁵S. The apparent molar ratio 4-methylumbelliferone:³⁵S in peak A was 0.8–0.9, suggesting that it contained equimolar amounts of 4-methylumbelliferone and sulphate residues. These ratios tend to rule out the obvious possibility that Peak A has the structure Δ^4 -GlcA $\rightarrow$ Gal $\rightarrow$ Gal $\rightarrow$ Xyl $\rightarrow$ 4-methylumbelliferone, corresponding to the linkage tetrasaccharide of chondroitin sulphate covalently attached to methylumbelliferone (Fukunaga *et al.*, 1975), but they would be consistent with a hexasaccharide structure such as Δ^4 -GlcA $\rightarrow$ GalNAc(SO₃H) $\rightarrow$ GlcA $\rightarrow$ Gal $\rightarrow$ Gal $\rightarrow$ Xyl $\rightarrow$ 4-methylumbelliferone, in which the *N*-acetyl-galactosamine residue is sulphated at either the 4- or 6-position. Table 4 also shows the small amount of ³H that was present as Δ -di-0S, indicating that the chondroitin sulphate isolated from the incubation medium was more than 95% sulphated; by contrast, the newly synthesized chondroitin sulphate that remains in the tissue in these incubations is only about 60% sulphated (Gibson *et al.*, 1977). A further notable point is that the ratio Δ -di-4S: Δ -di-6S was somewhat less than 1.0 in the chondroitin sulphate isolated from the medium (Table 4), but equal to or greater than 1.0 in the chondroitin sulphate in the tissue (Gibson *et al.*, 1977).

Approximate estimates of the size and degree of polydispersity of the chondroitin sulphate isolated from the tissue and medium were calculated by applying the formulae of Wåsteson (1971) to calculate number and weight averages for the ratio ³⁵S:4-methylumbelliferone from the data in Figs. 3(b), 6 and 7. Molecular weights were then calculated from these averages, on the assumption that the chondroitin sulphate was fully sulphated (Table 5). These values should represent a reasonable approximation to the molecular weight of the highly sulphated chondroitin sulphate released into the medium. The average values of the ratio ³⁵S:4-methylumbelliferone, and the average molecular weights calculated from

Table 4. *Products of chondroitin lyase digestion of glycosaminoglycans isolated from the incubation medium*

Groups of five sterna were incubated in medium containing 0.1 mM 4-methylumbelliferyl β -xyloside, [^3H]glucose (7.5 or 12 mCi/mmol) and [^{35}S]sulphate (1.2 or 1.8 mCi/mmol) for 6 h. Glycosaminoglycans were isolated from the medium and digested with chondroitin lyase (see under 'Methods'), and the digestion products were separated by paper chromatography with butan-1-ol/acetic acid/1 M-NH₃ (2:3:1, by vol.) as solvent (see, e.g., Fig. 8). Methylumbelliferone and radioactivity were determined as described under 'Methods'. Apparent molar ratios are mean values of two determinations, using two preparations of glycosaminoglycans; other results are mean values of three determinations, using four preparations. Percentage of ^3H as Δ -di-OS was calculated as the ratio Δ -di-OS:(Δ -di-OS + Δ -di-4S + Δ -di-6S).

Additions to the incubation medium	Apparent molar proportions methylumbelliferone: ^3H : ^{35}S				^3H as Δ -di-OS (%)
	In Δ -di-4S	In Δ -di-6S	In peak A	Δ -Di-4S: Δ -di-6S	
None	0:0.70:1	0:0.75:1	0.88:2.27:1	0.84	3.3
20% (v/v) Human serum + 10 nM-tri-iodothyronine	0:0.77:1	0:0.86:1	0.82:2.32:1	0.83	4.5

them, were approximately twice as large for the chondroitin sulphate that remained in the tissue as for the chondroitin sulphate that was released into the medium. Direct determinations of the average molecular weights of the chondroitin sulphate that remained in the tissue, obtained from the elution pattern of ^{35}S from the calibrated column of Sepharose 6B in Fig. 3(b), gave considerably higher values than the molecular weights calculated from the ^{35}S :methylumbelliferone ratio (Table 5), suggesting that the chondroitin sulphate in the tissue was undersulphated. Comparison of the calculated and observed number-average molecular weights of this material leads to an estimate of 70% for its degree of sulphation.

Discussion

In most of the studies with β -xylosides referred to in the introduction, synthesis of proteochondroitin sulphate was blocked by addition of puromycin or cycloheximide. Schwartz *et al.* (1976) presented evidence that embryonic-chicken chondrocytes, incubated in medium containing *p*-nitrophenyl β -xyloside and lacking protein-synthesis inhibitors, synthesize both proteochondroitin sulphate and free chondroitin sulphate. The work presented here extends these observations to whole embryonic cartilage, and shows in addition that more than one class of chondroitin sulphate is formed in such incubations. When embryonic sterna were incubated with [^{35}S]sulphate in medium containing 0.1 mM 4-methylumbelliferyl β -xyloside, label was incorporated into three distinct pools of chondroitin sulphate. Two of the pools were found in the cartilage, and could be separated when salt extracts of the tissue were chromatographed on Sepharose 6B (Figs. 3 and 4). On the basis of its chromatographic behaviour (Fig. 3) and the kinetics of labelling (Fig. 5b), one of these pools was identified as proteochondroitin sulphate. Identifi-

cation of the other tissue pool is less certain; however, on the basis of its chromatographic profile (Fig. 3b) and the products of digestion by chondroitin lyase (Fig. 2), it was probably chondroitin sulphate covalently linked to 4-methylumbelliferone. Chondroitin sulphate in both these pools had an average mol. wt. of 16000–19000 and was about 60% sulphated (Gibson *et al.*, 1977; Table 5). The third pool, isolated from the incubation medium, accounted for essentially all the labelled glycosaminoglycans present in the medium. This material was completely broken down by chondroitin lyase, yielding Δ -di-4S, Δ -di-6S and a trace of Δ -di-OS, together with a compound with molar proportions ^{35}S : ^3H :methylumbelliferone consistent with its being the terminal hexasaccharide of methylumbelliferyl-chondroitin sulphate. Taken together with the results of gel filtration (Fig. 6), the data allow identification of this material as chondroitin sulphate covalently attached to 4-methylumbelliferone. By contrast with the tissue chondroitin sulphate, this material has an average mol. wt. of 7000–8000 and was more than 95% sulphated (Tables 4 and 5).

Chondroitin sulphate containing 4-methylumbelliferone as aglycone has been isolated and characterized by Fukunaga *et al.* (1975) from cultures of chick-embryo chondrocytes incubated in the presence of the xyloside. This product was generally similar to ours, but differed in that it had an average of about 20 chondrosine residues per chain and was excluded from Sephadex G-50. After treatment of their product with chondroitin lyase, Fukunaga *et al.* (1975) isolated two fluorogenic compounds, one of which was $\Delta^4\text{-GlcA} \rightarrow \text{Gal} \rightarrow \text{Gal} \rightarrow \text{Xyl} \rightarrow 4\text{-methylumbelliferone}$. The other was a slightly larger oligosaccharide, whose structure was not determined. In our case, treatment with chondroitin lyase did not liberate a fluorogenic compound with the structure assigned by Fukunaga *et al.* (1975); this probably was not due to inadequate

Table 5. *Molecular weights of methylumbelliferyl-chondroitin sulphate*

Number- and weight-averages of the ratio ^{35}S :4-methylumbelliferone were calculated from the data in Figs. 3(b), 6 and 7, by using the formulae given by Wästeson (1971). Calculated molecular weights deduced from these values are for the sodium salt of chondroitin sulphate. Observed molecular weights were determined from the distribution of ^{35}S on a calibrated column of Sepharose 6B (Fig. 3b).

Additions to the incubation medium	Source of chondroitin sulphate	^{35}S :4-Methylumbelliferone		Calculated mol.wt.		Observed mol.wt.	
		Number-average	Weight-average	Number-average	Weight-average	Number-average	Weight-average
None	Tissue	25.1	29.1	13 000	14 800	19 500	21 300
None	Medium	13.0	15.7	7000	8300	—	—
20% (v/v) Human serum +10nM-tri-iodothyronine	Medium	15.6	19.8	8300	10300	—	—

Fig. 9. Time course of incorporation of sulphate into total chondroitin sulphate, proteochondroitin sulphate and methylumbelliferyl-chondroitin sulphate in tissue and medium

Data for incorporation into total chondroitin sulphate (a) and chondroitin sulphate in the medium (d) are mean values from Table 3. Data for incorporation into proteochondroitin sulphate (b) and methylumbelliferyl-chondroitin sulphate in tissue (c) were calculated from mean values for incorporation into glycosaminoglycans in tissue (Table 3) and the distribution of radioactivity between the two chondroitin sulphate pools (Fig. 4). The Figure shows incorporation in unsupplemented medium (Δ), in medium supplemented with 20% (v/v) human serum and 10nM-tri-iodothyronine ($\bullet$), in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside ($\circ$), or in medium containing 0.1 mM-4-methylumbelliferyl β -xyloside, 20% (v/v) human serum and 10nM-tri-iodothyronine ($\bullet$).

digestion, since the chromatographic pattern was not changed when the products of the first digestion were subjected to a second digestion with fresh enzyme. Possibly the unidentified fluorogenic oligosaccharide isolated by Fukunaga *et al.* (1975) had the same structure as the fluorogenic oligosaccharide that was liberated by chondroitin lyase in our case, and repre-

sented a slightly larger terminal oligosaccharide from chondroitin sulphate.

The kinetics of incorporation of [^{35}S]sulphate into the three pools of chondroitin sulphate are summarized in Fig. 9. The data for the total incorporation (Fig. 9a) and for incorporation into chondroitin sulphate in the medium (Fig. 9d) were

obtained from Table 3, and the data for incorporation into proteochondroitin sulphate (Fig. 9b) and into the pool of methylumbelliferyl-chondroitin sulphate in the tissue (Fig. 9c) were calculated from the incorporation of ^{35}S into the cartilage in Table 3 and the distribution between the two fractions in Fig. 4. Because the two sets of results on which this calculation is based were obtained by different techniques, Fig. 9 is an indication of the course of incorporation rather than an accurate measurement. Incorporation into proteochondroitin sulphate may be artificially low, since the data in Fig. 4 were obtained with guanidinium chloride extracts of sterna, and this solvent extracts only 80% of the proteoglycan in the tissue (Gibson *et al.*, 1977). With this caveat, some interesting conclusions can be drawn. Incorporation in sterna that were incubated in medium to which no xyloside was added was nearly all into proteoglycan that remained in the tissue. As Audhya *et al.* (1976), have shown stimulation by serum supplemented with tri-iodothyronine resulted in an approximate doubling of the incorporation. When the incubations were carried out in medium containing 0.1 mM 4-methylumbelliferyl β -xyloside, the rate of incorporation of sulphate into proteoglycan was dramatically decreased to about 20% of its value in control incubations (most of this fall in the rate of incorporation of sulphate is due to the decrease in chain length and degree of sulphation of the chondroitin sulphate synthesized under these conditions). The total incorporation into glycosaminoglycans was increased about 2-fold during the first 3 h of incubation compared with control incubations. This was the result of a very large incorporation into methylumbelliferyl-chondroitin sulphate that was released into the medium; the initial rate of this incorporation was about 10 times the rate of incorporation into proteoglycan. There was also a rapid initial burst of incorporation into methylumbelliferyl-chondroitin sulphate in the tissue, which levelled off to a slow steady rise within 1 h. When the incubation medium was supplemented with serum and tri-iodothyronine, the only parameter that changed significantly was the rate of incorporation into the proteoglycan, which increased more than 3-fold.

Fig. 9 indicates the kinetics of incorporation of sulphate. Allowing for the different molecular weights and degrees of sulphation of the chondroitin sulphate in the medium and tissue, we estimate that the average rate of polymerization of the chondroitin sulphate released into the medium would be about 6 times as great as the rate of polymerization of proteochondroitin sulphate, and the rate of chain initiation would be 2.5–3 times as great. In incubations in the presence of serum and tri-iodothyronine, the rates of polymerization would differ by a factor of only 2, and the rate of initiation of

chains of proteochondroitin sulphate would be slightly greater than the rate at which chains of methylumbelliferyl-chondroitin sulphate are initiated.

The large differences in chain length between the proteochondroitin sulphate and the methylumbelliferyl-chondroitin sulphate released into the medium in our incubations are in line with similar differences that have been noted by Okayama *et al.* (1973), Robinson *et al.* (1975) and Galligani *et al.* (1975), although in those cases the two classes of chondroitin sulphate were not formed simultaneously in the same incubations. The differences can be accounted for in terms of what is known about chondroitin sulphate synthesis in cell-free systems. Using microsomal preparations from embryonic-chicken epiphyseal cartilage, Richmond *et al.* (1973b) found a rapid co-ordinate addition of *N*-acetylgalactosamine and glucuronic acid residues on to a small endogenous primer, which resulted in the formation of chains of chondroitin sulphate of mol.wt. approx. 35000 with no detectable accumulation of products of intermediate size. The endogenous primer was shown to be a glycosaminoglycan linked to a peptide by an alkali-labile bond (Richmond *et al.*, 1973a). On the other hand, when the terminal pentasaccharide or hexasaccharide of chondroitin sulphate was added to an identical enzyme system as an exogenous primer, not more than 14–15 sugar residues could be added before polymerization ceased (Silbert & Reppucci, 1976). In our incubations, the enzymic addition of a few sugar residues to 4-methylumbelliferyl β -xyloside should produce oligosaccharides that resemble the exogenous primers used by Silbert & Reppucci (1976), whereas the addition of sugar residues to the peptide moiety of the sternal proteoglycan would produce compounds that resemble the endogenous primer identified by Richmond *et al.* (1973a,b). The oligosaccharides that contain methylumbelliferone would thus in general support only limited polymerization, whereas the oligosaccharides attached to peptide would prime the formation of much longer chains. This explanation accounts for the observed chain lengths of the proteochondroitin sulphate and of the chondroitin sulphate released into the medium in our incubations. The remaining pool of chondroitin sulphate, which was isolated from the tissue but probably contained 4-methylumbelliferone as aglycone, appeared to be a mixture of two types of molecule. From the time course of incorporation into this pool and its behaviour in pulse-chase experiments (Fig. 5), it is likely that in the early stages of the incubations most of the pool was a precursor to the short chains of chondroitin sulphate that were released into the medium. After longer times of incubation, the average size and degree of sulphation of the pool resembled that of proteochondroitin sulphate (Table 5). A possible explanation is that this material represents methylumbelliferyl-chon-

droitin that was initially formed as completed molecules of mol.wt. 7000–8000 and then served as primer for a second round of polymerization. Such an explanation is in line with an observation by Richmond *et al.* (1973b), who showed that completed chains of chondroitin sulphate could act as efficient primers for the addition of more chondroitin in their cell-free system.

The other marked difference between the pools of chondroitin sulphate lies in their degree of sulphation. In our previous study (Gibson *et al.*, 1977), we found that short chains of newly synthesized proteochondroitin sulphate were much less sulphated than chains of average length. From the relation between molecular weight and degree of sulphation that we observed, chains whose molecular weights were below 10000 were probably less than 60% sulphated (Gibson *et al.*, 1977). By contrast, in identical incubations all chains of the methylumbelliferyl-chondroitin sulphate that were released into the medium, including those whose size range overlapped that of the proteochondroitin sulphate, were nearly fully sulphated. Thus the failure of the cartilage to sulphate proteochondroitin to the same extent reflects some distinguishing property of this substrate other than its chain length. The distinguishing property is probably not the nature of the aglycone, since the methylumbelliferyl-chondroitin sulphate that remained in the tissue also appeared to be undersulphated. One possibility is that there may be a marked difference in lipid-solubility between proteochondroitin and methylumbelliferyl-chondroitin of low molecular weight, so that the latter might be able to penetrate the membranes of the Golgi apparatus and approach closely the sites of sulphate transfer (Godman & Lane, 1964), whereas the former must of necessity remain inside the cisternal space of the organelle. The apparent undersulphation of the tissue pool of methylumbelliferyl-chondroitin sulphate might then be due to its much greater chain length, which would make it resemble proteochondroitin in solubility properties.

In the light of this discussion, we can now suggest how 4-methylumbelliferyl β -xyloside influences the synthesis of proteochondroitin sulphate. When sterna are incubated in medium containing this xyloside, the rate of synthesis of the peptide moiety of the major proteoglycan is not greatly decreased compared with control incubations (Gibson *et al.*, 1977). Thus the rate of transport of the peptide from the rough endoplasmic reticulum, through the transitional elements of this system and the elements of the Golgi complex, and out to the extracellular space (Palade, 1975) cannot be greatly altered; and the average time spent by a molecule of peptide in each subcellular compartment will be nearly the same as in control incubations. However, at the subcellular site at which chain polymerization occurs there will be com-

petition between peptide-linked primers and primers linked to methylumbelliferone, so that the rate of polymerization on to the peptide-linked primers must necessarily be decreased. The combination of these two effects will result in premature termination of the chains of proteochondroitin. A similar phenomenon should occur at the sites at which sulphate is transferred, and lead to some decrease in the degree of sulphation of all chains of proteochondroitin sulphate, as we observed (Gibson *et al.*, 1977). Thus the decrease in sulphation of proteochondroitin sulphate is probably partly a direct result of competition at the site of sulphation, and partly a consequence of the premature termination of polymerization of the side chains. Our results support this explanation better than they do alternative hypotheses, such as direct interference with polymerization and sulphation by 4-methylumbelliferyl β -xyloside or a simple metabolite.

The phenomena described here and by Gibson *et al.* (1977) should be observable in other experiments in which cartilage or chondrocytes are incubated in the presence of β -xylosides. Schwartz *et al.* (1976), using cultured embryonic-chicken chondrocytes, reported that addition of *p*-nitrophenyl β -xyloside to the incubation medium brought about a large decrease in the incorporation of sulphate into proteoglycan, but did not change the amount of core protein in the cells. They interpreted the decrease in incorporation of sulphate as a decrease in the rate of synthesis of proteochondroitin sulphate, and they suggested that polysaccharide-chain addition was inhibited at a post-translational step of synthesis, perhaps because artificial β -xylosides compete with the natural peptidyl-xyloside in the first galactosyl-transferase reaction. However, the strong resemblance between our results and those of Schwartz *et al.* (1976) suggests that they were observing a phenomenon similar to the one described by us, namely a marked decrease in chain length and degree of sulphation of the proteochondroitin sulphate, with only a small change in the rate of synthesis of the peptide core of the proteoglycan, and no change in the number of polysaccharide chains attached to each molecule of peptide. This differs from the interpretation put forward by Schwartz *et al.* (1976) in that only the terminal steps of polysaccharide chain synthesis are inhibited.

It is evident from Fig. 9 that thyroid hormones and other serum factors stimulate only the synthesis of proteochondroitin sulphate, and not that of methylumbelliferyl-chondroitin sulphate. An obvious implication is that the serum factors have no effect on the activities of any of the enzymes involved in the synthesis of chondroitin sulphate, except possibly the first enzyme in the pathway. This offers strong support for our previous suggestion that the stimulation of proteoglycan synthesis by serum factors occurs at

the level of either peptide synthesis or transport to the sites of polymerization of the carbohydrate side chains (Gibson *et al.*, 1977).

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