Glycosyl transferases in chondroitin sulphate biosynthesis

Effect of acceptor structure on activity

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The *D*-glucuronosyl (GlcA)- and *N*-acetyl-*D*-galactosaminy (GalNAc)-transferases involved in chondroitin sulphate biosynthesis were studied in a microsomal preparation from chick-embryo chondrocytes. Transfer of GlcA and GalNAc from their UDP derivatives to ³H-labelled oligosaccharides prepared from chondroitin sulphate and hyaluronic acid was assayed by h.p.l.c. of the reaction mixture. Conditions required for maximal activities of the two enzymes were remarkably similar. Activities were stimulated 3.5–6-fold by neutral detergents. Both enzymes were completely inhibited by EDTA and maximally stimulated by MnCl₂ or CoCl₂. MgCl₂ neither stimulated nor inhibited. The GlcA transferase showed a sharp pH optimum between pH 5 and 6, whereas the GalNAc transferase gave a broad optimum from pH 5 to 8. At pH 7 under optimal conditions, the GalNAc transferase gave a velocity that was twice that of the GlcA transferase. Oligosaccharides prepared from chondroitin 4-sulphate and hyaluronic acid were almost inactive as acceptors for both enzymes, whereas oligosaccharides from chondroitin 6-sulphate and chondroitin gave similar rates that were 70–80-fold higher than those observed with the endogenous acceptors. Oligosaccharide acceptors with degrees of polymerization of 6 or higher gave similar *K_m* and *V_{max}* values, but the smaller oligosaccharides were less effective acceptors. These results are discussed in terms of the implications for regulation of the overall rates of the chain-elongation fractions in chondroitin sulphate synthesis *in vivo*.

The CS chains of PCS have hybrid sequences that contain both 4- and 6-sulphated GlcA-GalNAc disaccharide units (Kimata *et al.*, 1974; Seno *et al.*, 1975; Michelacci & Dietrich, 1976; Faltynek & Silbert, 1978; Knudson *et al.*, 1984). The CS chain elongation on the core protein takes place in the Golgi (Peterson & Leblond, 1964; Godman & Lane, 1964; Neutra & Leblond, 1966; Horwitz & Dorfman, 1968; Revel, 1970) and involves the alternating actions of glucuronosyl- and *N*-acetylgalactosaminy-transferases and chain sulphation by the 4- and 6-sulphotransferases (Perlman *et al.*, 1964; Telser *et al.*, 1966;

Horwitz & Dorfman, 1968; Silbert & DeLuca, 1969; Richmond *et al.*, 1973*a,b*; DeLuca *et al.*, 1973; Silbert & Repucci, 1976). Regulation of the sulphation patterns and chain lengths of CS depends on the acceptor specificities and the relative activities and the organelle localizations of the CS biosynthesis enzymes. If chain elongation and sulphation occur concurrently, the non-reducing terminal of the growing chain may vary in its positions of sulphation or its degree of sulphation and could have a profound influence on the rate or extent of chain elongation.

Silbert and his collaborators (Richmond *et al.*, 1973*a,b*) have shown that microsomal preparations from chick-embryo cartilage can catalyse CS chain elongation without concurrent sulphation. They also showed that the sulphotransferases could transfer sulphate to polymeric sulphate acceptors, a finding that was confirmed by Habuchi & co-workers (Habuchi & Miyata, 1980; Habuchi

Abbreviations used: CS, chondroitin sulphate; PCS, proteochondroitin sulphate; C4S, chondroitin 4-sulphate; C6S, chondroitin 6-sulphate.

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& Miyashita, 1982). Thus the activities of the enzymes involved in CS chain biosynthesis are such that chain elongation and chain sulphation may occur *in vivo* either separately or concurrently and the question of simultaneous as against sequential chain elongation and chain sulphation *in vivo* remains unresolved.

The acceptor specificities of the glycosyltransferases were reported in the early work of Perlman *et al.* (1964), Telser (1968) and Helting & Rodén (1969), who demonstrated that the galactosaminyltransferase could transfer GalNAc from UDP-GalNAc to the non-reducing terminal GlcA residue of a non-sulphated, a 4-sulphated or a 6-sulphated oligosaccharide, whereas the glucuronosyltransferase could transfer GlcA from UDP-GlcA only to the non-reducing terminal GalNAc residues of non-sulphated or 6-sulphated oligosaccharides and not to the corresponding 4-sulphated oligosaccharide. The relative rates of transfer to different acceptors were not reported, and the assay conditions used were shown later to be suboptimal for both enzymes.

The present study was undertaken to extend the information available for assessing the effects of substrate structure and assay conditions on the chain-elongation reaction in the synthesis of CS chains. The results obtained show significant differences from those reported previously. The reasons for these differences and the implications of these results for the chain-elongation reaction *in vivo* are discussed.

Experimental

Chromatography and electrophoresis

Radiolabelled samples were spotted on 2.5 cm × 57 cm strips of Whatman no. 3 paper and analysed by determining the rates of migration of the labelled products on chromatograms or electrophoretograms. Chromatograms were developed for the times indicated in Solvent I (butan-1-ol/acetic acid/1 M-NH₃ (2:3:2, by vol.) or Solvent II (butan-1-ol/acetic acid/1 M-NH₃ (2:3:3, by vol.). Electrophoresis was carried out in pyridine/acetic acid/water (1:5:400, by vol., pH 3.8) for 2 h at 1400 V on a water-cooled horizontal electrophoresis apparatus. Chromatography and electrophoresis strips were cut into 1.25 cm segments and analysed for radioactivity by liquid-scintillation counting (Conrad *et al.*, 1973).

H.p.l.c. was performed on a Partisil 10 SAX column (Whatman) under the conditions described previously (Delaney *et al.*, 1980).

Oligosaccharide preparation and characterization

Oligosaccharides having GlcA residues at their non-reducing terminals were generated by diges-

tion of C4S (Sigma Type A), C6S (Sigma Type C), hyaluronic acid (Sigma Grade I), and chondroitin with ovine testicular hyaluronidase (Sigma Type V, 1250 units/mg). The chondroitin was prepared by desulphation of C4S as described by Kantor & Schubert (1956). Each incubation mixture contained 50 mg of polysaccharide and 1 mg of hyaluronidase/ml of 0.1 M-sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl. For hyaluronic acid, the digestion mixture contained 25 mg of polysaccharide and 0.5 mg of hyaluronidase/ml of the same buffer. These incubations were scaled up to obtain larger amounts of oligosaccharides. Incubations were carried out at 37°C for 14 h for hyaluronic acid and for 24 h for the other polymers. The other polymers were digested for an additional 24 h after adding 1 mg of solid hyaluronidase/ml of reaction mixture. Each mixture was then heated at 100°C for 5 min to inactivate the enzyme, freeze-dried to a powder and resuspended in a minimum volume of water. Solutions derived from up to 1 g of the original polymer were chromatographed on a 2.6 cm × 160 cm Sephadex column prepared and developed in 0.1 M-NaCl for hyaluronic acid, 0.5 M-NaCl for chondroitin, 1.0 M-NaCl for C6S and 1.5 M-NaCl for C4S. Fractions (10 ml) were collected and were assayed by the carbazole procedure (Bitter & Muir, 1962), and the fractions containing the tetra-, hexa-, octa- and deca-saccharide peaks were combined separately, freeze-dried and desalted on Bio-Gel P-2 columns developed in water. These preparations of partially purified oligosaccharides were reduced with NaB³H₄ (424 Ci/mol, Amersham Corp.) under the 0°C reduction conditions developed previously for prevention of alkaline degradation (Glaser & Conrad, 1979). The [³H]oligosaccharides were finally purified by h.p.l.c. on a Magnum 9 Partisil SAX column (Whatman) under conditions similar to those described previously (Delaney *et al.*, 1980). Each oligosaccharide preparation was purified by elution from the SAX column with KH₂PO₄ solutions under isocratic conditions. For the sulphated tetra-, hexa-, octa- and deca-saccharides, the concentrations of KH₂PO₄ used were 150, 215, 340 and 350 mM respectively. The KH₂PO₄ concentrations used for the corresponding unsulphated oligosaccharides were 15, 35, 50 and 70 mM respectively. The ³H-labelled fractions obtained from the centres of the major peaks in each oligosaccharide preparation (see Delaney *et al.*, 1980) were combined, freeze-dried and desalted as described above. By analytical h.p.l.c., each purified oligosaccharide gave a single, symmetrical peak with a purity equal to that described previously (Delaney *et al.*, 1980).

Portions of each oligosaccharide purified as described above were treated with β -glucuronidase

(Sigma Type B-3, 3 500 000 units/mg) to generate a series of oligosaccharides having *N*-acetylhexosamine residues at their non-reducing terminals. In each case, approx. 0.1 mmol was incubated with 1 mg of β -glucuronidase at 37°C for 5 h in 400 ml of 0.05 M-sodium acetate buffer. Sulphated oligosaccharides were digested in pH 4.0 buffer containing 0.5 mM-2-acetamido-2-deoxy-D-galactono-lactone as described previously (Delaney *et al.*, 1980) to prevent the action of any contaminating hexosaminidase in the β -glucuronidase preparation. The products were purified by preparative paper chromatography in chromatography solvents appropriate for the individual oligosaccharide (see Table 1 below). The homogeneity of each product was confirmed by analytical h.p.l.c. (Delaney *et al.*, 1980). All of the oligosaccharides contained 2.35×10^8 d.p.m./ μ mol. This value was used with the counting efficiencies determined for each counting condition to calculate the concentrations of the substrates described below.

Preparation of UDP-GalNAc

A UDP-GlcNAc 4'-epimerase prepared from a *Bacillus subtilis* strain LC168⁻, obtained from Dr. R. L. Switzer of this Department, was grown for 24 h with shaking in 1 litre of a supplemented nutrient-broth medium (Hanson *et al.*, 1964). The cells were recovered by centrifugation at 800g and the cell paste was washed once with 100 ml of 25 mM-Tris/HCl, pH 8.8. The cells were resuspended in 100 ml of the same buffer and disrupted by sonication. The cellular debris was removed by centrifugation at 15 000g and the supernatant was then centrifuged at 100 000g for 60 min. The soluble fraction was concentrated with a PM-10 membrane (Anspec) and used as the epimerase preparation. UDP-GlcNAc (Sigma, 1.0 mmol) was incubated for 12 h at 37°C in 8.4 ml of 25 mM-Tris/HCl, pH 8.8, containing 8.4 μ mol of NAD⁺ and 187 mg of the *B. subtilis* enzyme protein. The reaction was treated at 100°C to inactivate the enzyme and precipitate the protein, and the pH was adjusted to 7.0 with 1 M-HCl. The reaction mixture was filtered through a 0.45 μ m-pore-size disposable filter (Anspec) to remove the precipitated protein and a portion of the filtrate was analysed for UDP-GlcNAc and UDP-GalNAc by measuring the amount of GlcNAc and GalNAc formed by acid hydrolysis. This was done by hydrolysing the mixture in 0.01 M-HCl at 100°C for 1 h, conditions found to convert the UDP derivatives quantitatively into free GlcNAc and GalNAc without removal of the *N*-acetyl groups. The hydrolysate was reduced with NaBH₄ and assayed for the reduced forms of GlcNAc and GalNAc by radiochromatography (Conrad *et al.*, 1973). The product was an equilibrium mixture of UDP-

GlcNAc containing 22.4 μ mol of UDP-GalNAc/ml.

Enzyme preparation

The glycosyltransferase preparation used was prepared from cultured chondrocytes. The cells were taken from zones 1–3 of the chick-embryo tibiotarsus (Kim & Conrad, 1977) and cultured in monolayers as described by Kim & Conrad (1977, 1980, 1982). Confluent cells were harvested by treatment with trypsin, and washed with phosphate-buffered saline [containing (in g/l): CaCl₂, 0.1; KCl, 0.2; KH₂PO₄, 0.2; MgCl₂·6H₂O, 0.1; NaCl, 8.0; and Na₂HPO₄·7H₂O, 2.16; final pH 7.2]. The cells were suspended in 1 mM-Tris, pH 7.4, at a density of 5×10^6 cells/ml, pelleted at 500g, and resuspended in 3–5 ml of the Tris buffer for 10 min. Then an equal volume of 0.5 M-sucrose in Tris buffer was added to swell and lyse the cells. Unbroken cells, lysosomes, mitochondria, nuclei, and plasma membranes were pelleted by centrifugation at 5000g for 30 min. The supernatant was then centrifuged at 100 000g for 60 min to obtain a microsomal pellet that contained the glycosyltransferases at a specific activity 4.5-fold greater than that of the original cell homogenate. The pellet was resuspended in 59 mM-imidazole buffer, pH 7.0, containing 50 mM-KCl. This enzyme preparation, which contained 4.27 mg of protein/ml (Lowry *et al.*, 1951), was quick-frozen in small portions in a solid-CO₂/ethanol bath and stored at –70°C without loss of activity for at least 6 months. Chondrocytes from 57 tibiotarsi yielded 21.4 mg of microsomal protein. Portions were thawed as needed and used for all of the work reported here.

GalNAc transferase assay

For the standard assay in which the rate of GalNAc transfer to exogenous oligosaccharide acceptors was measured, 50 nmol of an [³H]oligosaccharide with a non-reducing terminal GlcA residue and 350 nmol of the UDP-GalNAc were concentrated to dryness in a stream of air in a 0.5 ml Eppendorf microfuge tube and resuspended in 5 μ l of a stock solution that contained 50 mM-imidazole buffer, pH 7.0, 50 mM-KCl, 60 mM-MnCl₂ and 12% Brij 58, adjusted to pH 7.0. The reaction was started by adding 5 μ l of enzyme (21.4 μ g of protein) and the assay mixture was incubated at 37°C for the desired time interval (usually 1 h). The reaction was stopped by addition of 10 μ l of cold 10% (w/v) trichloroacetic acid. The contents of the tube were diluted by the addition of 80 μ l of water and filtered through a 0.45 μ m-pore-size disposable filter (Anspec). A 20 μ l portion of the filtrate was analysed by h.p.l.c. using isocratic elution with KH₂PO₄ concentrations chosen according to the oligosaccharide used as the acceptor

in the assay (see above and Table 1). The amounts (nmol) of products formed in the assays were calculated from the ^3H c.p.m. in the product peaks. As a control the assay was repeated with UDP-GlcNAc (Sigma) in place of the UDP-GalNAc (see the results section).

GlcA transferase assay

The UDP-GlcA used in these assays was obtained from Sigma and was analysed for its UDP-GlcA content by radiochromatographic determination (Conrad *et al.*, 1973; Conrad, 1980) of the amount of GlcA formed after hydrolysis in 0.1M-HCl at 100°C for 1h. For the standard glucuronosyltransferase assay in which the rate of GlcA transfer to exogenous oligosaccharide acceptors was measured, 25 nmol of an [^3H]oligosaccharide with a non-reducing terminal GalNAc residue and 500 nmol of UDP-GlcA were dried in a 0.5 ml Eppendorf microfuge tube and resuspended in 2.5 μl of water and 5 μl of a stock solution that contained 50 mM-imidazole buffer, pH 7.0, 50 mM-KCl, 60 mM-MnCl₂ and 12% Brij 58, adjusted to pH 7.0. The reaction was started by adding 2.5 μl of enzyme (10.7 μg of protein) and the assay mixture was incubated at 37°C for the desired time period (usually 1h). The reaction was stopped and the mixture was analysed by h.p.l.c. as described above.

To measure GlcA transfer to endogenous acceptors, the assay was repeated with the addition of 3.1×10^6 d.p.m. of UDP-D-[^{14}C]GlcA (New England Nuclear Corp.; 31 Ci/mol) in the presence or absence of the [^3H]oligosaccharide. After incubation, the reaction mixture was analysed for ^{14}C -labelled product at the origin of a paper chromatogram as described for the GlcA transferase above. In this same assay the stability of the UDP-D-[^{14}C]GlcA was assessed by determining the ^{14}C c.p.m. in the UDP-GlcA, the GlcA 1-phosphate and the free GlcA on the chromatogram.

Results

Enzyme assays

A new assay procedure was developed for use in the kinetic studies reported here. Oligosaccharides having NaB $^3\text{H}_4$ -reduced GalNAc or GlcNAc residues on their reducing terminals and GalNAc, GlcNAc or GlcA residues on their non-reducing terminals were incubated with a microsomal enzyme preparation in the presence of the appropriate glycosyl donor (UDP-GlcA or UDP-GalNAc), and the resulting end-labelled products were separated from the labelled acceptors by h.p.l.c.

The characteristic chromatography and electrophoretic properties of the oligosaccharide acceptors are shown in Table 1. Typical separations of

acceptors and products in the galactosaminyl- and glucuronosyl-transferase assays are shown in Fig. 1. The rates of both assays were linear with time and enzyme concentration.

Optimal assay conditions

In the comparison of oligosaccharide acceptors reported below, both glycosyltransferase activities were assayed at pH 7 in imidazole buffer containing MnCl₂, Brij 58 and saturating concentrations of the glycosyl donors and acceptors. By using these same assay conditions, the pH, metal ion and detergent concentrations were varied separately to observe the effects of these assay parameters on the activities of the enzymes. Fig. 2 shows the effects of pH on the glycosyltransferase activities. The pH optimum for the glucuronosyltransferase is between pH 5 and 6 for both the 6-sulphated and the non-sulphated heptasaccharide acceptors. Activities were higher in the imidazole buffer than in the acetate buffer. The galactosaminyltransferase exhibited a much broader pH optimum, with near-maximal rates from pH 5 to 8 for both the 6-sulphated and the non-sulphated hexasaccharide acceptors. Activities in the acetate and imidazole buffers were similar for this enzyme. For the assays described below, pH 7 was chosen as a common pH for assay of both enzymes. In part, this pH was chosen to avoid the competing activities of any contaminating lysosomal enzymes that might degrade both the substrate and the products of the glycosyltransferase reactions (Glaser & Conrad, 1979a).

In an initial study of the effect of detergents on the transferase activities, it was found that Brij 58 and Triton X-100, both neutral detergents, gave increases in both glycosyltransferase activities ranging from 3.5- to 6-fold. The activities were inhibited by cholate. Since Brij 58 gave somewhat higher activities than Triton X-100, it was chosen for use in the routine assays. For both enzymes, activity increased rapidly as the detergent concentration was raised to 0.1% (w/v) and continued to increase more gradually up to 6% (w/v) detergent, the highest concentration tested. For both enzymes the activity with the non-sulphated oligosaccharide acceptor was stimulated by detergent to a greater extent than was the activity with the 6-sulphated acceptor.

Activities in the absence of bivalent-metal ions were low, and were reduced to zero by addition of EDTA. The responses of both transferases to added metal ions were similar. At 10 mM concentrations Mn²⁺ and Co²⁺ gave the greatest stimulations of activity, whereas Mg²⁺ and Ca²⁺ had no effect on the enzyme activities. By assaying the enzymes over a range of MnCl₂ concentrations, it was found that rates for both enzymes increased up

Table 1. *Chromatographic and electrophoretic properties of glycosyl acceptors*

Oligosaccharides having degrees of polymerization (DP) from 4 to 10 were prepared from C4S, C6S, chondroitin (C0S) and hyaluronic acid (HA). Oligosaccharides were characterized by h.p.l.c. and by paper chromatography and paper electrophoresis. H.p.l.c. was run on a Whatman Partisil 10 SAX column (0.45 cm × 25 cm) developed isocratically in aqueous KH_2PO_4 containing 15% (v/v) methanol. The flow rate was 1 ml/min. Paper electrophoresis was run at 25 V/cm for 2 h on 2.5 cm × 57 cm strips of Whatman no. 3 paper in pyridine/acetic acid/water (1:5:400, by vol.).

Oligosaccharide		Paper chromatography		H.p.l.c.		Electrophoresis distance (cm)
		Solvent	Migration	KH_2PO_4 (mM)	Retention (min)	
DP	Source		Time (h) Distance (cm)			
4	C4S	I	24 19.1	170	17.5	34.2
	C6S		24 15.2		21.0	34.2
5	C4S		48 12.7		14.0	—
	C6C		48 19.1		12.0	—
6	C4S		48 10.2	240	26.5	33.0
	C6S		48 8.9		22.0	33.0
7	C4S	II	48 43.2		18.0	—
	C6S		48 26.7		14.0	—
8	C4S		48 17.8	300	32.0	34.2
	C6S		48 16.5		22.5	35.6
9	C4S		72 44.5		—	—
	C6S		72 30.5		8.0	—
10	C4S		72 27.9	350	28.0	33.0
	C6S		72 16.5		26.0	33.0
4	C0S	I	18 26.7	20	20.5	17.8
	HA		18 16.5		20.5	19.1
5	C0S		24 36.8		8.0	—
	HA		24 25.4	42	7.5	—
6	C0S		24 21.6		18.5	20.3
	HA		24 11.4		20.0	20.3
7	C0S		48 45.7		9.5	—
	HA		48 20.3	60	8.5	—
8	C0S		48 20.3		21.0	19.1
	HA		48 5.1		21.0	19.1
9	C0S	II	48 27.9		10.5	—
	HA		24 20.3	75	9.5	—
10	C0S	I	48 8.9		22.5	19.1
	HA	II	24 7.6		22.0	20.3

to 30 mM- MnCl_2 and that rates decreased at higher MnCl_2 concentrations.

Kinetic properties

The K_m and $V_{\max}$ values obtained from Eadie-Hofstee plots of rate data obtained with various concentrations of donors and acceptors are shown in Table 2. Similar results were obtained for both transferases. In the absence of any added oligosaccharide acceptors the rates of glycosyl transfer to endogenous acceptors in the enzyme preparation were very low. Both the 6-sulphated and the non-sulphated oligosaccharides served as good acceptors. In general, the $V_{\max}$ values were low and the K_m values were high for the smallest oligosaccharide acceptors tested. In fact, the GalNAc transfer-

ase rates with the 6-sulphated tetrasaccharide were too low to obtain valid kinetic data. However, the kinetic properties were similar for the acceptors having six or more monosaccharide units. The acceptor activities of the 4-sulphated oligosaccharides approached zero (see the Discussion section) for both transferases, whereas the acceptor activities of the hyaluronic acid oligosaccharides were also very low.

The K_m values for the UDP-sugar donors were similar when measured with the 6-sulphated and the non-sulphated oligosaccharide acceptors. When the galactosaminyltransferase assay was run with UDP-GlcNAc as the only glycosyl donor, the amount of product formed with the 6-sulphated hexasaccharide as the acceptor was only 3% of that

Fig. 1. *H.p.l.c. separations used in glycosyltransferase assays*

Assays were carried out as described in the Experimental section, and the ^3H -labelled products (peaks II) were separated from the ^3H -oligosaccharide substrates (peaks I) by h.p.l.c. on an anion-exchange column. GlcA transferase assays were run with ^3H -heptasaccharides prepared from chondroitin (a) and C6S (c) as glycosyl acceptors. GalNAc transferase assays were run with ^3H -hexasaccharides prepared from chondroitin (b) and C6S (d) as glycosyl acceptors. Isocratic elutions were carried out with KH_2PO_4 buffers containing 10% (v/v) methanol. The KH_2PO_4 concentrations used were 84 mM (a and b), 240 mM (c) and 290 mM (d).

obtained with UDP-GalNAc. This may have been due to the presence of a small amount of UDP-GlcNAc 4'-epimerase in the transferase enzyme preparation, which could give a slow conversion of the UDP-GlcNAc into UDP-GalNAc. This was not investigated further.

The extents of degradation of UDP-GalNAc and UDP-GlcA during the course of these assays was tested by adding donors labelled with ^{14}C in the sugar residue to the standard assay mixtures containing the hexasaccharide (GalNAc transferase) or heptasaccharide (GlcA transferase). The

Fig. 2. Effect of pH on the activities of GlcA transferase assayed with $[^3\text{H}]$ heptasaccharide acceptors (a) and GalNAc transferase assayed with $[^3\text{H}]$ hexasaccharide acceptors (b)

Standard assays of mixtures containing Brij 58, MnCl_2 and the appropriate glycosyl donors and acceptors prepared from chondroitin (○, ●) or C6S (□, ■) were run. Sodium acetate (●, ■) or sodium imidazole (○, □) buffers at the pH values indicated were present at a concentration of 50 mM.

results showed that more than 60% of the starting UDP-GalNAc and more than 90% of the starting UDP-GlcA were still present at the end of the incubation period. The degradation products formed were $[^{14}\text{C}]\text{GalNAc}$ and $[^{14}\text{C}]\text{GlcA}$ 1-phosphate respectively. Calculations using the K_m value obtained for the UDP-GalNAc show that, if 60% of the UDP-GalNAc added to a standard assay mixture remained, the GalNAc transferase reaction would run at a velocity that was greater than 90% of V_{max} . The linearity of the GalNAc transferase reaction with time indicates that the slow loss of UDP-GalNAc during the assay did not affect the validity of the assay.

Discussion

The previous descriptions of the substrate specificities and optimal assay conditions for the glycosyltransferases from chick-embryo cartilage, summarized by Rodén *et al.* (1972), are derived largely from the early work of Telser *et al.* (1966) and Perlman *et al.* (1964), who assayed the enzymes in Tris buffer containing 1 mM-EDTA but

no detergent. Under these conditions, it was found that both enzymes gave maximum activity at pH 7 and required MgCl_2 for activity. Silbert & DeLuca (1969) also used MgCl_2 to activate these enzymes in early work, but found in later studies that MnCl_2 was a better activator (Richmond *et al.*, 1973b; Silbert & Repucci, 1976). Reports on the glycosyltransferases from chick cartilage have indicated that both enzymes are optimally active in the presence of 30 mM- MnCl_2 , but that MgCl_2 can partially replace Mn^{2+} for the GalNAc transferase (Rodén *et al.*, 1972). In the present work it was found that Mg^{2+} had no effect on the activity of either of the glycosyltransferases, whereas the enzymes were stimulated 2.5–10-fold when Mn^{2+} was added. The activation by Mg^{2+} in the original studies may have been due to its effect in overcoming the inhibitory action of the EDTA. It may also be noted that the results for the GalNAc transferase in the early studies were obtained using the hexasaccharide prepared from C4S as the acceptor. As shown in present work, the latter is a very poor substrate for the GalNAc transferase.

The marked stimulation of the activities of the

Table 2. *Kinetic properties of glycosyltransferases*

K_m and V_{max} values for the glycosyltransferases were determined by Eadie-Hofstee plots. Values for the endogenous acceptors were measured in the absence of exogenous oligosaccharide acceptors as described in the Experimental section. The abbreviations used for the exogenous acceptors are given in the legend to Table 1.

Acceptor	GlcUA transferase			GalNAc transferase		
	K_m (mM)		V_{max}	K_m (mM)		V_{max}
	Source DP	UDP-GlcUA Acceptor		UDP-GalNAc Acceptor		
Endogenous		-	16	-	-	<7
COS	4	-	-	-	6.8	113
	5	-	2.2	-	-	-
	6	-	-	-	1.6	580
	7	0.53	3.5	1.7	-	-
	8	-	-	-	2.0	504
	9	-	3.0	-	-	-
	10	-	1125	-	1.7	441
C6S	5	-	6.5	-	-	-
	6	-	-	-	5.6	522
	7	0.35	1.4	1.2	-	-
	8	-	-	-	3.0	361
	9	-	1.2	-	-	-
	10	-	-	-	5.0	531
C4S	4	-	-	-	-	19
	5	-	1	-	-	-
	6	-	-	-	-	27
	7	-	14	-	-	-
	8	-	-	-	-	65
	9	-	12	-	-	-
HA	5	-	7	-	-	-
	6	-	-	-	-	62
	7	-	11	-	-	-
	8	-	-	-	-	85
	9	-	25	-	-	-
	10	-	-	-	-	101

Golgi-associated glycosyltransferases by neutral detergents suggests that disruption of the Golgi membrane by detergent allows free access of the enzymes to the exogenous substrates. Under the optimized assay conditions used here, the specific activities of the glycosyltransferases were 10–30-fold higher than those reported in the literature (Perlman *et al.*, 1964; Telser, 1968; Helting & Rodén, 1969; Silbert & DeLuca, 1969; Silbert & Freilich, 1980). The pH optima for both the GalNAc transferase and the GlcA transferase were reported previously to be 7.0 (Rodén *et al.*, 1972). However, in the presence of the optimal concentrations of $MnCl_2$ and detergent used in the present study, maximal activity for the GalNAc transferase was obtained from pH 5 to 8, whereas the GlcA transferase gave maximal activity from pH 5 to 6. Although transferase activities were measured at pH 7 in the present study, rates expected for other pH values can be estimated by

using data from the pH-optimum plots. The relative specific activities at pH 7 (Table 2) suggest that the GalNAc transferase activity should be the rate-limiting step in chain elongation at the lower pH values. However, as the pH is raised, the GlcA transferase activity falls, whereas the GalNAc transferase rate remains high. These data suggest that regulation of the internal pH of the Golgi compartment may affect both the relative amounts of GlcA and GalNAc at the non-reducing terminals of the growing polysaccharide chain and the overall rate of chain elongation.

K_m and V_{max} values for the glycosyl donors and for most of the glycosyl acceptors were not measured in the earlier work (Rodén *et al.*, 1972). The results in Table 2 show that the K_m values of the acceptors tend to decrease with increasing size of the acceptor, whereas the V_{max} values increase. The K_m values for the donors are in the millimolar range and are similar whether non-sulphated or

sulphated acceptors are used. The previous kinetic properties that have been reported for these enzymes have been measured in the absence of detergents and may therefore represent parameters describing the membrane transport of the substrates rather than values for the interaction of the substrates with the enzymes.

It is not known whether sulphation occurs *in vivo* concurrently with chain elongation or after the chain-elongation process has been completed. If, as found for heparin biosynthesis (Rodén, 1980), the sulphation reactions in CS biosynthesis take place after completion of the chain elongation, only those glycosyltransferase rates observed for the non-sulphated acceptors are pertinent in any consideration of the regulation of chain elongation under physiological conditions. A maximum rate for the GalNAc transferase was reached with the hexasaccharide, whereas a maximum rate for the GlcA transferase was approached with the heptasaccharide. These observations suggest that the acceptor-binding sites of these enzymes recognize at least three disaccharide units at the non-reducing terminal of the acceptor and that maximal rates of chain elongation on non-sulphated chains will occur when the size of the growing chain reaches a degree of polymerization of 6. However, it is only a tentative conclusion, since the acceptors *in vivo* are clearly more complex structures than the exogenous acceptors used here.

If elongation and sulphation occur concurrently, the effects of acceptor structure on the growth of the CS chain are much more complex. The rates for both transferases with the 6-sulphated oligosaccharides are somewhat lower than those for the non-sulphated acceptors, but the enzymes are nearly (or completely, see below) inactive with the 4-sulphated oligosaccharides. In contrast with these results, the literature reports that, whereas 4-sulphated oligosaccharides will not serve as acceptors for the GlcA transferase, they are good acceptors for the GalNAc transferase (Telser, 1968; Rodén *et al.*, 1972). In fact, the recommended substrate for use in the assay of the GalNAc transferase is the hexasaccharide prepared from C4S (Rodén *et al.*, 1972). A possible reason for the difference between the present and the previous results is discussed below. In any event, it is clear that prior sulphation of the growing CS chain can have a profound effect on the chain-elongation process, since chain termination will occur with either glycosyltransferase when the chain becomes 4-sulphated.

A possible explanation for the previously observed apparent acceptor activity of the 4-sulphated hexasaccharide may lie in the structural features of the oligosaccharide substrate used. In early work it was believed that C4S and C6S were distinct

polymers and that, on cleavage of these polymers with hyaluronidase and purification of the resulting oligosaccharides according to size, each oligosaccharide size class would contain all of the ester sulphate groups in the same position on the GalNAc residues. With the use of improved analytical tools for the analysis of CS and its fragments, it has been recognized within the past few years that CS chains are hybrid structures containing both 4-sulphated and 6-sulphated disaccharide units (Kimata *et al.*, 1974; Seno *et al.*, 1975; Michelacci & Detrich, 1976; Faltynek & Silbert, 1978; Knudson *et al.*, 1984). The CS that is obtained from any source, including that from rat chondrosarcoma (Mason *et al.*, 1982), contains both types of disaccharides, even though it may be highly enriched in one of the types. Limited digestion of CS will yield a mixture of oligosaccharides that can be separated by gel filtration to obtain products that contain a mixture of hybrid oligosaccharides within single size classes. C4S oligosaccharides prepared by this methodology, including those used in the earlier acceptor studies, contain a significant proportion of oligosaccharides which contain 6-sulphated disaccharide units (Delaney *et al.*, 1980). In fact, the specificity of hyaluronidase is such that the smaller oligosaccharides obtained in such digests are more enriched in the 6-sulphated disaccharide units than the starting polymer (Knudson *et al.*, 1984). If the oligosaccharide acceptors prepared from C4S in the early acceptor-specificity studies contained significant amounts of the active 6-sulphated disaccharide units, it is possible that the activity observed with these preparations could have been due to these 6-sulphated structures. Although each oligosaccharide size class can be largely resolved into species that contain a single type of disaccharide unit by h.p.l.c., as in the present work, the final products still retain small amounts of some of the hybrid sequences (Delaney *et al.*, 1980). The low activities measured with the 4-sulphated oligosaccharides in the present study may have been due to these remaining 6-sulphated structures. If this is the case, the 4-sulphated oligosaccharides may be totally inactive as glycosyl acceptors.

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