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Biosynthesis of heparin

The D-glucuronosyl- and *N*-acetyl-D-glucosaminyltransferase reactions and their relation to polymer modification

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Oligosaccharides with the general structure [GlcA-GlcNAc]$_n$-GlcA-aMan (aMan is 2,5-anhydro-D-mannose), derived from the *Escherichia coli* K5 capsular polysaccharide, were found to serve as monosaccharide acceptors for a GlcNAc-transferase, solubilized from a mouse mastocytoma microsomal fraction and implicated in heparin biosynthesis. Digestion of these oligosaccharides with β-D-glucuronidase yielded acceptors for the GlcA-transferase that acts in concert with the GlcNAc-transferase. Assays based on the oligosaccharide acceptors showed broad pH optima for the two enzymes, centred around pH 6.5 for the GlcNAc-transferase and around pH 7.0 for the GlcA-transferase. The GlcNAc-transferase showed an absolute requirement for Mn^{2+}, whereas the GlcA-transferase was stimulated by Ca^{2+} and Mg^{2+} but not by Mn^{2+}. The GlcNAc acceptor ability of the [GlcA-GlcNAc]$_n$-GlcA-aMan oligosaccharides increased with increasing chain length, as reflected by the apparent K_m, which was 60 μM for a hexasaccharide but 6 μM for a hexadecasaccharide. By contrast, the K_m for [GlcNAc-GlcA]$_n$-aMan oligosaccharides in the GlcA-transferase reaction was higher, ~ 0.5 mM, and unaffected by acceptor size. After chemical modification of the oligosaccharides to obtain mixed *N*-substituents (*N*-unsubstituted, *N*-acetylated or *N*-sulphated GlcN residues), GlcNAc transfer was found to be virtually independent of the *N*-substituent pattern of the acceptor sequence. The GlcA-transferase, on the other hand, showed marked preference for an acceptor with a non-reducing-terminal GlcNAc-GlcA-GlcNSO$_3^-$ sequence, which would thus have a lower K_m for the enzyme than the corresponding fully *N*-acetylated structure. These results, along with our previous finding that chain elongation in a mastocytoma microsomal system is strongly promoted by concomitant *N*-sulphation of the nascent chain [Lidholt, Kjellén & Lindahl (1989) Biochem. J. **261**, 999–1007], raise the possibility that the glycosyltransferases and the *N*-deacetylase/*N*-sulphotransferase act in concert during chain elongation, assembled into an enzyme complex.

INTRODUCTION

Heparin and heparan sulphate are synthesized as proteoglycans. The formation of the actual glycosaminoglycan chains is initiated by *O*-D-xylosylation of selected serine residues in the appropriate proteoglycan core proteins and is followed by the addition of two D-galactose units (Rodén, 1980). The resulting galactosyl-galactosyl-xylosyl trisaccharide sequence serves as an acceptor for the first GlcA monosaccharide unit. The glycosaminoglycan chain proper is then generated by stepwise alternating transfer of GlcNAc and GlcA residues from the corresponding UDP-monosaccharide derivatives to the non-reducing terminus of the nascent polymer. The final glycosaminoglycan structures are acquired by a series of polymer-modification reactions that is initiated by *N*-deacetylation and *N*-sulphation of GlcNAc units, proceeds through C-5 epimerization of GlcA to IdoA units and is concluded by incorporation of *O*-sulphate groups at various positions (for reviews see Lindahl *et al.*, 1986; Gallagher *et al.*, 1986; Lindahl & Kjellén, 1987; Lindahl, 1989). The structural heterogeneity of the final products, heparin and heparan sulphate, and, indeed, the structural difference between these species, are ascribed to the mode of biosynthetic polymer modification; the reactions involved in this process are generally incomplete in the sense that a fraction of the potential substrate residues escape modification.

Most of the previous studies on the chain-elongation and modification reactions utilized microsomal fractions from mouse mastocytoma tissue (Silbert, 1963; Lindahl *et al.*, 1973; Lidholt *et al.*, 1988). Such preparations contain all the (membrane-bound) enzymes involved in the process and, in addition, the preformed 'primer', i.e. a core protein presumably substituted with the Gal-Gal-Xyl trisaccharide sequence, required to generate glycosaminoglycan chains from added UDP-GlcA and UDP-GlcNAc. Under optimal conditions the microsomal chain-elongation reaction occurs rapidly, the individual glycosaminoglycan chain growing by at least 40 disaccharide units/min (Lidholt *et al.*, 1988). The rate of elongation can be modulated by changing the relative proportions of the two sugar nucleotides (Lidholt *et al.*, 1988). Moreover, the polymerization reaction was found to be markedly stimulated by concomitant *N*-sulphation of the nascent chain (Lidholt *et al.*, 1989).

Attempts have been made to characterize the individual glycosyltransferase reactions by using exogenous saccharides as acceptors for GlcA or GlcNAc monosaccharide units (Helting & Lindahl, 1971, 1972; Helting, 1972; Forsee & Rodén, 1981). The acceptors were oligosaccharides or carbohydrate–serine compounds derived either from the polysaccharide–protein linkage region or from more peripheral portions of heparin/heparan sulphate chains. Although the expected transfer reactions could be demonstrated, the structures of the acceptors utilized differed significantly from those assumed to prevail *in vivo*; for instance, the exogenous acceptors generally contained various amounts of non-reducing-terminal IdoA units. In the present study we have avoided this problem by preparing acceptor oligosaccharides

Abbreviations used: GlcA, D-glucuronic acid; IdoA, L-iduronic acid; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; GlcNAc, 2-deoxy-2-acetamido-D-glucose (*N*-acetyl-D-glucosamine); GalNAc, 2-deoxy-2-acetamido-D-galactose (*N*-acetyl-D-galactosamine); aMan, 2,5-anhydro-D-mannose; -NSO$_3$, *N*-sulphate group.

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from a microbial source. The capsular polysaccharide from *Escherichia coli* K5 thus has the same $[GlcA\text{-}GlcNAc]_n$ structure as the non-sulphated precursor polysaccharide generated during heparin/heparan sulphate biosynthesis (Vann *et al.*, 1981), and can in fact be modified into heparin-like sequences by the action of enzymes (GlcNAc *N*-deacetylase/*N*-sulphotransferase, GlcA C-5 epimerase, various *O*-sulphotransferases) solubilized from mouse mastocytoma tissue (Riesenfeld *et al.*, 1987; Kusche *et al.*, 1991). Use of oligosaccharides, derived from the *E. coli* K5 polysaccharide, as acceptors in glycosyltransferase assays enabled a systematic study of the basic kinetics properties of the GlcA- and GlcNAc-transferases present in mouse mastocytoma. The results open novel insights into the relationship between the chain elongation and *N*-sulphation reactions in heparin/heparan sulphate biosynthesis. Furthermore, this information provides the basis for a new model of the overall biosynthetic process.

EXPERIMENTAL

Materials

A microsomal fraction was prepared as described (Jacobsson *et al.*, 1979) from a transplantable mouse mastocytoma (Furth *et al.*, 1957).

UDP-[^{14}C]GlcA was prepared enzymically from D-[U-^{14}C]glucose (321 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) by the procedure of Jacobsson *et al.* (1979). UDP-[6-^{3}H]GlcNAc (27 Ci/mmol) was obtained from New England Nuclear. Unlabelled UDP-GlcA and UDP-GlcNAc and bovine liver β-D-glucuronidase (type B-10) were from Sigma.

Capsular polysaccharide from *E. coli* K5, with the structure $[GlcA\beta1,4\text{-}GlcNAc\alpha1,4]_n$, was generously given by Dr. G. van Deedem, Diosynth, Oss, The Netherlands. A 0.5 g batch of the polysaccharide was purified by ion-exchange chromatography on a column (130 ml) of DEAE-cellulose (Whatman DE-52), equilibrated with 50 mM-NaCl/50 mM-Tris/HCl, pH 8. The polysaccharide was dissolved in 10 ml of water and applied to the column, which was subsequently washed, first with 0.5 litre of the equilibration buffer and then with 0.5 litre of 50 mM-NaCl/50 mM-sodium acetate, pH 4. The polysaccharide was eluted with a linear salt gradient (total volume 1 litre) from 50 mM- to 1.5 M-NaCl in 50 mM-acetate buffer, pH 4. Fractions ($\sim$ 15 ml) were collected at a rate of $\sim$ 30 ml/h and were analysed for hexuronic acid by the carbazole reaction. Pooled effluent fractions were dialysed against water and freeze-dried.

Oligosaccharides for use as acceptors in glycosyltransferase reactions were prepared from the purified K5 polysaccharide by partial *N*-deacetylation followed by deaminative cleavage (Höök *et al.*, 1982; Shaklee & Conrad, 1984) as follows. Polysaccharide ($\sim$ 400 mg) was dissolved in 40 ml of hydrazine, containing 1% hydrazine sulphate, and the mixture was heated at 100 °C for 30 min. The hydrazine was removed by repeated evaporation to dryness in the presence of toluene, and the partially *N*-deacetylated polysaccharide was dialysed against water. After evaporation to dryness, deaminative cleavage of *N*-unsubstituted GlcN units was achieved by treatment with 40 ml of HNO_2 reagent, pH 3.9 (Shively & Conrad, 1976), for 60 min. The reaction was interrupted by increasing the pH to $\sim$ 7 with Na_2CO_3. The resulting $[GlcA\text{-}GlcNAc]_n$-GlcA-aMan ($n \geqslant 0$) oligosaccharides were applied to a column (240 cm × 3 cm) of Sephadex G-50, equilibrated with 1 M-NaCl, and eluted at a rate of $\sim$ 17 ml/h. Effluent fractions (8.5 ml) were collected and analysed for hexuronic acid by the carbazole reaction. Fractions corresponding to peaks of oligosaccharides ranging from tetra- to octadeca-saccharides were pooled, concentrated by evaporation, and re-applied separately to the Sephadex G-50 column. The purified oligosaccharides were desalted by passage through a column (70 cm × 1 cm) of Sephadex G-15, equilibrated with 0.2 M-NH_4HCO_3, followed by freeze-drying. To obtain odd-numbered $[GlcNAc\text{-}GlcA]_n$-aMan oligosaccharides, a portion of each isolated even-numbered oligosaccharide species was digested with β-D-glucuronidase from bovine liver. Incubation mixtures containing 1–10 mg of oligosaccharide, 0.4 mg of enzyme and 2 mg of BSA in 1 ml of 0.05 M-sodium acetate, pH 5, were maintained at 37 °C for 24 h and were then heated at 100 °C for 3 min. After centrifugation to remove precipitated protein, oligosaccharides in the supernatant were separated from released GlcA monosaccharide by chromatography on Sephadex G-15, as indicated above, and were finally freeze-dried.

Oligosaccharide preparations with mixed *N*-substituents were obtained as follows. A portion (100 mg) of the partially *N*-deacetylated and depolymerized K5 polysaccharide was further *N*-deacetylated by treatment with hydrazine/hydrazine sulphate, as above, for 2 h (known from pilot experiments to produce $\sim$ 70% *N*-deacetylation). The resulting *N*-unsubstituted GlcN units were partially *N*-sulphated, by treatment (arbitrary trial-and-error approach) of the re-isolated material with 78 mg of trimethylamine/SO_3 complex (Aldrich Chemie, Steinheim, Germany) in 1.5 ml of water, at pH 9.5 for 24 h at 55 °C, as described by Levy & Petracek (1962). The modified saccharide was recovered by gel filtration on Sephadex G-15. To assess the *N*-substitution pattern of the product, a fraction containing molecules essentially larger than octasaccharides (isolated by gel chromatography on Sephadex G-50) was subjected to selective deamination at pH 1.5 only (cleavage at *N*-sulphated GlcN residues) or at pH 1.5, followed by deamination at pH 3.9 (cleavage at *N*-sulphated as well as *N*-unsubstituted GlcN units) (Riesenfeld *et al.*, 1982). The cleavage patterns, observed by gel chromatography (Fig. 1), allowed approximative estimations of the various *N*-substituents, based on calculations of peak areas corresponding to di- and oligo-saccharide deamination products (see Jacobsson *et al.*, 1979). The GlcN residues of the modified oligosaccharide preparation thus were found to be $\sim$ 45% *N*-sulphated, $\sim$ 30% *N*-acetylated and $\sim$ 25% *N*-unsubstituted.

Methods

Glycosyltransferase assays. Assays for GlcNAc-transferase activity were conducted by incubating various amounts (based on hexuronic acid contents as determined by the carbazole reaction) of the appropriate even-numbered K5 oligosaccharide acceptors (containing non-reducing-terminal GlcA units) with 0.5 μCi of UDP-[^{3}H]GlcNAc and 0.25 mg of mastocytoma microsomal protein in a total volume of 25 μl of 10 mM-$MnCl_2$/10 mM-$MgCl_2$/5 mM-$CaCl_2$/1% Triton X-100/50 mM-Hepes, pH 7.2. GlcA-transferase was assayed in similar incubations, but with β-D-glucuronidase-digested odd-numbered oligosaccharides (having non-reducing-terminal GlcNAc residues) and 0.68 μCi of UDP-[^{14}C]GlcA. After incubation at 37 °C for 10 min, 25 μl of 10% trichloroacetic acid was added to precipitate protein-bound endogenous saccharide acceptors, the mixtures were centrifuged and the supernatants were neutralized with 10 μl of 1 M-NaOH. After addition of 0.5 mg of carrier heparin, and repeated centrifugation, the samples were applied to columns (1 cm × 40 cm) of Sephadex G-25 (superfine grade, Pharmacia), equilibrated with 1 M-NaCl/0.1% Triton X-100/0.05 M-Tris/HCl, pH 8. Effluent fractions (0.5 ml) were collected at a rate of 15 ml/h, and analysed for radioactivity. Alternatively, gel chromatography was performed on a h.p.l.c. column (1.6 cm × 60 cm) packed with a gel-filtration matrix prototype gel developed by Pharmacia with separation properties intermediate between those of Sephadex G-25 and G-50. The column was eluted with the above buffer at

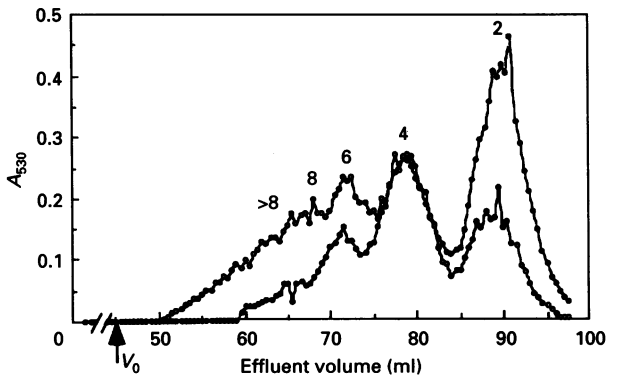

Fig. 1. Analysis of *N*-substituent patterns of partially *N*-deacetylated *N*-sulphated oligosaccharides

Oligosaccharides prepared from *E. coli* K5 polysaccharide were chemically modified as described under 'Materials'. A sample (0.5 mg) of the products was treated with HNO_2 at pH 1.5 (○), and another sample first at pH 1.5 and then at pH 3.9 (●), and the deamination products were analysed by h.p.l.c. gel chromatography (see under 'Methods'). Effluent fractions were analysed for hexuronic acid by the carbazole reaction. The number of monosaccharide units per molecule is shown above each of the various oligosaccharide peaks. The undegraded material was polydisperse, with a peak eluted at 50 ml and material tailing to 80 ml (results not shown). For additional information see the text.

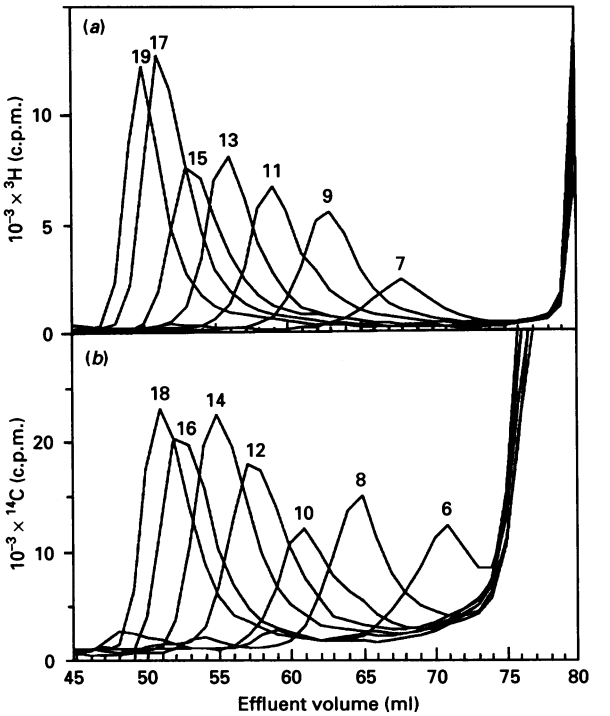

Fig. 2. Analysis of products of glycosyltransferase reactions with K5 oligosaccharides as sugar acceptors

Oligosaccharides, derived from the *E. coli* K5 polysaccharide, belonging to the (*a*) [GlcA-GlcNAc]$_n$-GlcA-aMan or (*b*) [GlcNAc-GlcA]$_n$-aMan series, were tested as acceptors in the (*a*) GlcNAc-transferase (0.5 μCi of UDP-[^{3}H]GlcNAc per incubation; 10 μM acceptor) and (*b*) GlcA-transferase (0.68 μCi of UDP-[^{14}C]GlcA per incubation; 1 mM acceptor) reactions, respectively, by the standard procedures described under 'Methods'. The labelled reaction products were fractionated by h.p.l.c. gel chromatography; effluent fractions were analysed for radioactivity. The numbers above the various peaks indicate the number of monosaccharide units per molecule, including the labelled incorporated sugar residue; in each case the corresponding acceptor tested contained one monosaccharide unit less.

a rate of 60 ml/h, and fractions (~ 1 ml) were collected. The amounts of radioactivity incorporated into oligosaccharides, separated from labelled sugar nucleotides, were taken to indicate transferase activity. Appropriate control incubations without added oligosaccharides were done in parallel.

Analytical methods. Hexuronic acid was determined by the carbazole method of Bitter & Muir (1962) with D-glucuronolactone as a standard. Concentrations of saccharides derived from the K5 polysaccharide were estimated assuming (as verified by actual analysis of a weighed sample of polysaccharide) that GlcA accounted for half of the dry weight of the material. Protein was measured by the method of Lowry *et al.* (1951). Radioactivity was measured by liquid-scintillation spectrometry in a Beckman model LS 3800 liquid-scintillation spectrometer.

Treatment of saccharides with HNO_2 at pH 1.5 or pH 3.9, resulting in deamination of *N*-sulphated or *N*-unsubstituted GlcN residues respectively, and cleavage of the corresponding glucosaminidic linkages (Shively & Conrad, 1976), was performed as described by Pejler *et al.* (1987) (see also Riesenfeld *et al.*, 1982).

RESULTS

Oligosaccharides with the general structures [GlcA-GlcNAc]$_n$-GlcA-aMan and [G]cNAc-GlcA]$_n$-aMan, derived from *E. coli* K5 capsular polysaccharide, were used as sugar acceptors in the GlcNAc-transferase and GlcA-transferase reactions respectively, and the basic kinetics of the reactions were studied. In addition, analogous oligosaccharide preparations with mixed *N*-substituents (sulphate groups, acetyl groups, unsubstituted amino groups) were used to evaluate the role of the *N*-substituent patterns of the acceptor saccharide sequences.

Oligosaccharide acceptors containing *N*-acetylated glucosamine units

Even-numbered oligosaccharides were obtained by partial *N*-deacetylation of the K5 polysaccharide, followed by deaminative cleavage at the sites of the resulting *N*-unsubstituted GlcN units, as described under 'Materials', and were separated by gel chromatography. Oligosaccharide species composed of 4–18 monosaccharide units were separately incubated with UDP-[^{3}H]GlcNAc and detergent-solubilized mastocytoma microsomal enzymes (see under 'Methods'), and the products were analysed by gel chromatography (Fig. 2*a*). Each acceptor compound yielded a labelled component which emerged at the elution position of the next-higher odd-numbered oligosaccharide. Conversely, the odd-numbered [GlcNAc-GlcA]$_n$-aMan oligosaccharides did not serve as acceptors for labelled GlcNAc units (results not shown), but incorporated labelled GlcA from UDP-[^{14}C]GlcA, and thus were converted into even-numbered saccharide products (Fig. 2*b*).

Both enzymes were active within a fairly broad pH range, appreciable transfer of monosaccharide units occurring even at the extreme pH values tested, 5.2 and 8.2 (Fig. 3). The GlcNAc-transferase displayed a relatively pronounced optimum between pH 6.0 and 7.2 (Fig. 3*a*), whereas the GlcA-transferase showed near-maximal activity between pH 6.0 and 8.2 (Fig. 3*b*). In subsequent experiments both enzymes were routinely assayed at pH 7.2.

The cation requirements differed markedly between the two enzymes. The GlcNAc-transferase, as previously observed (Helting & Lindahl, 1972; Forsee & Rodén, 1981), showed an

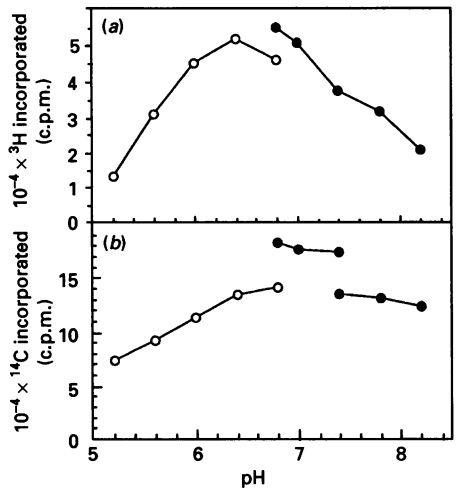

Fig. 3. pH-dependence of glycosyltransferase reactions

(*a*) GlcNAc-transferase (10 μM decasaccharide acceptor) or (*b*) GlcA-transferase (800 μM nonasaccharide acceptor) activities were determined by the standard procedures (see under 'Methods'), except that the pH was varied as indicated by using either 0.05 M-Mes (○) or 0.05 M-Hepes (●) buffer.

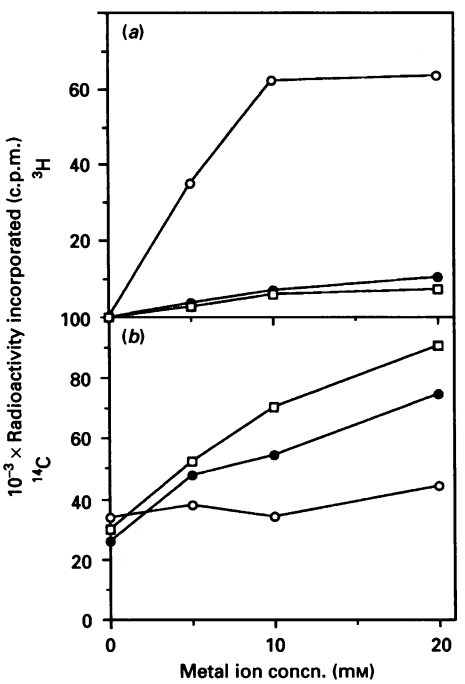

Fig. 4. Metal-ion dependence of glycosyltransferase reactions

(*a*) GlcNAc-transferase (10 μM decasaccharide acceptor) or (*b*) GlcA-transferase (800 μM undecasaccharide acceptor) activities were determined by the standard procedures (see under 'Methods'), except that Mn^{2+} (○), Ca^{2+} (●) or Mg^{2+} (□) was added as indicated.

absolute requirement for Mn^{2+}, that could not to any significant extent be substituted for by, e.g. Ca^{2+} or Mg^{2+} (Fig. 4*a*). In contrast, the GlcA-transferase was quite active also in the absence of added metal ions (Fig. 4*b*); moreover, transfer of GlcA units was promoted by both Ca^{2+} and Mg^{2+}, but not by Mn^{2+}. The standard assay mixtures for both enzymes contained all three cations, as indicated under 'Methods'. Under the conditions of the assays, the incorporation of radioactivity into the appropriate oligosaccharide acceptors was linear with time for ~ 10 min and

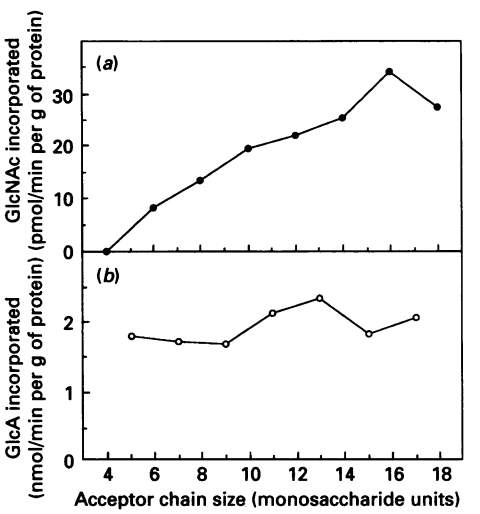

Fig. 5. Effect of acceptor chain length on acceptor ability

Oligosaccharides containing the number of monosaccharide units indicated in the figure were tested as acceptors in (*a*) the GlcNAc-transferase reaction (10 μM acceptor) or (*b*) the GlcA-transferase reaction (1 mM acceptor) by the standard procedures (see under 'Methods'). The amounts of labelled products obtained are plotted against acceptor chain size.

then slowly levelled off, presumably owing to breakdown of the UDP-sugar precursors. Standard incubations thus were maintained for 10 min. Linearity of incorporation was maintained up to 10 mg of microsomal protein/ml of incubation mixture.

A series of experiments was undertaken to investigate the role of acceptor chain length in the sugar transfer reactions. K_m was initially determined for a decasaccharide acceptor in the GlcNAc-transferase reaction (at 0.7 μM-UDP-[^{3}H]GlcNAc) and found to be 8 μM (results not shown; see below). Somewhat unexpectedly, the K_m for a nonasaccharide acceptor in the GlcA-transferase reaction (at 80 μM-UDP-[^{14}C]GlcA) was almost two orders of magnitude higher, 0.6 mM. The low K_m for the decasaccharide GlcNAc acceptor, and hence the difference as compared with the nonasaccharide GlcA acceptor, was maintained also when the UDP-[^{3}H]GlcNAc was diluted with unlabelled UDP-GlcNAc to yield the same overall sugar nucleotide concentration as applied to the GlcA-transferase assay.

From the above K_m determinations, series of GlcNAc and GlcA acceptors of different molecular size were tested in the two transferase reactions, at 10 μM and 1 mM respectively. The results indicated that the incorporation of [^{3}H]GlcNAc increased with increasing size of the even-numbered oligosaccharide acceptors (Fig. 5*a*), whereas the GlcA acceptor ability of the odd-numbered oligosaccharides was essentially independent of acceptor chain length (Fig. 5*b*). These findings led us to perform separate K_m determinations for a high- and a low-M_r member of each oligosaccharide series. In accord with the results shown in Fig. 5(*a*), the K_m for the hexasaccharide GlcNAc acceptor was 60 μM, whereas that of the hexadecasaccharide was appreciably lower, ~ 5 μM (Figs. 6*a* and 6*b*). By contrast, a pentasaccharide and a tridecasaccharide both gave the same high K_m, 0.5 mM, in the GlcA-transferase reaction (Figs. 6*c* and 6*d*). These findings suggest that, although the affinity of the GlcNAc-transferase for the growing polysaccharide chain may increase markedly in the early stages of polymerization (see Forsee & Rodén, 1981), the GlcA-transfer reaction is more likely to be the overall rate-limiting step of the polymerization process.

By using the appropriate oligosaccharide acceptors, at concentrations [2 mM for the nonasaccharide (GlcA acceptor) and 1 mM

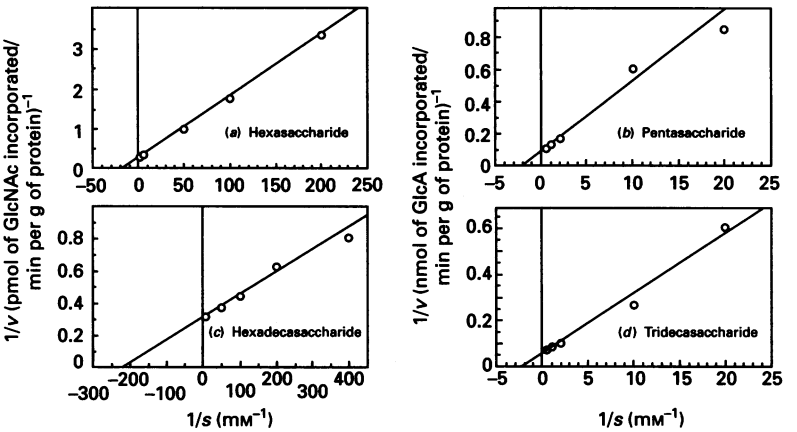

Fig. 6. Lineweaver–Burk plots for acceptors of different size

The K5-derived (*a*) hexasaccharide and (*b*) hexadecasaccharide were tested as acceptors at different concentrations in the GlcNAc-transferase reaction (0.74 μM-UDP-[^{3}H]GlcNAc), and the (*c*) pentasaccharide and (*d*) tridecasaccharide were tested as acceptors in the GlcA-transferase reaction (84 μM-UDP-[^{14}C]GlcA) by the standard procedures. The labelled products formed were isolated by gel chromatography and quantified by scintillation counting.

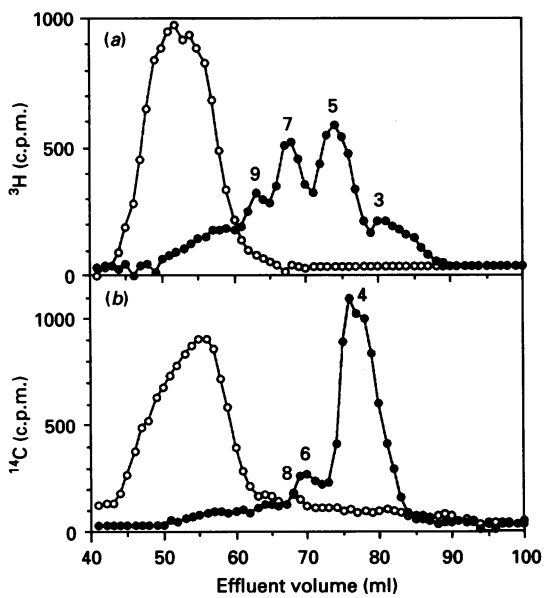

Fig. 7. *N*-Substituent patterns of acceptor sequences

E. coli K5 polysaccharide was partially depolymerized by *N*-deacetylation/deamination, and the resulting (even-numbered) oligosaccharides were further partially *N*-deacetylated and *N*-sulphated, as described under 'Materials'. A portion of the products was digested with β-D-glucuronidase to yield odd-numbered species. A sample (5 μg) of the even-numbered oligosaccharides was incubated with 1 μCi of UDP-[^{3}H]GlcNAc and 0.5 mg of solubilized microsomal protein in a total volume of 50 μl under otherwise standard conditions. Similarly, 100 μg of the odd-numbered oligosaccharides was incubated with 1.35 μCi of UDP-[^{14}C]GlcA. The appropriate labelled products of (*a*) GlcNAc-transferase or (*b*) GlcA-transferase reactions were isolated, and separated by h.p.l.c. gel chromatography into fractions of larger or smaller molecular size (not shown). ○, Re-runs of the high-molecular-mass portions; ●, the same materials after treatment with HNO_2 at pH 1.5. The numbers above the various peaks indicate the number of monosaccharide units per molecule.

for the decasaccharide (GlcNAc acceptor)] well above the K_m values determined for the oligosaccharides, the K_m values for UDP-GlcA and UDP-GlcNAc were found to be 400 μM and 60 μM respectively. [The latter value is somewhat uncertain, owing to the presence of endogenous UDP-GlcNAc in the microsomal preparation (Lidholt *et al.*, 1988)]. Finally, it may be noted that addition of 5 mM unlabelled UDP-GlcA to a GlcNAc-transferase assay mixture containing 0.7 μM-UDP-[^{3}H]GlcNAc resulted in ~ 90% inhibition of the [^{3}H]GlcNAc incorporation into an octasaccharide acceptor, presumably owing to competition at the UDP-binding site of the GlcNAc-transferase (Lidholt *et al.*, 1988).

Oligosaccharide acceptors with mixed *N*-substituents

Our previous finding, that polymerization in the intact microsomal system is promoted by concomitant *N*-sulphation of the nascent polysaccharide chain (Lidholt *et al.*, 1989), prompted us to examine the effects of *N*-substituents on the monosaccharide acceptor properties of K5-derived oligosaccharides. The approach chosen involved incubations of heterogeneous oligosaccharides, containing mixtures of *N*-unsubstituted, *N*-acetylated and *N*-sulphated GlcN residues (see under 'Materials'), followed by structural analysis of radiolabelled products so as to identify recognition sequences preferentially selected by the glycosyl-transferases.

The ^{3}H-labelled products obtained by incubating the GlcA-terminal oligosaccharides, containing the mixed *N*-substituents, with solubilized microsomal enzymes and UDP-[^{3}H]GlcNAc were first separated by h.p.l.c. gel chromatography (see 'Glycosyltransferase assays' under 'Methods'), and components larger than nonasaccharides were recovered (results not shown). Cleavage of these saccharides at sites occupied by *N*-sulphated GlcN units, by treatment with HNO_2 at pH 1.5, yielded labelled degradation products with the size-distribution shown in Fig. 7(*a*). Major peaks of penta- and hepta-saccharides were seen, along with smaller peaks of trisaccharide, nonasaccharide and larger oligosaccharides. Formation of labelled trisaccharide following deamination at pH 1.5 requires that substituent R′ in Fig. 8 (upper sequence) be a sulphate group; a pentasaccharide is generated when R′ = -H or -$COCH_3$, and R″ = -SO_3^-, and a heptasaccharide when R′ = R″ = -H or -$COCH_3$ and the next GlcN unit is *N*-sulphated. Similar components were generated by treatment with HNO_2 at pH 3.9, conditions resulting in deamination of GlcN residues with unsubstituted amino groups (results not shown). Taken together, these findings indicate that GlcNAc transfer will occur to acceptor sequences in which the

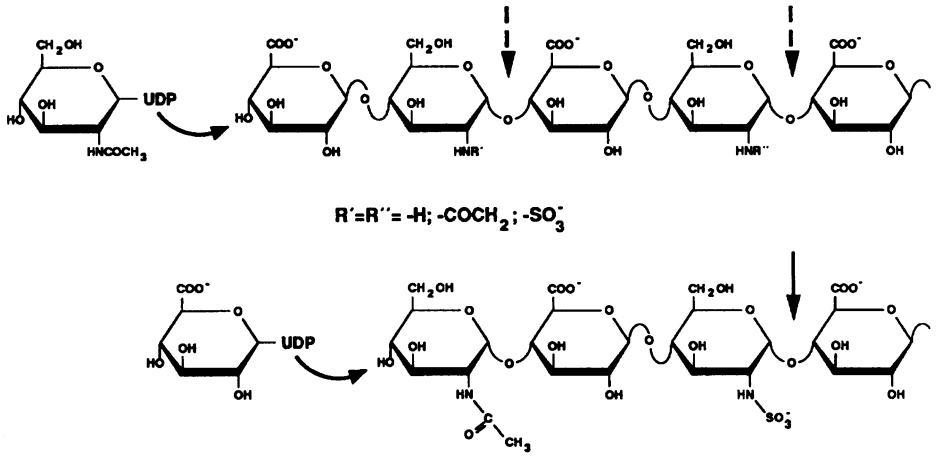

Fig. 8. Favoured acceptor sequences in glycosyltransferase reactions

The structures illustrate the results of glycosyl transfer to acceptors with mixed *N*-substituents (see Fig. 7). The upper sequence shows the incorporation of GlcNAc units into saccharide structures without any apparent preference for type of *N*-substituent. The broken arrows indicate points of deaminative cleavage by HNO_2 at pH 1.5, but only when the corresponding GlcN units are *N*-sulphated. After incorporation of a radiolabelled terminal GlcNAc residue, HNO_2 (pH 1.5) treatment will release a labelled trisaccharide if R′ = $-SO_3^-$ and a labelled pentasaccharide if R′ = $-COCH_3$ and R″ = $-SO_3^-$. The lower sequence shows the predominant acceptor structure for GlcA transfer, the recipient GlcN unit being *N*-acetylated and the next one *N*-sulphated. Cleavage by HNO_2 at pH 1.5 (solid arrow) will, in an analogous fashion, give rise to a tetrasaccharide.

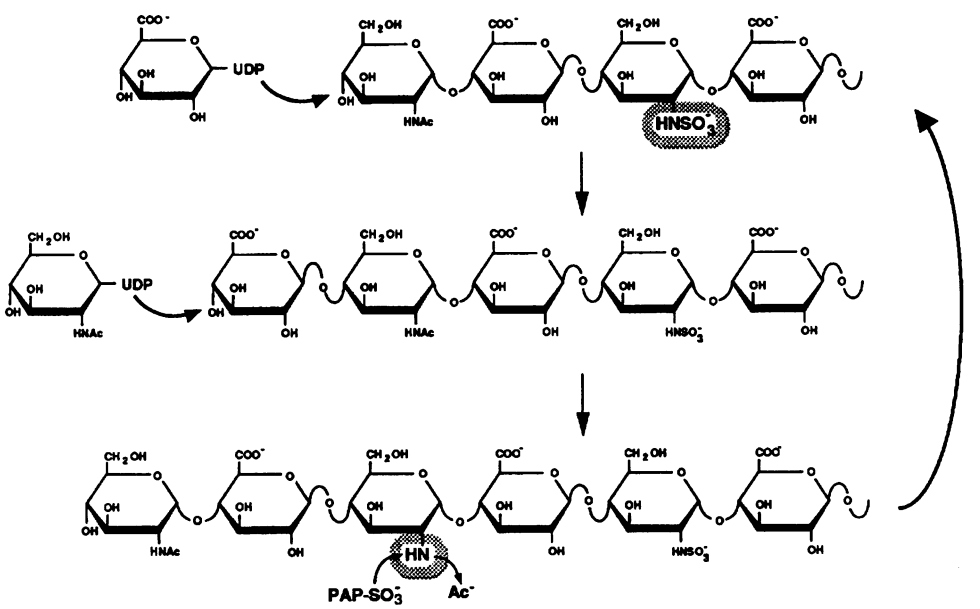

Fig. 9. Proposed scheme for concerted chain elongation and *N*-sulphation

The scheme illustrates GlcA transfer to an acceptor sequence that carries an *N*-sulphate group on the penultimate GlcN unit, and further, how such a structure will be regenerated after completed elongation by a GlcNAc-GlcA- disaccharide unit. For additional information see the text. Abbreviation: PAP-SO_3^-, adenosine 3′-phosphate 5′-phosphosulphate.

adjacent as well as the penultimate GlcN residue may be *N*-unsubstituted, *N*-acetylated or *N*-sulphated.

The corresponding experiments relating to transfer of GlcA units gave an entirely different picture. Before incubation with microsomal enzymes and UDP-[^{14}C]GlcA, potential acceptor sequences were generated by digesting the oligosaccharide mixture with β-D-glucuronidase. After enzymic labelling of the oligosaccharides, the products were again fractionated by h.p.l.c. gel chromatography and components larger than octasaccharides were recovered (results not shown). This fraction was essentially resistant toward deamination at pH 3.9, indicating lack of *N*-unsubstituted GlcN residues in the preferred acceptor sequences for the GlcA-transferase (results not shown). In contrast, treatment with HNO_2 at pH 1.5 resulted in almost quantitative conversion of the labelled saccharide into a tetrasaccharide, presumably having the structure [^{14}C]GlcA-GlcNAc-GlcA-aMan (Fig. 7*b*). These findings, reproducible in separate experiments, indicate that the GlcA-transferase expresses strong preference for an acceptor with the structure of the lower sequence shown in Fig. 8, the non-reducing-terminal and penultimate GlcN units being *N*-acetylated and *N*-sulphated respectively.

An attempt was made to compare the K_m of the partially *N*-sulphated oligosaccharides in the GlcA-transferase reaction with that of the corresponding fully *N*-acetylated oligosaccharides (after β-D-glucuronidase digestion of both oligosaccharide preparations). In parallel assays with the same enzyme prep-

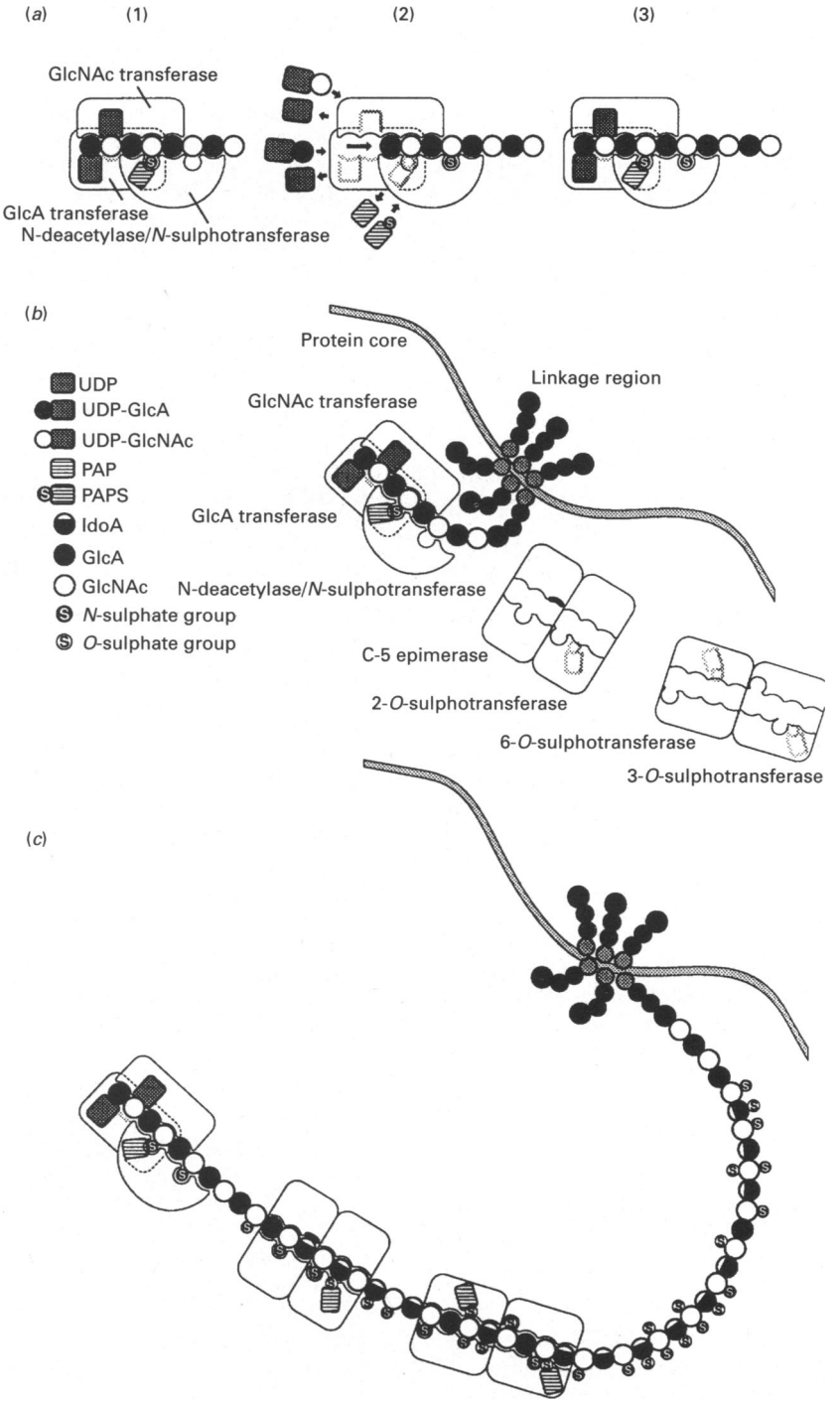

Fig. 10. Model for the formation and modification of polysaccharide chains in heparin biosynthesis

(*a*) Scheme of the concerted action of glycosyltransferases and the GlcNAc *N*-deacetylase/*N*-sulphotransferase. The reducing terminus is to the right. (1) GlcNAc and GlcA are transferred to the saccharide acceptor from the corresponding UDP-sugars, and the penultimate *N*-acetyl group is exchanged for an *N*-sulphate group. The *N*-sulphate residue is introduced before the GlcA unit. (2) The saccharide moves to a new acceptor position; UDP and PAP (adenosine 3′,5′-bisphosphate) are released. (3) New UDP-sugars and PAPS attach to the enzymes for another round of chain elongation and *N*-deacetylation/*N*-sulphation. (*b*, *c*) Model of interaction of nascent polysaccharide chain with enzymes. The protein core with attached tetrasaccharide (or pentasaccharide) linkage region is the first substrate to be recognized by the GlcA-/GlcNAc-transferases (*b*). The Figure depicts only one polysaccharide chain; it is not known how many chains on the same core protein may be formed simultaneously, nor whether all preformed linkage regions will actually serve as initiation sites. At a later stage of polymerization the glycosyltransferase/*N*-deacetylase/*N*-sulphotransferase complex will interact with a [-GlcA-GlcNAc-]$_n$ sequence, which is further elongated, along with *N*-sulphation. (*c*) Once the nascent, partially *N*-sulphated, chain has reached a certain length it will interact also with the GlcA C-5 epimerase and with the *O*-sulphotransferases that catalyse the final modification reactions. The substrate specificities of the epimerase (Jacobsson *et al.*, 1984) and of the 3-*O*-sulphotransferase (Kusche *et al.*, 1988) have been superficially outlined. The model accommodates previous findings suggesting that GlcA C-5 epimerization and IdoA 2-*O*-sulphation take place concurrently (Jacobsson *et al.*, 1979, 1984) and before the GlcN 6-*O*- and 3-*O*-sulphation reactions (Jacobsson & Lindahl, 1980; Kusche *et al.*, 1988). However, the information available does not exclude the occurrence of a single enzyme complex comprising all the enzyme species shown in the Figure.

aration the apparent K_m for the partially *N*-sulphated acceptor was ~0.5 mM (assuming an average octasaccharide size), compared with ~1.3 mM for the fully *N*-acetylated species (results not shown). The validity of these determinations may be questionable, considering the heterogeneous nature of the acceptor preparations, particularly the partially *N*-sulphated one, and the difference may seem unimpressive. However, it appears reasonable to assume that only a minor fraction of the total population of partially *N*-sulphated oligosaccharides displays the terminal GlcNAc-GlcA-GlcNSO$_3^-$ acceptor sequence so clearly favoured by the GlcA-transferase; hence the K_m for this particular species would be appreciably lower. Presumably optimal integration of the polymerization and *N*-deacetylation/*N*-sulphation processes will yield a nascent chain that contains several contiguous *N*-sulphated units adjacent to the non-reducing terminal GlcNAc. Such a structure would escape detection by the analytical procedure used here.

DISCUSSION

Exogenous oligosaccharides or carbohydrate–serine compounds have been used as sugar acceptors in the GlcA- and GlcNAc-transferase reactions involved in heparin biosynthesis (see the Introduction). However, owing to lack of the appropriate acceptors it has previously not been possible to compare the two enzymes with regard to basic catalytic properties under unbiased conditions. This problem has been circumvented by introducing the oligosaccharide acceptors derived from the *E. coli* K5 polysaccharide, by which the same carbohydrate sequence, save for the actual non-reducing-terminal target unit, is readily applied to assay of either activity. The present results of such a comparative study reveal striking differences between the two enzymes, with regard to substrate-recognition properties (apart from the terminal unit) and cofactor requirements.

Firstly, the two transferases differ with regard to metal ion requirement. The Mn^{2+} requirement of the GlcNAc-transferase is not shared by the GlcA-transferase, which is instead stimulated by (without being strictly dependent on) Ca^{2+} and Mg^{2+}. Interestingly, the corresponding enzymes involved in chondroitin sulphate biosynthesis, a GlcA-transferase and a GalNAc-transferase, were both reported to be stimulated by Mn^{2+}, whereas Mg^{2+} and Ca^{2+} had no effect on either enzyme activity (Gundlach & Conrad, 1985). Further, the GlcNAc-transferase showed a generally lower K_m for its saccharide acceptors than the GlcA-transferase, and this difference increased with increasing acceptor chain length. It is recognized that these data are derived from assays in solubilized systems of enzymes that are normally inserted into the Golgi membrane; hence they should be evaluated with caution. However, it seems clear that both enzymes accept saccharide substrates with mixed *N*-acetyl/*N*-sulphate substituents. Although the GlcNAc-transferase appeared not to discriminate between differently *N*-substituted acceptor sequences, the GlcA-transferase showed marked preference for a specific sequence in which the non-reducing-terminal and penultimate GlcN units were *N*-acetylated and *N*-sulphated respectively.

These findings, along with our previous observation that chain elongation is promoted by concomitant *N*-sulphation of the nascent chain (Lidholt *et al.*, 1989), can be tentatively rationalized into a scheme for the co-ordination of the polymerization and early polymer-modification reactions (Fig. 9). Transfer of a GlcA to a terminal GlcNAc unit, i.e. the presumably rate-limiting step of chain elongation, is greatly facilitated by the occurrence of a *N*-sulphate group on the adjacent GlcN residue. The incorporated GlcA accepts a GlcNAc unit at C-4. The initially terminal GlcNAc unit (first internal GlcNAc unit after the GlcA transfer reaction) undergoes *N*-deacetylation/*N*-sulphation, thus regenerating the favoured GlcA acceptor structure. Alternatively, all or part of the *N*-deacetylation/*N*-sulphation step takes place already after GlcA transfer, before the GlcNAc transfer reaction. Chain elongation not obligately coupled to *N*-deacetylation/*N*-sulphation is, of course, entirely feasible, as evidenced by the formation of non-sulphated polysaccharide in a microsomal system incubated with UDP-sugars in the absence of PAPS (Silbert, 1963; Höök *et al.*, 1975; Lidholt *et al.*, 1988, 1989), by the transfer of GlcA and GlcNAc monosaccharides to non-sulphated acceptor oligosaccharides, and, indeed, by the occurrence of *N*-acetylated block (or even extended polymer; Iozzo, 1989) sequences in heparan sulphate (Gallagher *et al.*, 1986; Lindahl, 1989). Modulation of *N*-deacetylation is obviously required to generate the variously *N*-acetylated and *N*-sulphated regions found in heparan sulphates. The mechanism behind such modulation is unknown, but may conceivably involve temporary reversible separation of the *N*-deacetylase/*N*-sulphotransferase from its (growing) polymer substrate. *N*-Deacetylation is known to be promoted by previously incorporated *N*-sulphate groups (Riesenfeld *et al.*, 1982). Other potentially regulatory elements include a protein 'cofactor' as well as Mn^{2+}, both of which are required for *N*-deacetylase activity (Pettersson *et al.*, 1991). Conversely, inhibition of *N*-deacetylation was induced by treatment of heparin-producing mastocytoma cells with n-butyrate (Jacobsson *et al.*, 1985).

Indirect evidence for coupling between chain polymerization and sulphation was presented previously by Dietrich *et al.* (1988), and was based on the use of selenate as a sulphation inhibitor in heparan sulphate biosynthesis. A similar coupling in chondroitin sulphate biosynthesis was proposed by Sugumaran & Silbert (1990), who observed 4-*O*-sulphation of GalNAc units during formation of the polysaccharide chain.

The scheme proposed in Fig. 9 is partly at odds with a previous model of heparin biosynthesis (Lindahl *et al.*, 1986), which depicts chain polymerization and modification as separate processes. The rationale behind this model derived from the demonstration of polysaccharide intermediates at various stages of modification, which had apparently taken place in a stepwise fashion. The proteoglycan molecule under modification thus was visualized as traversing a series of membrane-bound enzyme complexes, arranged in a 'station-wise' fashion, such that the individual polysaccharide chain would move across rather than along the enzyme molecules. This model does not seem to describe appropriately the initial stages of the process, i.e. formation and *N*-deacetylation/*N*-sulphation of the polysaccharide chains, as elucidated through more recent experiments (Lidholt *et al.*, 1989). In particular, pulse–chase incubations using the mastocytoma microsomal system demonstrated that sulphated chains could continue to grow and, in fact, became longer than chains grown in the absence of sulphation. The findings of the present study, as interpreted in Fig. 9, provide a mechanism to account for the concerted chain elongation and *N*-sulphation. Conceivably, this process is catalysed by an enzyme complex that contains the GlcA- and GlcNAc-transferases as well as a *N*-deacetylase/*N*-sulphotransferase. While the features of such an enzyme complex are still unclear, it may be noted that the GlcNAc deacetylase and *N*-sulphotransferase activities are linked to the same protein, as demonstrated by the isolation of a ~110 kDa mouse mastocytoma protein required for the expression of either activity (Pettersson *et al.*, 1991). Furthermore, a presumably single mutational event was found to eliminate both the GlcA- and the GlcNAc-transferase activities of cultured Chinese-hamster ovary cells, suggesting that the two enzymes might share at least a common subunit (Lidholt *et al.*, 1992).

Clearly, attempts to rationalize the accumulated information

on heparin biosynthesis into a model still remain speculative. On the other hand, as noted above, the previously postulated model (Lindahl *et al.*, 1986) appears at least in part untenable, and it thus seems justified to introduce modifications that will accommodate more recent experimental findings (Fig. 10). The main novel concept is that of the glycosyltransferase/*N*-deacetylase/*N*-sulphotransferase complex outlined above, and schematically illustrated in Fig. 10(*a*). It seems reasonable to assume that such a complex would continue to extend and modify the same polysaccharide chain rather than move from one chain to another. Moreover, also the subsequent polymer-modification events, C-5-epimerization/2-*O*-sulphation of unspecified hexuronic acid moieties and 6-*O*-sulphation/3-*O*-sulphation of GlcN units, may be catalysed by enzyme complexes that operate in a similar fashion, presumably simultaneously on the same polysaccharide chain (Figs. 10*b* and 10*c*). All that is required to explain the formation of the observed polysaccharide 'intermediates' (Höök *et al.*, 1975; Jacobsson & Lindahl, 1980) that provided the conceptual foundation of the previous model (Lindahl *et al.*, 1986), is the (artifactual) occasional loss of enzyme complexes during the preparation of microsomal fractions. An 'assembly line' lacking, say, the putative GlcN 6-*O*-sulphotransferase complex will generate an 'intermediate' species containing *N*-sulphated GlcN units and 2-*O*-sulphated IdoA residues, but no GlcN 6-*O*-sulphate groups.

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