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Biosynthesis of heparin

Modulation of polysaccharide chain length in a cell-free system

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The formation of heparin-precursor polysaccharide (*N*-acetylheparosan) was studied with a mouse mastocytoma microsomal fraction. Incubation of this fraction with UDP-[^{3}H]GlcA and UDP-GlcNAc yielded labelled macromolecules that could be depolymerized, apparently to single polysaccharide chains, by alkali treatment, and thus were assumed to be proteoglycans. Label from UDP-[^{3}H]GlcA (approx. 3 μM) is transiently incorporated into microsomal polysaccharide even in the absence of added UDP-GlcNAc, probably owing to the presence of endogenous sugar nucleotide. When the concentration of exogenous UDP-GlcNAc was increased to 25 μM the rate of incorporation of ^{3}H increased and proteoglycans carrying polysaccharide chains with an M_r of approx. 110000 were produced. Increasing the UDP-GlcNAc concentration to 5 mM led to an approx. 4-fold decrease in the rate of ^{3}H incorporation and a decrease in the M_r of the resulting polysaccharide chains to approx. 6000 (predominant component). When both UDP-GlcA and UDP-GlcNAc were present at high concentrations (5 mM) the rate of polymerization and the polysaccharide chain size were again increased. The results suggest that the inhibition of polymerization observed at grossly different concentrations of the two sugar nucleotides, UDP-GlcA and UDP-GlcNAc, may be due either to interference with the transport of one of these precursors across the Golgi membrane or to competitive inhibition of one of the glycosyltransferases. The maximal rate of chain elongation obtained, under the conditions employed, was about 40 disaccharide units/min. The final length of the polysaccharide chains was directly related to the rate of the polymerization reaction.

INTRODUCTION

The sulphated glycosaminoglycans generally occur in the tissues as proteoglycans composed of polysaccharide chains covalently linked to a protein core (Rodén, 1980; Gallagher *et al.*, 1986; Poole, 1986). For all proteoglycan species, except keratan sulphate, the linkage region consists of a galactosyl-galactosyl-xylosyl trisaccharide sequence, which connects the polysaccharide chain to a serine residue in the core protein. Analysis of single polysaccharide chains released from proteoglycans by alkaline β-elimination has revealed striking variation in chain length, not only between proteoglycans of different origin or polysaccharide structure, but also within single proteoglycan preparations. The polysaccharide chain length influences the physicochemical properties of the proteoglycan and thus is a parameter of potential functional importance.

The biosynthesis of proteoglycans is believed to be initiated by formation of the core polypeptide, which then becomes glycosylated (Rodén, 1980). After formation of the trisaccharide linkage region the actual polysaccharide is assembled by alternate transfer of D-glucuronosyl and *N*-acetyl-D-hexosaminyl moieties from the appropriate UDP-sugars to the non-reducing end of the growing chain. Following these glycosylation reactions, the polysaccharide chains undergo modification, which includes the incorporation of sulphate groups and yields the mature proteoglycan. During the polymerization and polymer-modification reactions both the proteoglycan intermediates and the enzymes involved appear to be bound to the endoplasmic, presumably Golgi, membranes. Although some basic features of these reactions have been defined (Rodén, 1980; Lindahl & Kjellén, 1986), major aspects of regulation remain unclear. This applies also to the mechanisms that control the polymerization reactions and determine the length of the resulting polysaccharide chains.

The present paper is concerned with chain-elongation reactions in a microsomal fraction from a heparin-synthesizing mouse mastocytoma. It is shown that the rate of polymerization can be modulated extensively by varying the concentrations of the UDP-sugar substrates added to the system. Furthermore, this modulation is strikingly reflected in the length of the synthesized polysaccharide chains.

EXPERIMENTAL

Materials

A microsomal fraction was prepared as described previously (Jacobsson *et al.*, 1979*a*) from a transplantable mouse mastocytoma (Furth *et al.*, 1957). UDP-[^{3}H]GlcA was synthesized enzymically from D-[1-^{3}H]glucose (15 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) as reported previously (Jacobsson *et al.*, 1979*a*). Unlabelled UDP-GlcA, UDP-GlcNAc, other nucleo-

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tides, chondroitin ABC lyase (chondroitinase ABC) and bovine liver β-D-glucuronidase (type B-10) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hyaluronan [see Balazs *et al.* (1986) for nomenclature] fractions ranging in average M_r from approx. 6600 to approx. 43000, isolated from a partially hydrolysed sample (Cleland, 1983), were provided by R. Cleland (Dartmouth College, Hanover, NH, U.S.A.). Two fractions, M_r approx. 170000 and approx. 210000, obtained by autoclaving hyaluronan at 128 °C, were given by O. Wik (Pharmacia Fine Chemicals, Uppsala, Sweden).

Methods

The formation of heparin-precursor polysaccharide was studied by incubating 50 μCi (approx. 3 nmol) of UDP-[^{3}H]GlcA and various amounts of UDP-GlcNAc with approx. 10 mg of microsomal protein per ml of 50 mM-Hepes buffer, pH 7.4, containing 10 mM-$MnCl_2$, 10 mM-$MgCl_2$ and 5 mM-$CaCl_2$. After incubation at 37 °C for various periods of time the reactions were interrupted by the addition of SDS to a final concentration of 2% (w/v), and were then heated at 100 °C for 3 min. Labelled polysaccharide was isolated along with 0.5 mg of carrier heparin from portions (100–300 μl) of incubation mixtures by passage through columns (1 cm × 35 cm) of Sephadex G-50 (medium grade) equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 0.1% SDS. Fractions (2 ml) were collected at a rate of 12 ml/h and analysed for radioactivity by liquid-scintillation counting; labelled proteoglycan appeared as a distinct peak at the excluded volume of the column.

To convert proteoglycan into single polysaccharide chains NaOH was added to a final concentration of 0.5 M and the sample was kept at room temperature for 20 h. Control experiments using gel chromatography showed that the released labelled polysaccharide chains were not depolymerized to any significant extent during alkali treatment. After completed β-elimination the samples were passed through Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.) in order to eliminate the SDS. The samples were concentrated as required for further analysis by using a Speed Vac evaporation centrifuge (Savant Instruments, Hickville, NY, U.S.A.). An alternative method for preparing single polysaccharide chains, involving digestion of the microsomal incubation mixtures with Pronase followed by alkali treatment, was abandoned because of consistently lower recoveries of the largest molecules.

Gel chromatography was performed on a column (1 cm × 80 cm) of Sepharose 2B-CL equilibrated with 1 M-NaCl. Fractions (approx. 1.7 ml) were collected at a rate of 5 ml/h. Free polysaccharide chains were also analysed on columns (0.75 cm × 60 cm) of Ultropac TSK G4000 SW and TSK G2000 SW (LKB, Bromma, Sweden) equilibrated with 1 M-NaCl in 10 mM-Tris/HCl buffer, pH 7.4. The columns were eluted at a flow rate of 0.5 ml/min by means of an LKB model 2150 h.p.l.c. pump, and 1 ml fractions were collected. V_0 and V_t were determined by using hyaluronan (Healon; Pharmacia) and ^3H_2O respectively. The columns were calibrated with hyaluronan fractions of defined M_r (see the Experimental section), which were detected by the carbazole reaction (Bitter & Muir, 1962). Differences in polysaccharide chain size revealed by this system (see the Results section) were maintained also when the eluent was changed to 4 M-guanidinium chloride (results not shown).

RESULTS

Previous studies with mastocytoma microsomal fractions have demonstrated synthesis of a heparin-precursor polysaccharide from the appropriate nucleotide sugars (Silbert 1963; Lindahl *et al.*, 1973; Höök *et al.*, 1975; Jacobsson & Lindahl, 1980). Accordingly, the labelled macromolecular product (excluded from Sephadex G-50) formed by incubating a microsomal preparation with UDP-[^{3}H]GlcA and UDP-GlcNAc was largely (invariably > 85%) resistant to degradation by chondroitin lyase ABC, but susceptible to deaminative cleavage by HNO_2 at pH 3.9 (results not shown). In previous experiments the incorporation of radioactivity from labelled UDP-GlcA into microsomal polysaccharide remained essentially linear with time for more than 1 h, provided that the concentration of UDP-GlcNAc was sufficiently high (> 2.5 mM; Lindahl *et al.*, 1973). This system has now been used to study in more detail the kinetics of the polymerization reaction.

Incorporation of [^{3}H]glucuronic acid into microsomal polysaccharide

Incubation of the mastocytoma microsomal fraction with UDP-[^{3}H]GlcA in the presence of 5 mM unlabelled UDP-GlcNAc yielded labelled polysaccharide at a constant rate for at least 1 h (Fig. 1; cf. Lindahl *et al.*, 1973). When the concentration of UDP-GlcNAc was decreased to 25 μM the incorporation of label into polysaccharide started to level off after about 10 min and ceased altogether after 30–40 min (Fig. 1). However,

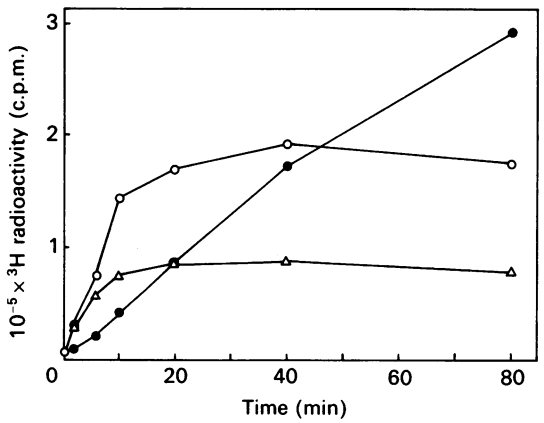

Fig. 1. Time course of incorporation of radioactivity from UDP-[^{3}H]GlcA into microsomal polysaccharide at different UDP-GlcNAc concentrations

Mastocytoma microsomal fractions was incubated at 37 °C with UDP-[^{3}H]GlcA and 25 μM- (○), 5 mM- (●) or no (△) exogenous UDP-GlcNAc as described in the Experimental section. After the indicated periods samples of 300 μl were withdrawn and labelled polysaccharide was isolated by gel chromatography (see the Experimental section). The values are corrected for non-specific incorporation of radioactivity (approx. 25000 c.p.m. of ^{3}H/sample), which was quantified by using a control incubation with heat-inactivated enzyme. For further information see the text.

during the period of linear incorporation, i.e. the first 10 min of incubation, the rate of polysaccharide synthesis was 4.3-fold higher (average of 12 experiments; S.D. ± 1.3) in the presence of 25 μM-UDP-GlcNAc than in the presence of 5 mM-UDP-GlcNAc. Significant polymerization was observed even in the absence of added UDP-GlcNAc, suggesting the presence of endogenous UDP-GlcNAc in the microsomal preparation (Fig. 1). Again the rate of polysaccharide formation was initially higher than that observed with 5 mM-UDP-GlcNAc but levelled off after about 10 min of incubation, the final amount of polysaccharide synthesized being about half of that produced in the presence of 25 μM exogenous UDP-GlcNAc. The decrease in incorporation rate noted at low UDP-GlcNAc concentration, following the initial 10 min incubation period, coincided with depletion of the UDP-[^{3}H]GlcA pool, as indicated by high-voltage paper electrophoresis of samples of the incubation mixtures (results not shown). During incubation the UDP-[^{3}H]GlcA was gradually replaced by a faster-migrating labelled component, presumably [^{3}H]GlcA 1-phosphate. Apparently, the labelled UDP-GlcA was protected from enyzmic degradation at higher concentrations of UDP-GlcNAc.

The evaluation of these results was complicated by the presence of labelled material that appeared to be formed through a non-enzymic mechanism. This material was recovered from incubations containing microsomal protein that had been heated in 2% SDS at 100 °C for 10 min before the addition of sugar nucleotides. It was excluded from Sephadex G-50 in the initial isolation step, but appeared as a small molecular component, diffusible on dialysis and well-retarded on renewed Sephadex G-50 chromatography after alkali treatment of the proteoglycan fraction. The amounts of this component formed were remarkably constant, approx. 25000 c.p.m. in a 300 μl standard incubation, and were related to the amounts of microsomal protein present. This apparent artifact, which has not been identified, is indicated in gel chromatograms of free polysaccharide chains as retarded shaded peaks (see Figs. 5 and 6), and has been appropriately corrected for in the kinetic analyses (Figs. 1 and 2 and Table 1).

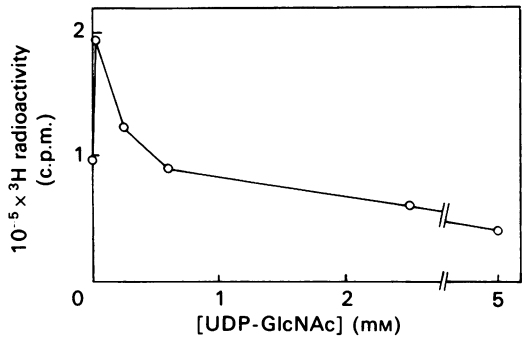

Fig. 2. Formation of microsomal polysaccharide as a function of UDP-GlcNAc concentration

Mastocytoma microsomal fraction was incubated in a total volume of 300 μl with UDP-[^{3}H]GlcA and UDP-GlcNAc at the concentrations indicated, as described in the Experimental section. After 5 min at 37 °C the incubations were interrupted and labelled polysaccharide was isolated. Values are corrected for non-specific incorporation of radioactivity (see the legend to Fig. 1).

Table 1. Effects of nucleotides on polysaccharide formation

Standard incubation mixtures (see the Experimental section) of 200 μl, containing UDP-[^{3}H]GlcA and 25 μM-UDP-GlcNAc (control), as well as similar mixtures with the additions indicated, were kept at 37 °C for 10 min and labelled polysaccharide was isolated by gel chromatography on Sephadex G-50. The control incubation mixture incorporated 290 000 c.p.m. of ^{3}H from UDP-[^{3}H]GlcA into microsomal polysaccharide. The values indicated are averages of two separate experiments and are corrected with regard to non-enzymic incorporation of radioactivity into macromolecular material (see the legend to Fig. 1).

Addition	[^{3}H]Polysaccharide formed (% of control)
None (control)	100
5 mM-UDP-GlcNAc	29
5 mM-UTP	10
5 mM-UDP	12
5 mM-UMP	25
5 mM-Uridine	100
5 mM-ATP	62
5 mM-ADP	48
5 mM-AMP	42
5 mM-Pyrophosphate	101

The effect of UDP-GlcNAc concentration on the rate of polymerization was studied in more detail by determining the amounts of labelled polysaccharide formed in the presence of UDP-[^{3}H]GlcA (approx. 3 μM; see below) during the initial 5 min of incubation. The resulting activity profile showed a sharp highly reproducible maximum at about 25 μM-UDP-GlcNAc, with a consistent decline in rate with higher concentrations of this precursor (Fig. 2). Maximal rate of polymerization was maintained even with 2.5 μM-UDP-GlcNAc (results not shown), whereas a lower rate was obtained in the absence of exogenous UDP-GlcNAc (significance uncertain because of apparent deviation from linear incorporation kinetics; see Fig. 1). In a particular experiment, the actual rate of polysaccharide formation at 25 μM-UDP-GlcNAc was calculated as approx. 0.6 pmol of hexuronic acid incorporated (disaccharide unit formed)/min per mg of microsomal protein, assuming a specific radioactivity for the added UDP-[^{3}H]GlcA of 15 Ci/mmol and no endogenous UDP-GlcA, hence a UDP-GlcA concentration of approx. 3 μM. In the presence of 5 mM-UDP-GlcNAc the corresponding rate declined to ~ 0.15 pmol/min per mg of protein. When unlabelled UDP-GlcA was added to incubation mixtures containing UDP-[^{3}H]GlcA and 5 mM-UDP-GlcNAc, the amount of ^{3}H incorporated into polysaccharide decreased, owing to the decrease in specific radioactivity of the UDP-[^{3}H]GlcA pool. However, the decrease in the rate of ^{3}H incorporation was much smaller than was expected from the dilution of the UDP-[^{3}H]GlcA pool, suggesting a substantial increase in the overall rate of polysaccharide synthesis. An incubation mixture containing 5 mM-UDP-GlcA along with 5 mM-UDP-GlcNAc thus synthesized polysaccharide at an apparent rate of approx. 15 pmol/min per mg of protein. These findings suggest that the UDP-[^{3}H]GlcA concentration utilized in most of the experiments fell well below the saturation value for the glucuronosyltransferase; in fact, a K_m of approx. 50 μM was

calculated for UDP-GlcA in a previous similar experiment at 2.5 mM-UDP-GlcNAc concentration (see Lindahl *et al.*, 1973). (It should be realized, however, that the reliability of such K_m determinations is limited in view of the particular nature of the biosynthetic system.)

Various nucleotides were tested with regard to their effects on polysaccharide synthesis in the microsomal system (Table 1). The nucleotides were added at 5 mM to standard incubation mixtures containing UDP-[^{3}H]GlcA and 25 μM-UDP-GlcNAc. Under these conditions not only UDP-GlcNAc (at 5 mM), but also, and to a larger extent, the uridine mono-, di- and tri-phosphates lacking the sugar residue, lowered the rate of polysaccharide synthesis. Uridine and pyrophosphate had no effect. The adenosine-based nucleotides showed significant inhibitory effect, albeit less pronounced than that of uridine nucleotides.

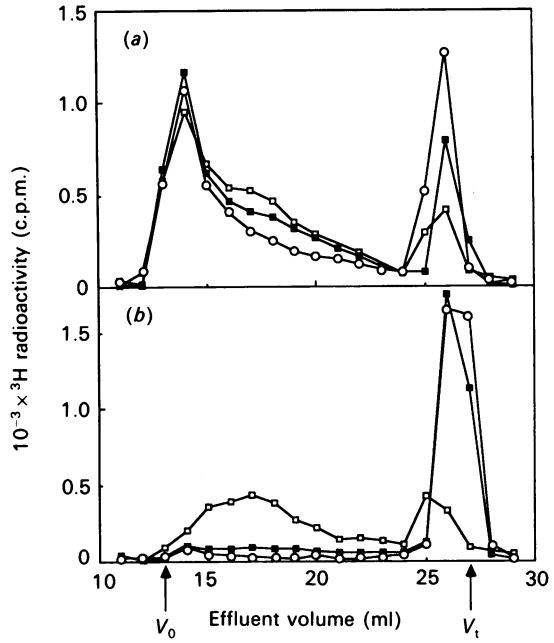

Fig. 3. Effect of Triton X-100 on the formation of microsomal polysaccharide

Mastocytoma microsomal fraction was incubated (500 μl incubation mixtures) for 30 min (*a*) with UDP-[^{3}H]GlcA and 5 mM-UDP-GlcNAc under standard conditions (see the Experimental section) or (*b*) with UDP-[^{3}H]GlcA alone in the presence of 1% Triton X-100. Labelled macromolecules were isolated by gel chromatography and were then treated with alkali, as described in the Experimental section. The products were adsorbed on small columns (1 cm × 2 cm) of DEAE-cellulose (Whatman DE-52), equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, which were then washed with 10 ml of 50 mM-LiCl in 50 mM-acetate buffer, pH 4.0. This procedure eliminated most of the artifact product(s) due to non-enzymic incorporation of label (see the Results section). Remaining labelled material [approx. 300000 c.p.m. from experiment (*a*) and 50000 c.p.m. from experiment (*b*)] was eluted with 1.5 M-LiCl in acetate buffer, pH 4.0, desalted by passage through Sephadex G-25 (PD-10; Pharmacia) columns, and analysed by gel chromatography on TSK G2000 SW as described in the Experimental section (□). Before chromatography, samples of labelled material were digested with β-glucuronidase (in accordance with Jacobsson *et al.*, 1979*b*) (■) or with chondroitin ABC lyase (in accordance with Jacobsson & Lindahl, 1987) (○). The retarded components appearing at approx. 25 ml effluent volume have not been identified. They may represent a remainder of the artifactual labelled material (see the Results section), most of which was removed with the breakthrough and washing of the fractions off the DEAE-cellulose column; in fact, separate analysis of each of these fractions on the TSK 2000 column showed exclusively low-M_r components, with an elution position similar to those displayed in the Figure.

Experiments were undertaken to ascertain that the incorporation of labelled glucuronic acid did indeed represent a true polymerization reaction, and not merely capping of preformed primer molecules with a labelled monosaccharide residue at the non-reducing end. Labelled polysaccharide isolated after incubation of microsomal fraction with UDP-[^{3}H]GlcA and 5 mM-UDP-GlcNAc for 30 min was essentially resistant to digestion with bovine liver β-glucuronidase (Fig. 3*a*), showing that most of the labelled glucuronic acid units had become incorporated into internal positions of the polysaccharide chain. The product obtained in the absence of exogenous UDP-GlcNAc released only a small portion, about 15%, of the incorporated uronic acid (results not shown), in agreement with the notion that the microsomal preparation contained appreciable amounts of endogenous UDP-*N*-acetylhexosamine. For comparison, a corresponding incubation was performed in the presence of 1% Triton X-100, a non-ionic detergent previously shown to preclude elongation of the endogenous microsomal polysaccharide without interfering with the individual glycosyltransferase reactions (Riesenfeld *et al.*, 1987). The resulting labelled product released free [^{3}H]glucuronic acid monsaccharide in almost quantitative yield on digestion with β-glucuronidase (Fig. 3*b*), as predicted for a non-reducing terminal glucuronic acid residue. This product was of particular interest, since it represented endogenous glycosyl acceptors ('primers') serving as potential sites of polysaccharide chain elongation in the intact microsomal system, and was therefore analysed further. The end-group-labelled species, M_r approx. 12000, emerged as a sulphated polysaccharide on DEAE-cellulose ion-exchange chromatography (results not shown), and was virtually all susceptible to degradation by chondroitin ABC lyase (Fig. 3*b*). These findings suggested that the major component capable of serving as a glucuronosyl acceptor in the solubilized system was a chondroitin sulphate. In contrast, most of the labelled polysaccharide produced in the intact microsomal system (in the presence of UDP-GlcNAc) resisted chondroitin lyase digestion (Fig. 3*a*), as expected for a heparin precursor. Lacking any demonstrable heparin-related glycosyl acceptor in the microsomal fraction, it is conceivable that such structures were formed *de novo* during the incubations, starting from preformed polysaccharide–protein linkage regions.

Effects of UDP-sugar concentration on polysaccharide chain length

The macromolecular properties of the microsomal polysaccharide were initially investigated by using gel chromatography on Sepharose 2B. The product labelled by UDP-[^{3}H]GlcA in the presence of 25 μM-UDP-GlcNAc emerged considerably before that synthesized

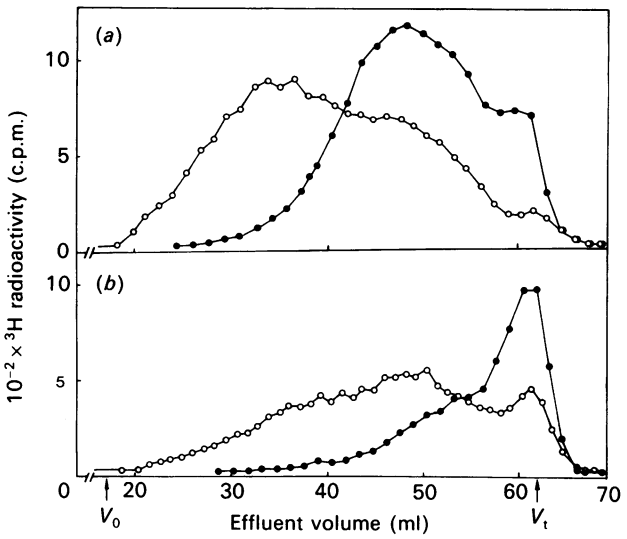

Fig. 4. Effect of alkali treatment on the macromolecular properties of microsomal polysaccharide

Microsomal fraction was incubated under standard conditions (see the Experimental section) in a total volume of 300 μl with UDP-[^{3}H]GlcA and either (*a*) 25 μM- or (*b*) 5 mM-UDP-GlcNAc. After 5 min at 37 °C the incubations were interrupted and labelled macromolecules were isolated by gel chromatography on Sephadex G-50 (see the Experimental section). Distinct fractions of excluded material (not shown) were recovered and were then analysed by gel chromatography on Sepharose 2B, either before (○) or after (●) alkali treatment (see the Experimental section). Each sample applied contained approx. 17000 c.p.m. of ^{3}H. Part of the most retarded peaks of the alkali-treated samples (○) represents non-specific incorporation of label. For further information see the text.

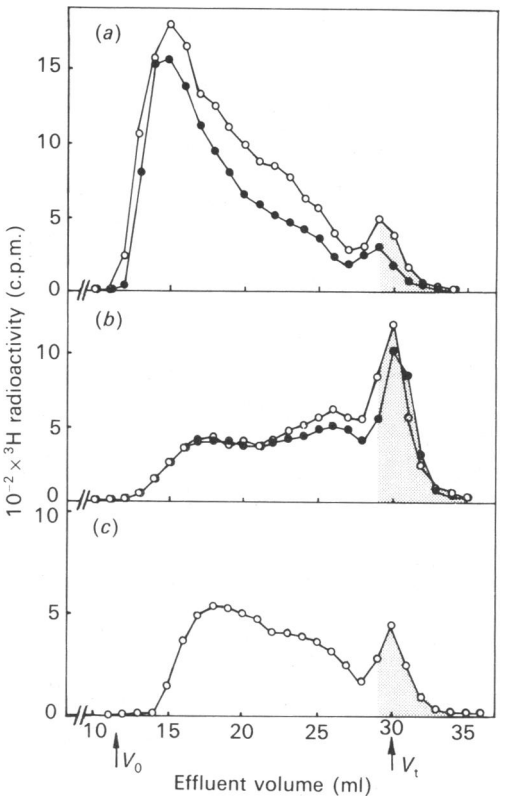

Fig. 5. Gel chromatography of polysaccharide chains synthesized at different UDP-GlcNAc concentrations

Standard incubation mixtures of 300 μl containing UDP-[^{3}H]GlcA and (*a*) 2.5 μM- (○) or 25 μM- (●), (*b*) 2.5 mM- (○) or 5 mM- (●), or (*c*) no exogenous UDP-GlcNAc, were kept at 37 °C for 5 min and labelled microsomal polysaccharide was isolated. After alkali treatment the resulting free polysaccharide chains were subjected to chromatography on a column of Ultropac TSK G4000 SW. The stippled peaks represent at least partly non-specific incorporation of label that was recovered with the macromolecular fraction and released as (*a*) small-molecular component(s) on alkali treatment. For further information see the text.

in the presence of 5 mM-UDP-GlcNAc (Fig. 4). The polysaccharide chains released from the macromolecules by alkali treatment appeared at more retarded elution positions, with the chains synthesized in the presence of 25 μM-UDP-GlcNAc again being appreciably larger than those obtained with 5 mM-UDP-GlcNAc (Fig. 4). These findings indicate, as expected (Robinson *et al.*, 1978), but not previously demonstrated, that the heparin-precursor polysaccharide is synthesized as a proteoglycan in which the individual polysaccharide chains are bound by alkali-sensitive linkages. However, they also point to an unexpected correlation between the rate of the polymerization reaction and the molecular size of the resulting proteoglycan, since proteoglycan molecules synthesized at a high rate (in the presence of 25 μM-UDP-GlcNAc) were larger than those synthesized at a lower rate (in the presence of 5 mM-UDP-GlcNAc). The concomitant difference in elution position of the corresponding alkali-treated products indicates that the modulation of proteoglycan size is due, at least partly, to a change in length of the constituent polysaccharide chains.

A more detailed analysis of polysaccharide chain length revealed striking differences related to the concentration of UDP-GlcNAc during polymerization (Fig. 5). After 5 min of incubation in the presence of UDP-[^{3}H]GlcA (approx. 3 μM) and low concentrations of UDP-GlcNAc (2.5 μM or 25 μM exogenous sugar nucleotide) the main fraction of the resulting labelled polysaccharide chains emerged shortly after the excluded volume from a TSK G4000 SW column (Fig. 5*a*). Calibration of the column with hyaluronan standards of known M_r (results not shown) indicated an M_r of approx. 110000 (corresponding to about 280 disaccharide units) for this fraction. On the other hand, the chains produced at high UDP-GlcNAc concentration (2.5 mM or 5 mM) emerged appreciably retarded on the same column (Fig. 5*b*), as two broad poorly separated peaks corresponding to M_r approx. 45000 (about 110 disaccharide units) and less than 6000 (about 15 disaccharide units) respectively. These findings corroborate the relationship between rate of polymerization and polysaccharide chain length. Polysaccharide synthesized at an apparently intermediary rate (in the absence of exogenous UDP-GlcNAc; see Fig. 2) showed an elution pattern (Fig. 5*c*) intermediate between those of polysaccharides synthesized at high (Fig. 5*a*) or at low (Fig. 5*b*) rate.

Fig. 6 illustrates the effects of incubation time on polysaccharide chain length. The low rate of monomer

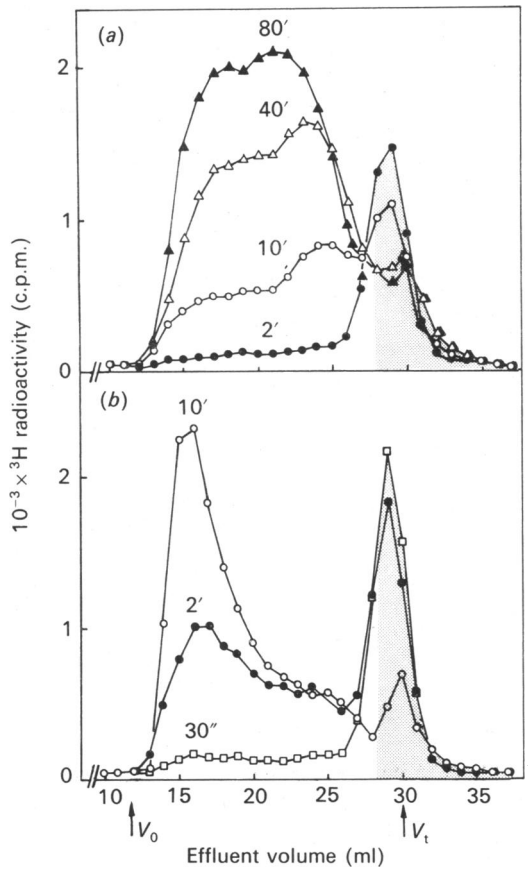

Fig. 6. Gel chromatography of polysaccharide chains synthesized for different periods of time at (*a*) high or (*b*) low UDP-GlcNAc concentration

Mastocytoma microsomal fraction was incubated under standard conditions at 37 °C (final volume 300 μl) with UDP-[^{3}H]GlcA and either (*a*) 5 mM- or (*b*) 25 μM-UDP-GlcNAc for periods of time ranging from 30 s (30″) to 80 min (80′) as indicated. Labelled polysaccharide was isolated and treated with alkali, and the resulting free polysaccharide chains were subjected to chromatography on a column of Ultropac TSK G4000 SW (see the Experimental section). The amounts of labelled polysaccharides in the chromatograms do not indicate the yields of the various incubations. For explanation of the stippled peaks see the legend to Fig. 5.

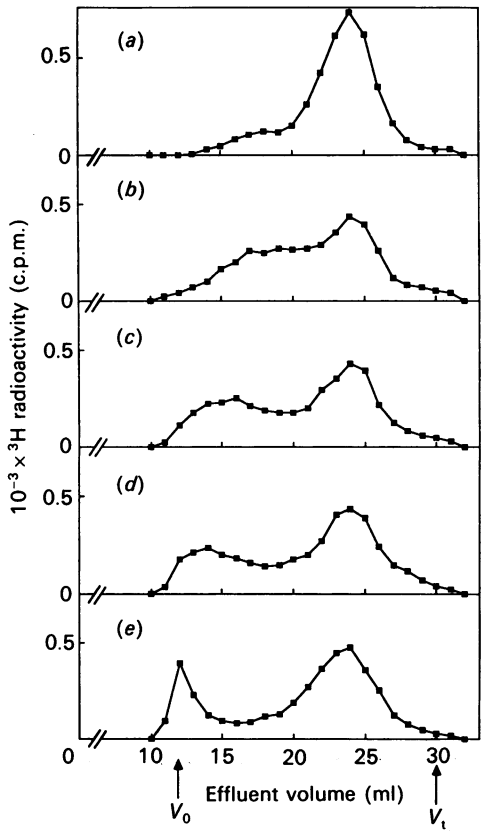

Fig. 7. Polysaccharide chain elongation at high concentrations of both UDP-GlcA and UDP-GlcNAc

Microsomal fraction was incubated at 37 °C with UDP-[^{3}H]GlcA and 5 mM-UDP-GlcNAc. After 5 min of incubation, unlabelled UDP-GlcA was added to 5 mM concentration, and 300 μl samples were withdrawn after (*a*) 0 min, (*b*) 1 min, (*c*) 5 min, (*d*) 10 min and (*e*) 30 min of continued incubation. Each sample, mixed with an equal volume of 4% SDS, was treated at 100 °C for 3 min, and microsomal polysaccharide was isolated, treated with alkali, dialysed (to eliminate non-specifically incorporated label; see the legend to Fig. 4) and analysed by chromatography on Ultropac TSK G4000 SW as described in the Experimental section.

incorporation observed at high UDP-GlcNAc and low UDP-GlcA concentrations was reflected by slow chain elongation, as shown by gel chromatography of samples isolated after different periods of incubation (Fig. 6*a*). Chains isolated after 10 min showed a broad size distribution and tended to accumulate in two poorly resolved peaks. During prolonged incubation this pattern was slowly shifted towards lower elution volume, yet with the major peak at a $K_{av.}$ value of approx. 0.5 (M_r approx. 20000) still after 80 min of incubation. In contrast, the chains formed in the presence of 25 μM-UDP-GlcNAc were large, with a peak $K_{av.}$ of approx. 0.25 (M_r approx. 75000), after only 2 min of incubation (Fig. 6*b*).

An attempt was made to determine quantitatively the rate of chain elongation obtained in the presence of high concentrations of both UDP-GlcNAc and UDP-GlcA. A pulse–chase approach was utilized, as illustrated in Fig. 7. Chains were labelled for 5 min at a low rate in the presence of UDP-[^{3}H]GlcA and 5 mM-UDP-GlcNAc. The resulting retarded elution pattern was only slightly altered when the incubation time was extended by an additional 10 min period (results not shown). However, addition of UDP-GlcA at high concentration (5 mM), following the initial 5 min of pulse-labelling, resulted in a marked change in the elution profile of the pulse-labelled material (Figs. 7*b*–7*e*). After 1 min of chase incubation a major portion of less-retarded label was already observed (Fig. 7*b*), which was then progressively shifted towards the elution positions of larger molecules. Interestingly, only a fraction, varying between 30% and 60% in different experiments, of the pulse-labelled polysaccharide chains appeared to be amenable to elongation during the chase. The significance of this finding is unclear. The change in elution position of the major nascent chains would correspond to an increase in M_r of 150000–200000 during a 10 min period of chase

incubation. Assuming an M_r for the repeating glucuronosyl-*N*-acetylglucosaminyl disaccharide unit of approx. 400, these results can be roughly approximated to indicate a rate of chain elongation during the chase of 30–40 disaccharide units/min. The fraction of the chased polysaccharide that remained small-molecular could not be eliminated by further prolonging the chase period (results not shown).

DISCUSSION

Addition of UDP-GlcNAc to reaction mixtures containing mastocytoma microsomal fraction resulted in an increase in the rate of incorporation of label from UDP-[^{3}H]GlcA into heparin-precursor polysaccharide. However, this effect applied only up to a point where the concentrations of the two UDP-sugars were approximately equal; a further increase in the UDP-GlcNAc concentration caused a decrease in the polymerization rate. A corresponding effect was observed when UDP-[^{14}C]GlcNAc was added as the labelled precursor, together with unlabelled UDP-GlcA at different concentrations (results not shown). The inhibitory effect of either UDP-sugar on the polymerization reaction was noted only when the alternate precursor was present at relatively low concentration. Apparently, the inhibitory effect is not caused by the high UDP-sugar concentration as such but rather reflects a disproportion in the concentrations of the two sugar nucleotides and hence, presumably, a competition phenomenon.

Competition might be exerted at the glycosyltransferase level, UDP-GlcNAc at high concentration interacting with a nucleotide-binding site in the glucuronosyltransferase and thus interfering with the binding of the labelled UDP-GlcA to this enzyme; the reverse situation would apply to the *N*-acetylglucosaminyltransferase. On the other hand, if the catalytic sites of the glycosyltransferases are located within the vesicles of the Golgi system (Nuwayhid *et al.*, 1986), the nucleotide sugars will have to be translocated across these membranes before they can interact with the enzymes. Capasso & Hirschberg (1984*a*,*b*) have shown that the membranes contain specific transporters which mediate the equimolar exchange of cytosolic sugar nucleotides and 3′-phosphoadenylyl sulphate with the corresponding luminal nucleoside monophosphates. The nucleotide components, but not the sugar moieties, provide the recognition features prerequisite for binding to the Golgi membranes, but are not sufficient for overall translocation, which also depends on the type of sugar. The role of sugar nucleotide transport in the mastocytoma microsomal preparation used in the present study has not been evaluated. However, assuming the involvement of transporters for UDP-GlcNAc and UDP-GlcA that both bind UDP, the inhibition of polysaccharide chain formation observed at grossly disparate concentrations of the two sugar nucleotides may be explained in terms of mutual competition for binding to the appropriate transporter. At high concentration UDP-GlcNAc will not only saturate the UDP-GlcNAc transporter (resulting in a high rate of translocation of this sugar nucleotide) but will also compete with UDP-GlcA for binding to its transporter. If the latter component is present at low concentration it thus will be unable to penetrate the membrane, resulting in a lack of UDP-GlcA at the site of the polymerization reaction. Conversely, at high concentration of UDP-GlcA and low concentration of UDP-GlcNAc translocation of the latter compound will be prevented. In accord with this interpretation, uridine-based nucleotides (but not uridine alone; see Table 1) were also able to inhibit polysaccharide synthesis, whereas adenosine-based nucleotides were less effective (cf. Capasso & Hirschberg, 1984*b*). Again, regulation of glycosyltransferase activity through direct inhibition by intra-Golgi UDP or UMP remains a possibility.

Two principally different hypotheses have been advanced to define the factors that determine polysaccharide chain length in proteoglycan biosynthesis. One proposal implies that a specific structural modification of non-reducing terminal sugar unit, such as the incorporation of sulphate groups at certain positions, might serve as a chain-termination signal (Telser *et al.*, 1966; Otsu *et al.*, 1985). Effects of sulphation were not considered in the present study and thus cannot be evaluated. Alternatively, the length of polysaccharide chains is kinetically determined by the relation between polymerization activity and the number of available primer sites (Mitchell & Hardingham, 1982). In accord with the latter hypothesis, the length of polysaccharide chains formed in our cell-free biosynthetic system could be modulated in a systematic fashion by varying the concentrations of the UDP-sugar precursors and thus the rate of the polymerization reaction.

Unfortunately, the mode of primer utilization remains obscure. The microsomal fraction obviously contains an appreciable pool of preformed primer sites for the major chain-elongation reaction, i.e. the formation of GlcA–GlcNAc disaccharide units. Although chondroitin sulphate chains, apparently capable of further chain elongation, could be demonstrated in the microsomal preparation, the experiments did not reveal any heparin-related polysaccharide. We therefore suggest that heparin-precursor chains were synthesized *de novo* in the cell-free system on primers containing the galactosyl-galactosyl-xylosyl trisaccharide sequence of the polysaccharide–protein linkage region (such components would presumably have escaped detection by the analyses performed) but little or no additional saccharide. On the other hand, the experimental data imply that the primer pool is functionally heterogeneous, such that different sets of primer molecules are utilized under different conditions of chain elongation. Incubation of the microsomal fraction with UDP-[^{3}H]GlcA thus yielded the same amounts of labelled polysaccharide after 20 min in the presence of 25 μM-UDP-GlcNAc as after 40 min in the presence of 5 mM-UDP-GlcNAc (Fig. 1); yet the polysaccharide chains isolated after such incubations differed drastically in size, being much larger after synthesis at the lower UDP-GlcNAc concentration (Fig. 6). This finding indicates that fewer chains were formed and hence that a smaller proportion of primer sites was utilized under the latter conditions. Could it be that different primer pools give rise to separate subpopulations of proteoglycans, synthesized with different kinetics? Indeed, such a possibility would be consistent with the marked polydispersity of the liberated polysaccharide chains, maintained also after prolonged incubation, as well as with the accumulation of these chains into discernible, albeit poorly resolved, subfractions (Figs. 5 and 6). Similar considerations apply to the experiment illustrated in Fig. 7, where only a fraction of the polysaccharide chains initiated during 5 min of

pulse-labelling remained accessible for chase elongation. Did all pulse-labelled chains originate from a single pool of primer? A better characterization of the structure, distribution and commitment of primer saccharides will obviously promote our understanding of the overall process of proteoglycan biosynthesis.

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