Effects of detergent on the sulphation of chondroitin by cell-free preparations from chick-embryo epiphyseal cartilage

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The effects of the non-ionic detergent Triton X-100 on 6-sulphation of two species of endogenous nascent proteochondroitin by a chick-embryo cartilage microsomal system was examined. Sulphation of the larger (Type I) species with adenosine 3'-phosphate 5'-phosphosulphate was slightly diminished when Triton X-100 was present, whereas sulphation of the smaller (Type II) species was slightly enhanced. An ordered rather than random pattern of sulphation was obtained for the smaller proteoglycan, but with a considerably lower degree of sulphation than that of the larger proteochondroitin. These differences were consistent with other differences between these two species as described previously. Sulphation of exogenous [^{14}C]chondroitin and exogenous proteo[^3H]chondroitin by the microsomal system with Triton X-100 present produced ordered rather than random sulphation patterns. When a 100 000 *g* supernatant fraction was utilized for sulphation of [^{14}C]chondroitin or proteo[^3H]chondroitin, Triton X-100 was not needed, and ordered sulphation was still obtained. When hexasaccharide was used, sulphation of multiple *N*-acetylgalactosamine residues of the individual hexasaccharides resulted. This was relatively independent of Triton X-100 or the concentration of the hexasaccharide acceptors. With soluble enzyme, sulphation of multiple *N*-acetylgalactosamine residues on the individual hexasaccharide molecules was even greater, so that tri-sulphated products were found. This suggests that ordered rather than random sulphation of chondroitin with these enzyme preparations is due to enzyme-substrate interaction rather than to membrane organization.

INTRODUCTION

We have previously shown that two species of nascent proteochondroitin are formed when chick embryo cartilage microsomal preparations are incubated with UDP-[^{14}C]GlcA and UDP-[^3H]GalNAc [1,2]. One species (Type I) consists of the formation of multiple entire large chondroitin chains on a protein core of undetermined size [3]. The second (Type II) consists of the formation of multiple large chondroitin oligosaccharides or short chondroitin chains on proteochondroitin sulphate already containing sulphated chondroitin chains of substantial size, and therefore at a later stage of synthesis, than the Type I nascent proteochondroitin [3].

Significant differences between these two species of nascent proteochondroitin has been demonstrated. The Type I proteochondroitin was shown to be much larger in size than the Type II [1,2], since it was mainly excluded from Sepharose 2B, thus resembling the PG-M proteochondroitin sulphate that has been described in chick limb buds [4]. The Type II proteochondroitin was smaller than the PG-H proteochondroitin found in chick embryo cartilage [5] and was also different from the PG-Lb and PG-Lt proteochondroitin sulphate found in the same source [6,7]. The Type I proteochondroitin had characteristics of extractability from the microsomes that differed from the characteristics of extractability of the Type II [1]. The Type I proteochondroitin required a higher concentration of sugar nucleotides for maximal synthesis [2], and the synthesis was not affected by the presence of detergent (Triton X-100) during the course of the microsomal incubations [8]. In contrast, maximal synthesis of the smaller Type II proteochondroitin could be achieved with lower sugar nucleotide concentrations [2] and was stimulated several-fold by the presence of the detergent [8]. These results suggested that the

two species were synthesized in two different locations in the microsomes. This was confirmed by demonstrating a relative inaccessibility of the Type I proteochondroitin when microsomal preparations were preincubated with chondroitin lyase [9] and by demonstrating that the membranes containing these two nascent species could be separated on a sucrose density gradient [9]. The gradient indicated that chondroitin polymerization and sulphation of Type I took place in a heavier *medial* fraction of Golgi, whereas chondroitin polymerization and sulphation of the Type II proteochondroitin took place in a lighter *trans* fraction.

Since the two species of microsomal nascent proteoglycans had separate locations for chondroitin synthesis, and polymerization was affected differently by the presence of detergent, we considered the possibility that detergent treatment of the microsomal preparation during sulphation would also result in different or modified patterns. In particular, we questioned whether patterns of ordered sulphation as previously described for chondroitin sulphate synthesis when no detergent was present [10,11] might be affected. Such disruption of an ordered sulphation pattern by Triton X-100 has been reported for heparin synthesis with a microsomal preparation for heparin-producing cultured mast cells [12]. In contrast, we have found that Triton X-100 had essentially no effect on the organization of sulphation during synthesis of chondroitin 4-sulphate by microsomal preparations from cultured mast cells [13]. We also noted that, in the mast-cell microsomal system, Triton X-100 increased the accessibility of exogenous oligosaccharide substrates to the sulphating site, resulting in a several-fold increase in the amount of sulphate incorporated. Thus the effects of detergent on sulphation of proteoglycans appeared to vary with the source and type of nascent proteoglycan.

We have now extended our studies with Triton X-100 to

Abbreviations used: PAPS, adenosine 3'-phosphate 5'-phosphosulphate; $\Delta\text{Di-6S}$, 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; $\Delta\text{Di-4S}$, 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; $\Delta\text{Di-OS}$, 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyranosyluronic acid)-D-galactose.

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examine its effect on the sulphation of the two species of proteochondroitin in the more complex 6-sulphating chick cartilage system. Our results indicated that the extent of sulphation of the larger Type I species of proteochondroitin was decreased when Triton X-100 was present during synthesis, whereas there was an increase in the degree of sulphation of the smaller Type II species. Nevertheless, the ordered sulphation pattern did not appear to be affected to any great extent.

MATERIALS AND METHODS

UDP-[¹⁴C]GlcA, UDP-[³H]GalNAc and adenosine 3'-phosphate 5'-phosphol[³⁵S]sulphate (PAP³⁵S) were purchased from du Pont-New England Nuclear (Boston, MA, U.S.A.). Non-radioactive UDP-GlcA, UDP-GalNAc and unlabelled PAPS were purchased from Sigma (St. Louis, MO, U.S.A.). Chondroitin 6-sulphate, chondroitin 4-sulphate, chondroitin ABC lyase, and various disaccharides produced by degradation with bacterial enzyme (Δ Di-0S, Δ Di-4S, Δ Di-6S) were purchased from ICN Immunobiologicals (Lisle, IL, U.S.A.). Chondroitin was obtained by desulphation of chondroitin 4-sulphate [14]. Chondroitin hexasaccharide was obtained as previously described [15]. Mast-cell proteo[³H]chondroitin or [¹⁴C]chondroitin glycosaminoglycan were obtained as described below.

A microsomal fraction sedimenting between 10000 *g* and 105000 *g* and the 105000 *g* supernatant was prepared from the epiphysis of 100 17-day-old chick embryos as previously described [16,17]. Microsomal preparations, as well as 105000 *g* supernatants, were used immediately after preparation or were stored at -20°C until used.

Reaction mixtures containing 0.05 M-Mes buffer, pH 6.5, 0.015 M-MnCl₂, 0.2 mM-UDP-[¹⁴C]GlcA (350 × 10⁶ c.p.m./μmol) plus 0.2 mM-UDP-GalNAc or 0.2 mM-UDP-[³H]GalNAc (930 × 10⁶ c.p.m./μmol) plus 0.2 mM-UDP-GlcA and 12 μl of chick cartilage microsomal preparation in a total volume of 45 μl were incubated in the absence or presence of Triton X-100 (1%) and in the absence or presence of PAPS (3.0 mM) at 37°C for various periods of time. Some incubations were first performed with labelled sugar nucleotides for 2 h. Then a 100-fold excess of unlabelled sugar nucleotides was added to dilute the label, and PAPS was added. Aliquots from reaction mixtures that contained PAPS from the beginning, as well as from reaction mixtures that had PAPS added only after the initial 2 h incubation, were incubated for various time intervals, spotted on Whatman no. 1 paper and chromatographed in ethanol/1 M-ammonium acetate (pH 7.8) (5:2, v/v) overnight. After chromatography, the origins were eluted with 1% pancreatin in 0.05 M-Tris, pH 8.0 at 37°C for 2 h, followed by 0.5 M-NaOH at room temperature overnight to obtain the [¹⁴C]glycosaminoglycan or [³H]glycosaminoglycan chains. After neutralization with acetic acid, each eluate was freeze-dried and then desalted on a Sephadex G-25 column. Aliquots of [¹⁴C]glycosaminoglycans obtained from incubations performed in the absence of Triton X-100, together with [³H]glycosaminoglycans obtained from incubations performed in the presence of Triton X-100, were co-chromatographed along with standards on DEAE-cellulose (DE-52) columns (0.7 cm × 5 cm) using an exponential gradient of 0.05–1.0 M-LiCl. Aliquots from the fractions were assayed for radioactivity and uronic acid [18]. Other aliquots of the [¹⁴C]-glycosaminoglycans or [³H]glycosaminoglycans plus standard chondroitin 4-sulphate, chondroitin 6-sulphate and chondroitin were treated with chondroitin ABC lyase (0.15 unit, 1 h, 37°C) and chromatographed on Whatman no. 1 paper with a descending system of butanol/acetic acid/aq. 1 M-NH₃ (2:3:1, by vol.) overnight [19]. Locations of Δ Di0S, Δ Di-4S and Δ Di-6S

were determined by observation of the paper strips under u.v. light. These areas were eluted with water and counted by liquid-scintillation spectrometry.

Sulphation of exogenous proteo[³H]chondroitin and [¹⁴C]-chondroitin was performed as follows. Proteo[³H]chondroitin was obtained by incubating mast-cell microsomes with UDP-[³H]GalNAc (0.2 mM) and UDP-GlcA (0.2 mM) in Mes buffer as described previously [13]. After incubation for 2 h, the reaction mixture was placed in a boiling-water bath for 1 min to inactivate the microsomes, and a 100-fold excess of cold UDP-GalNAc was added.

Proteo[¹⁴C]chondroitin was obtained similarly with use of UDP-[¹⁴C]GlcA and UDP-GalNAc, and [¹⁴C]chondroitin was then obtained from the proteo[¹⁴C]chondroitin by alkali treatment. Aliquots of this [¹⁴C]chondroitin plus proteo-[³H]chondroitin were then incubated together for 2 h with 3.0 mM-PAPS plus fresh chick cartilage microsomes and 1% Triton X-100, or soluble enzyme and no 1% Triton X-100. The final concentrations of the labelled substrates were 0.0015 mM for glucuronic acid or hexosamine. The reaction mixtures were then spotted on Whatman no. 1 paper and chromatographed as described previously [13]. After sulphation, [³H]chondroitin sulphate was obtained from proteo[³H]chondroitin sulphate by alkali treatment. Aliquots of this [³H]chondroitin sulphate together with [¹⁴C]chondroitin sulphate were co-chromatographed on DEAE-cellulose columns as described above.

In other experiments, various concentrations of chondroitin hexasaccharides were incubated with 3.0 mM-PAP³⁵S (30 × 10⁸ c.p.m./μmol) in the presence or absence of Triton X-100 and with either microsomal or soluble enzyme for 2 h at 37°C (total volume 20 μl). These reaction mixtures were spotted on Whatman no. 1 paper and chromatographed in butanol/acetic acid/aq. 1 M-NH₃ (2:3:1, by vol.) overnight or longer. Origins were eluted with water to obtain ³⁵S-labelled hexasaccharides, which were then analysed on DEAE-cellulose columns along with non-sulphated hexasaccharide and tri-sulphated hexasaccharide standards to examine the degree of sulphation that had occurred. The DEAE-cellulose columns were run in 50 mM-Tris/HCl buffer, pH 7.4, and eluted with an exponential gradient of 0–0.5 M-LiCl. Aliquots from the fractions were assayed for radioactivity and uronic acids [18].

RESULTS

Synthesis of [¹⁴C]chondroitin by the chick cartilage microsomal system with UDP-[¹⁴C]GlcA plus UDP-GalNAc as substrates incubated with and without Triton X-100 and with or without PAPS is shown in Fig. 1. Only minimal incorporation of [¹⁴C]GlcA was observed in the absence of UDP-GalNAc, demonstrating that polymer formation had taken place when both sugar nucleotides were present. As previously described, using fresh non-frozen microsomes, formation of chondroitin was generally, but not always, linear for 2 h or more, with a 2–3-fold increase in the rate of synthesis when the detergent was present [8]. The presence of PAPS in the incubation mixture resulted in considerable 6-sulphation of the nascent chondroitin, accompanied by a consistent decrease of approx. 50% in synthesis of the chondroitin polymer. This decrease has been noted previously [2] and was approximately the same proportion whether or not Triton X-100 was present.

DEAE-cellulose co-chromatography of [¹⁴C]chondroitin/chondroitin sulphate formed in the absence of Triton X-100 together with [³H]chondroitin/chondroitin sulphate formed in the presence of Triton X-100 was performed to examine the degree and distribution of sulphation (Fig. 2). Similar DEAE-cellulose chromatography (results not shown) was performed

Fig. 1. Synthesis of chick cartilage microsomal [^{14}C]chondroitin and [^{14}C]chondroitin sulphate in the absence or presence of Triton X-100 and PAPS

Aliquots of [^{14}C]chondroitin and [^{14}C]chondroitin sulphate formed after various time intervals were assayed as described in the Materials and methods section. ●, No PAPS, no Triton; ○, no PAPS, 1% Triton; ▲, PAPS, no Triton; △, PAPS, 1% Triton.

using a reverse labelling with the [^3H]chondroitin/chondroitin sulphate formed in the absence of Triton X-100 together with [^{14}C]chondroitin/chondroitin sulphate formed in the presence of Triton X-100. Results with this reversal of labels were the same. In all cases the aliquots taken for chromatography were selected to adjust for differences in specific activities between the ^3H and ^{14}C . Thus the radioactivity shown in Fig. 2 directly reflects the amount of synthesis. As previously described [3], [^{14}C]chondroitin formed by this system in the absence of PAPS and in the absence of Triton X-100 was eluted as two peaks (Fig. 2a). The first peak (peak I, fractions 30–44), eluted near a hyaluronic acid standard, was previously shown [3] to be derived mainly from the large Type I proteochondroitin formed by polymerization of large non-sulphated chondroitin chains on to a small primer. The second peak (Peak II, fractions 45–74), eluted just before a chondroitin 4-sulphate standard, was previously shown [3] to be derived mainly from the smaller Type II proteochondroitin formed by polymerization of smaller non-sulphated chondroitin chains or chondroitin oligosaccharides on to sulphated proteochondroitin that already contained chondroitin sulphate chains of substantial size. As previously described [8], [^3H]chondroitin formed similarly in the presence of Triton X-100 had a different profile, with almost three times as much Peak II material, but little change in the amount of Peak I material.

When PAPS was present in the Triton-free incubation mixtures during chondroitin synthesis (Fig. 2b), the products chromatographed quite differently, with a time-related disappearance of Peak I, a significant shift of Peak II towards the area of the chondroitin 4-sulphate standard, and the appearance of a third peak (Peak III, fractions 75–90), mainly after the chondroitin 4-sulphate standard. As reported previously [10], sulphation occurred in an ordered fashion, so that most nascent chains were either highly sulphated or not sulphated at all. Even at earlier time intervals (10 min) there was a substantial appearance of Peak III, indicating little time lag in sulphation. [^3H]Chondroitin sulphate formed similarly when Triton X-100 was present had a profile on DEAE-cellulose showing considerably more formation of Peak II than that of the [^{14}C]chondroitin sulphate formed when Triton X-100 was absent. In contrast with the increased formation of Peak II, there was no increase in Peak III. The Peak III [^3H]chondroitin sulphate formed when Triton X-100 was

Fig. 2. DEAE-cellulose chromatograms of chondroitin and chondroitin sulphate formed in the presence or absence of Triton X-100 with PAPS present during polymerization

Aliquots of [^{14}C]chondroitin and [^{14}C]chondroitin sulphate formed at various time intervals in the absence of Triton X-100 (●) and [^3H]chondroitin and [^3H]chondroitin sulphate formed at various time intervals in the presence of Triton X-100 (○) together with hyaluronic acid (HA) and chondroitin 4-sulphate (C-4-S) standards were co-chromatographed on a DEAE-cellulose column (0.7 cm $\times$ 5 cm) with an exponential gradient of 0.05–1.0 M-LiCl as described in the Materials and methods section. Fractions (0.5 ml) were collected and assayed for radioactivity and uronic acids. (a) [^{14}C]Chondroitin and [^3H]chondroitin formed in the absence of PAPS; (b) [^{14}C]chondroitin sulphate and [^3H]chondroitin sulphate formed in the presence of PAPS during polymerization.

present was also eluted somewhat earlier than the Peak III [^{14}C]chondroitin sulphate formed in its absence.

The degree of Peak II and Peak III sulphation was determined by chondroitin ABC lyase degradation (Table 1). This indicated that there were distinct differences between Peak II and Peak III in sulphation of nascent chondroitin, so that the former was only one-third to two-thirds sulphated, whereas the latter was almost entirely sulphated. Moreover, there did not appear to be much chondroitin sulphation intermediate between these two peaks. There were also consistent differences in the extent of sulphation depending upon the presence or absence of Triton X-100 during the synthesis. Although the differences were small, the co-chromatography technique of ^3H -labelled (Triton present) and ^{14}C -labelled (Triton absent) permitted us to demonstrate that these differences were significant. This was also confirmed by chondroitinase degradation of the reversed label [^3H]chondroitin/chondroitin sulphate formed without Triton and the

Table 1. Sulphation of endogenous nascent proteochondroitin by the microsomal system

Sample	Triton X-100	Peak II (% Δ Di-6S)	Peak III (% Δ Di-6S)
Fig. 2			
(a)			
^{14}C	—	0	—
^3H	+	0	—
(b)			
10 min			
^{14}C	—	38	88
^3H	+	41	84
120 min			
^{14}C	—	37	89
^3H	+	43	85
Fig. 3			
120 min			
^{14}C	—	65	92
^3H	+	70	86

Fig. 3. DEAE-cellulose chromatograms of chondroitin sulphate formed in the absence or presence of Triton X-100 with PAPS added subsequent to polymerization

Aliquots of [^{14}C]chondroitin sulphate formed in the absence of Triton X-100 (●) and [^3H]chondroitin sulphate formed in the presence of Triton X-100 (○) were chromatographed as described in Fig. 2. Abbreviations are defined in Fig. 2.

[^{14}C]chondroitin/chondroitin sulphate formed with Triton. Peak II formed in the presence of Triton X-100 had a slightly higher sulphate content than the Peak II chondroitin formed in its absence. Conversely, Peak III formed in its presence had a lower sulphate content than the Peak III formed in its absence. As previously described [8] there was a slight diminution in the molecular size of the chondroitin/chondroitin sulphate of each peak formed in the presence of Triton X-100. Thus the earlier appearance of the Peak III on DEAE-cellulose chromatography was due to a combination of less sulphation and slightly smaller size.

Chromatograms showing the sulphation of preformed [^{14}C]chondroitin and preformed [^3H]chondroitin are shown in Fig. 3. In this case the microsomal preparation was incubated with labelled sugar nucleotides for 2 h before non-labelled sugar nucleotides plus PAPS were added. Under these conditions sulphation proceeded rapidly, so that the pattern observed after a 10 min incubation with PAPS did not differ to a significant

Fig. 4. DEAE-cellulose chromatograms of sulphated exogenous [^{14}C]chondroitin and sulphated [^3H]chondroitin obtained from sulphated exogenous proteo[^3H]chondroitin

Aliquots of sulphated exogenous [^{14}C]chondroitin (●) and [^3H]chondroitin (○) derived from sulphated exogenous proteo[^3H]chondroitin together with hyaluronic acid and chondroitin 4-sulphate standards were co-chromatographed on a DEAE-cellulose column (0.7 cm $\times$ 5 cm) as described in the legend to Fig. 2. (a) Sulphation by microsomal system; Triton X-100 present; (b) sulphation by 100000 g supernatant; no Triton present.

extent from that of the 120 min incubation. Moreover, the Peak II material was more highly sulphated than the Peak II material that had been sulphated concurrently with polymerization (Table 1). The presence of Triton X-100 slightly altered the degree of sulphation (Table 1) similarly to that seen when sulphation accompanied polymerization, with a slight increase in sulphation of Peak II and slight decrease in sulphation of Peak III.

Exogenous [^{14}C]chondroitin and proteo[^3H]chondroitin was incubated for 2 h with the chick cartilage microsomal preparation and PAPS in the presence of Triton X-100. The labelled chondroitin and proteochondroitin used for these experiments was taken from mast-cell microsomal incubations, since the mast-cell microsomal system produces a single form of proteoglycan representing *de novo* synthesis of entire chains. Chondroitin formed by this system is found only in the Peak I region on chromatography with DEAE-cellulose [11,13]. As previously described [20], minimal sulphation of exogenous glycosaminoglycan and no sulphation of proteoglycan took place if the detergent were omitted from the incubation mixture. DEAE-cellulose chromatography of sulphated exogenous [^{14}C]chondroitin together with [^3H]chondroitin sulphate derived by alkali treatment of sulphated exogenous proteo[^3H]chondroitin is shown in Fig. 4(a). Overall sulphation of the exogenous [^{14}C]chondroitin and proteo[^3H]chondroitin was less (25 and 30% respectively) than overall sulphation of endogenous nascent chick cartilage chondroitin (55–62%). Nevertheless, the ordered pattern of sulphation was still present, so that exogenous substrates were either significantly sulphated or not sulphated at all. The Peak-II material chromatographed somewhat earlier than the Peak-II material derived from endogenous chondroitin sulphate (Fig. 3), and there was little or no

Fig. 5. DEAE-cellulose chromatograms of [³⁵S]hexasaccharide sulphated by microsomal system in the absence or presence of Triton X-100

Aliquots of hexasaccharide sulphated by chick cartilage microsomes in the absence (●) or presence (○) of Triton X-100 were separately chromatographed on a DEAE-cellulose column (0.7 × 5 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.4. Non-sulphated and tri-sulphated hexasaccharide (Hexa O-S and Tri-S) standards were present. The column was eluted with an exponential gradient of 0–0.5 M-LiCl. Aliquots from fractions were assayed for radioactivity and uronic acids. (a) 3.0 mM-hexasaccharide incubated with PAP³⁵S; (b) 0.003 mM-hexasaccharide incubated with PAP³⁵S.

Fig. 6. DEAE-cellulose chromatograms of [³⁵S]hexasaccharide sulphated by 100 000 g supernatant

Aliquots of hexasaccharide sulphated by 100 000 g supernatant in the absence of Triton X-100 were chromatographed and assayed as described in the legend to Fig. 5. (a) 3.0 mM-Hexasaccharide incubated with PAP³⁵S; (b) 0.003 mM-hexasaccharide incubated with PAP³⁵S.

Table 2. Sulphation of hexasaccharide by microsomal and supernatant systems

System	[Hexasaccharide] (mM)	Triton X-100	10 ⁻³ × Radioactivity (c.p.m.)	GalNAc sulphated (%)*
Microsomal	3.0	–	3200	0.57
	3.0	+	11800	2.1
	0.003	–	32	6.1
	0.003	+	95	18.1
Supernatant	3.0	–	666	0.13
	0.003	–	6	1.1

* This was calculated by using the specific radioactivity of the PAP³⁵S and the amount of hexasaccharide in the incubation mixtures.

Peak-III material. When the sulphated Peak-II (fractions 45–74) material was degraded by chondroitin ABC lyase, there was 58 % [¹⁴C]ΔDi-6S/42 % [¹⁴C]ΔDi-0S and 62 % [³H]ΔDi-6S/38 % [³H]ΔDi-0S. As in previous experiments with this system, there was no measurable ΔDi-4S. There was essentially no sulphation in the Peak-I (fraction 30–44) material.

We questioned whether the ordered sulphation pattern of exogenous [¹⁴C]chondroitin and proteo[³H]chondroitin was due to the membranous nature of this microsomal system. Therefore we performed similar incubations (no Triton X-100) with soluble (100 000 g supernatant) enzyme to see whether or not ordered sulphation would be lost, resulting in a completely heterogeneous

mixture of partially sulphated chains. DEAE-cellulose chromatography of the [¹⁴C]chondroitin sulphate together with the [³H]chondroitin sulphate derived from proteo[³H]chondroitin sulphate is shown in Fig. 4. The ordered sulphation persisted in the soluble system, with two discrete populations, although the sulphated chains were somewhat less sulphated than with the microsomal preparation. When the sulphated Peak II (fractions 45–74) material was degraded with chondroitin ABC lyase, there was 48 % [¹⁴C]ΔDi-6S, 52 % [¹⁴C]ΔDi-0S and 52 % [³C]ΔDi-6S, 48 % [³H]ΔDi-0S.

Chondroitin hexasaccharide sulphation by microsomal enzyme was examined to see if multiple sites would be sulphated. Amounts of [³⁵S]sulphate incorporated are shown in Table 2. DEAE-cellulose chromatograms of these [³⁵S]hexasaccharides are shown in Fig. 5, together with standards of non-sulphated hexasaccharide and tri-sulphated hexasaccharide. At high concentrations of hexasaccharide (3.0 mM) in the absence of Triton X-100, much of the [³⁵S]hexasaccharide was found to be di-sulphated, with the rest mono-sulphated hexasaccharide and no tri-sulphated hexasaccharide. This indicates a highly organized pattern of sulphation, since a completely random pattern with only 0.57% of the GalNAc sulphated would have resulted in a ratio of mono-sulphated/di-sulphated/tri-sulphated of approx. 30000:175:1. Lowering the hexasaccharide concentration decreased the incorporation markedly, but increased the GalNAc that was sulphated to 2.1% (Table 2). Nevertheless, there was only a slight effect on the nature of the products, resulting in a small amount of tri-sulphated hexasaccharide. If there had been a random pattern with 2.1% of the GalNAc sulphated, the ratio

of monosulphated/disulphated/trisulphated would have been approx. 2500:50:1. The presence of Triton X-100 during the incubations increased total sulphation 3–4 fold (Table 2), but, as seen in Fig. 5, there was no change in the percentage of disulphated hexasaccharide relative to the monosulphated hexasaccharide at high hexasaccharide concentration. There was, however, an increment of trisulphated hexasaccharide at this low hexasaccharide concentration. Thus the ordered sulphation of multiple residues rather than just a single residue in the hexasaccharide was almost entirely independent of hexasaccharide concentration and independent of Triton X-100.

As with the sulphation of exogenous chondroitin and proteo-chondroitin, we questioned whether the sulphation of multiple residues in individual hexasaccharides was related to the particulate nature of the sulphating system. Therefore, hexasaccharide and PAP³⁵S was incubated with soluble (supernatant) enzyme. Results were identical whether or not Triton X-100 was present. Even though there was considerably less sulphation than with the microsomal system (Table 2), the [³⁵S]hexasaccharide products (Fig. 6) indicated a much higher proportion of trisulphated hexasaccharide, even at the high hexasaccharide concentrations. Thus the supernatant enzyme was significantly more capable of sulphating multiple residues than was the particulate system.

DISCUSSION

The presence of PAPS during chondroitin formation was accompanied by a decrease in the amount of chondroitin formed by the microsomal preparation. However, the size of the proteo-chondroitin products [2] and the length of the individual chondroitin chains were not affected. Thus the decrease in synthesis was apparently due to a decrease in the rate with which proteo-chondroitin primers became available for the addition of chondroitin, or to the facility with which sugar nucleotides were made available to the sites for synthesis. These results differ from those reported with mast-cell microsomes [21], where the presence of PAPS leading to sulphation of *N*-acetylheparosan (heparin precursor) resulted in much longer glycosaminoglycan chains with little change in total amount of sugars incorporated. The explanation for our results is not clear, but they might be due to a competition of the PAPS for transport of sugar nucleotides. Noteworthy in this regard is the report [22] that increasing the concentration of UDP-GlcNAc while UDP-GlcA concentration was kept low resulted in a decrease in synthesis of *N*-acetylheparosan glycosaminoglycan by a mast-cell microsomal system. It was suggested that this was due to competition of the UDP-GlcNAc for transport of the UDP-GlcA. It is not known whether PAPS can inhibit transport of the sugar nucleotides in a similar fashion.

The present work concerning chondroitin 6-sulphation by microsomal preparations from chick cartilage is similar in some important respects to our previous work on chondroitin 4-sulphation by mast-cell microsomes [13]. Thus sulphation of exogenous proteo-chondroitin (Fig. 4), exogenous chondroitin (Fig. 4) and exogenous chondroitin hexasaccharide (Fig. 5) all resulted in organized rather than random patterns. However, as with the mast-cell system, the extent of sulphation did not approach that seen with the nascent material formed on endogenous acceptors. Sulphation of the same exogenous substrates by soluble enzyme (Figs. 4 and 6) was also similar to sulphation with the mast-cell soluble enzyme. As with the mast-cell enzymes, sulphation of chondroitin and proteo-chondroitin had a somewhat less ordered pattern with the soluble enzyme, whereas sulphation of hexasaccharide had a somewhat more ordered pattern than with the microsomal enzyme. As we previously

reported [20], the presence of Triton X-100 was required in the incubation with the microsomal preparation in order to obtain any sulphation of proteo-chondroitin.

As also described by us [23] and by other investigators [24], the amount of exogenous chondroitin and hexasaccharide subject to sulphation was increased considerably by the presence of neutral detergents with the microsomal preparation (Table 2), even though the extent of sulphation on individual acceptor molecules remained essentially the same. Our results (Figs. 5 and 6 and Table 2) indicated that this multiple sulphation occurred even at high hexasaccharide concentration and with soluble enzyme. These results disagree with those previously reported [24] with cultured chick chondrocytes as a microsomal source, where it was stated that multiple sulphation of single oligosaccharide acceptor molecules was not seen unless the oligosaccharide concentration was well below the K_m . Thus the mechanisms of 6-sulphation by the cartilage enzyme appeared similar to those we previously reported [13] for 4-sulphation by the mast-cell enzyme.

The chick cartilage microsomal chondroitin-synthesizing system differs significantly from that of the mast-cell microsomes, since there is formation of two separate species of proteo-chondroitin rather than a single species. Moreover, synthesis of one of these species (Type II) is markedly stimulated by Triton X-100, whereas the presence of this detergent had essentially no effect on the synthesis of chondroitin by the mast-cell system. Our present results now indicate that sulphation is similarly affected so that the presence of Triton slightly increased the extent of sulphation of the Type II proteo-chondroitin. In contrast, the presence of Triton X-100 resulted in somewhat less effective sulphation of the Type I proteo-chondroitin than in its absence (Figs. 2 and 3 and Table 1). This suggests that the Triton X-100 might have enabled an increased availability of PAPS at the site of the smaller Type II proteoglycan primer, whereas the detergent had the opposite effect at the site of the larger Type I species of proteoglycan primer. This interpretation is consistent with the delay that was noted in sulphation of the Type I material (Peak III) when PAPS was added subsequent to polymerization by Triton-containing microsomes (Fig. 3).

We previously have shown that the apparent K_m for the sugar nucleotides in biosynthesis of these two species are different [2]. However, we consider it to be highly unlikely that this reflects a difference in glycosyl transferases for these two proteo-chondroitin sulphates. It is more reasonable to suggest that this is a reflection of the two separate Golgi compartments or loci we have previously demonstrated [9] for synthesis of the two forms. It is not surprising that the Triton might affect these loci differently, and these findings are consistent with the differences we have previously shown [1] for extraction of the two forms from their microsomal sites of synthesis. These results in turn indicate that there might be considerable differences in the organization of the separate Golgi loci for sulphation of the glycosaminoglycan portions of the two species of proteoglycans. Thus we now have an indication that sulphation of the larger form is decreased (but still ordered), whereas the sulphation of the smaller form is enhanced. The results are also consistent with the likelihood that sulphation and polymerization occur together, as they do in mast-cell chondroitin sulphate synthesis [25]. In this sense one might expect that Triton X-100 would stimulate sulphation of the small Type II proteo-chondroitin species in the same way that polymerization was stimulated.

These results are all in marked contrast with observations on the synthesis of heparin by mast-cell microsomes [12], where glycosaminoglycan polymerization has been reported to be abolished by detergent, and sulphation has been reported to be changed from an ordered pattern to a completely variable pattern. These differences from the proteo-chondroitin-sulphate-

synthesizing systems suggest that there are variations from tissue to tissue and from product to product in the organization of membrane-bound sulphotransferases relative to the membrane-bound glycosaminoglycan glycosyltransferases.

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