BIOSYNTHESIS OF L-IDURONIC ACID IN HEPARIN: EPIMERIZATION OF D-GLUCURONIC ACID ON THE POLYMER LEVEL

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Received December 9, 1971

SUMMARY. Incubation of a microsomal fraction from mouse mastocytoma with UDP-^{14}C-glucuronic acid and unlabelled UDP-N-acetylglucosamine resulted in the incorporation of ^{14}C-glucuronic acid into endogenous polysaccharide. When 3'-phosphoadenosine 5'-phosphosulfate (PAPS) was included in such incubations the polysaccharide product contained ^{14}C-iduronic acid in addition to ^{14}C-glucuronic acid. Pulse-chase experiments showed that ^{14}C-glucuronic acid units incorporated into the polymer during the pulse period (in the absence of PAPS), were subsequently converted to ^{14}C-iduronic acid units during the chase period (in the presence of PAPS). It is concluded that the iduronic acid residues had been formed by epimerization on the polymer level at C-5 of glucuronic acid residues.

The biosynthesis of mammalian glycosaminoglycans is generally considered to occur by stepwise and alternate addition of hexosamine and uronic acid units from the requisite UDP-sugars[1)] to the non-reducing termini of growing chains (1, 2). Subsequent to polymerization the resulting macromolecules are sulfated, PAPS being the sulfate donor.

Two uronic acids, D-GlcUA and L-IdUA, have been identified as constituents of glycosaminoglycuronans. The role of UDP-GlcUA in the formation of GlcUA-containing polysaccharides has been amply verified (1, 2). Further, the conversion of UDP-GlcUA to UDP-IdUA in mammalian tissues has been demonstrated (3), suggesting a precursor-product relationship between the latter nucleotide-sugar and IdUA-containing polysaccharides. However, UDP-IdUA has never been isolated, nor has it been shown to participate in the biosynthesis of glycosaminoglycans. Indeed, alternative pathways are conceivable, as indicated by recent observations pertaining to the biosynthesis of bacterial alginic acid (4). In this process, L-guluronic acid is formed by epimerization of D-mannuronic acid units on the polymer level.

1) Abbreviations: UDP, uridine-diphosphate; GlcNAc, N-acetylglucosamine; GlcUA, glucuronic acid; IdUA, iduronic acid; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

In the present work the biosynthesis of heparin has been studied, with particular regard to the formation of IdUA residues. Evidence will be presented demonstrating that IdUA is formed by epimerization at C-5 of GlcUA units previously incorporated in the polymer.

MATERIALS

A heparin-producing (5,6) mouse mastocytoma[2] originally described by Furth et al. (7) was obtained from Dr. N. Ringertz, Karolinska Institutet, Stockholm, and has been maintained by routine intramuscular and subcutaneous transplantation on (Leaden X A)F_1 mice. A microsomal fraction, prepared from this tissue as described by Silbert (9), was used as a suspension in 0,25 M sucrose - 0.05 M Tris-HCl, pH 7.3 (approx. 30 mg of protein/ml).

^{35}S-PAPS (12 μCi/umole) was prepared by the method of Balasubramanian et al. (10). UDP-^{14}C-GlcUA (238 μCi/μmole) was purchased from New England Nuclear Co. Unlabelled UDP-GlcUA and UDP-GlcNAc were obtained from Sigma.

The sample of purified heparin used has been described previously (8). L-IdUA was prepared from dermatan sulfate (11).

Crystalline papain was prepared from a crude preparation according to the procedure of Kimmel and Smith (12).

METHODS

Analytical methods. Uronic acid was determined by the carbazole method of Bitter and Muir (13), and protein by the procedure of Lowry et al. (14), with human serum albumin as standard.

Radioactivity was determined with a Beckman Model LS-250 liquid scintillation counter; the scintillation liquid contained 5 g of 2,5-diphenyloxazole and 100 g of naphtalene per liter of dioxan. Under the conditions employed, ^{35}S and ^{14}C were counted at 40% and 54% efficiency, respectively, the same discriminator setting being used in the analysis of either isotope. Radioactive components on electrophoretograms or paper chromatograms were quantitated either by scintillation counting, after elution from the paper with water, or with a Packard Model 7201 strip scanner. In the latter procedure, ^{35}S and ^{14}C were determined with equal efficiency (for further details, see the legend to Fig. 1).

Incorporation of ^{14}C-uronic acid and ^{35}S-sulfate into microsomal glycosaminoglycan. Microsomal fraction was incubated with nucleotide sugars and/or PAPS, as described in detail in Table 1. After the addition of 0.5 mg of carrier heparin, the incubations were terminated by heating at 100°C for 3 min. The mixtures (0.3 ml) were diluted to 1.0 ml with 0.05 M acetate buffer, pH 5.5, containing 0.01 M EDTA and 0.01 M cystein-HCl, adjusted to 1.0 M with respect

[2] Analysis (8) of the uronic acid composition of mastocytomal glycosaminoglycan showed IdUA to be a major component (unpublished).

Table I. Incorporation of ^{14}C-uronic acid and ^{35}S-sulfate into microsomal glycosaminoglycan.

Exp. no.	Incubation scheme: Time after start	Component[a] added; Termination[b]	Incorporation into polysaccharide: Uronic acid[c] dpm ^{14}C	Uronic acid[c] pmole	Sulfate[c] dpm ^{35}S	Sulfate[c] pmole	^{14}C-IdUA[d] / (^{14}C-IdUA + ^{14}C-GlcUA)
	min						%
1	0	UDP-^{14}C-GlcUA					
	0	UDP-GlcNAc					
	30	Termination	17400	33	-	-	<10
2	0	UDP-^{14}C-GlcUA					
	0	UDP-GlcNAc					
	0	^{35}S-PAPS					
	30	Termination	21900	42	7200	270	40
3	0	UDP-^{14}C-GlcUA					
	0	UDP-GlcNAc					
	30	UDP-GlcUA					
	30	^{35}S-PAPS					
	90	Termination	22600	43	8400	320	45
4	0	UDP-^{14}C-GlcUA					
	0	UDP-GlcNAc					
	30	UDP-GlcUA					
	45	^{35}S-PAPS					
	105	Termination	18900	36	13200	500	50
5	0	^{35}S-PAPS					
	30	Termination	-	-	5200	200	-

a) Each reaction mixture contained, in a total volume of 0.3 ml, 0.05 ml of microsomal preparation, 3mM $MnCl_2$, 8 mM $MgCl_2$, 3 mM $CaCl_2$, 0.8 mM EDTA, 0.4 mM mercaptoethanol and 50 mM Tris, pH 7.3. In addition, the following components were added, as indicated: UDP-^{14}C-GlcUA, 17 nmoles (4 μCi); unlabelled UDP-GlcUA, 0.5 μmole; unlabelled UDP-GlcNAc, 0.5 μmole; and ^{35}S-PAPS, 0.4 μmole (5 μCi).

b) Heating at 100°C for 3 min.

c) Determined by relating the ^{35}S/^{14}C ratio (estimated as described in the legend to Fig. 1) to the total radioactivity incorporated (determined by scintillation counting of the polysaccharide sample obtained after precipitation with cetylpyridinium chloride; see Methods). The latter value was corrected for losses during isolation, as estimated by the recovery of carrier heparin (determined by the carbazole reaction, the color yield of the endogenous microsomal polysaccharide being disregarded). The appropriate counting efficiency factors (see Methods) were used in the calculations.

d) Determined by liquid scintillation counting, after elution of uronic acids from paper chromatograms (Fig. 2). The values given refer to peak III in Fig. 1 (free uronic acid).

to NaCl and digested with 2 mg of papain at 65°C over night. The digests were passed through columns (1 x 90 cm) of Sephadex G-50, eluted with pyridine-acetic acid-water (10:6:984) at a rate of 4 ml/hour. The labelled polysaccharides, emerging as distinct peaks with the void volume of the columns, were precipitated with 10 mg of cetylpyridinium chloride from 1.5 ml of 0.06 M NaCl and were finally converted to the sodium-polysaccharide salts (6).

Degradation of the polysaccharide samples to yield free uronic acid was

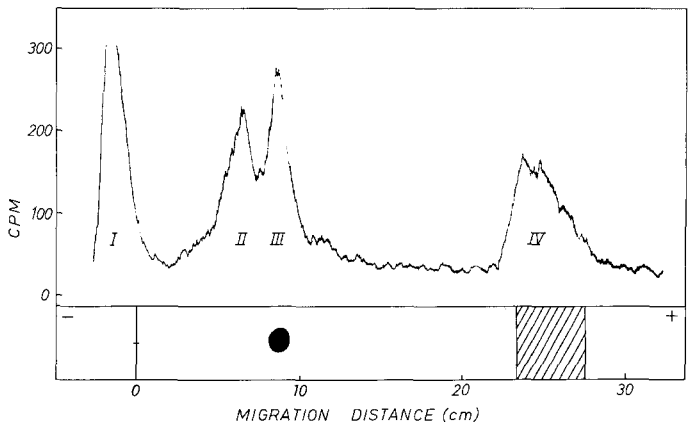

Fig. 1. High-voltage electrophoresis of degradation products (see Methods) of ^{35}S- and ^{14}C-labelled microsomal glycosaminoglycan (experiment 2; see Table 1). The electrophoresis was carried out on Whatman 3MM paper, in 0.08 M pyridine -0.05 M acetic acid (pH 5.3), at 70 V/cm for 20 min. The GlcUA standard was stained by a silver dip procedure (17).

The standards shown below the tracing, corresponding to peaks III and IV, were GlcUA (or IdUA) monosaccharide and inorganic ^{35}S-sulfate, respectively. Peak I represented neutral components (behaving like uronolactones on paper chromatography), whereas peak II migrated similar to uronosylanhydromannose disaccharide (8). Peak areas were determined by planimetry; the $^{35}S/^{14}C$ ratio was calculated as peak IV/peaks I+II+III. A sample of glycosaminoglycan (experiment 5) labelled with ^{35}S, but not with ^{14}C, yielded peak IV only, indicating complete desulfation during the degradation procedure. A comparison of the areas of peaks I, II and III shows that more than half of the uronic acid incorporated into microsomal glycosaminoglycan had been liberated as free monosaccharide.

carried out as follows. N-Acetyl and N-sulfate groups were removed by hydrolysis in 60 µl of 2 M trifluoroacetic acid at 100°C for 3 hours (unpublished). After evaporation of the hydrolysates to dryness, samples were deaminated by treatment at room temperature with 30 µl of 3.9 M $NaNO_2$ in 0.29 M acetic acid (15). After 10 min. the deamination mixtures were diluted with 0.6 ml of 1 M acetic acid, passed through columns (1 x 3 cm) of Dowex 50-X8, which had been equilibrated with 1 M acetic acid, and finally evaporated to dryness with several additions of methanol. The residues were dissolved in 1.0 ml of 2 M trifluoroacetic acid and heated at 100°C for 4 hours. The resulting uronic acids (L-IdUA and D-GlcUA) were isolated after preparative paper electrophoresis at pH 5.3 (see the legend to Fig. 1 for details) and were then separated by paper chromatography (Fig. 2).

RESULTS

Incorporation of uronic acid and sulfate into microsomal glycosaminoglycan (Table 1) occurred essentially as described previously by Silbert (16). In the presence of PAPS, sulfate was incorporated both into endogenous, preformed polysaccharide and into material synthesized in the cell-free system. The degree

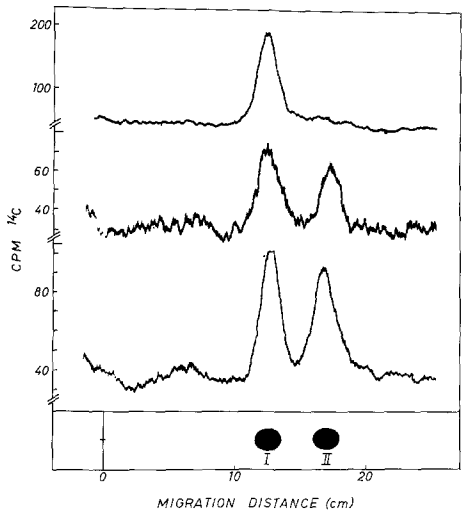

Fig. 2. Paper chromatography of uronic acids (peak III in Fig. 1) isolated in experiments 1, upper tracing; 2, middle tracing; and 4, lower tracing (see Table 1 for details of the incubation procedures). The standards of D-GlcUA (I) and L-IdUA (II) shown below the tracings were stained by a silver dip procedure (17). The solvent used was ethyl acetate - acetic acid - water (3:1:1), on Whatman 3MM paper; similar agreement between the migration distances of the radioactive components and those of the uronic acid standards were observed with butanol - acetic acid - water (4:1:2).

Paper chromatography of the lactone fractions (peak I in Fig. 1) gave essentially the same result; i.e. all ^{14}C-labelled samples yielded ^{14}C-GlcUA, whereas ^{14}C-IdUA was obtained from experiments 2, 3 and 4, only. However, the ^{14}C-IdUA/^{14}C-GlcUA ratio was generally lower for the lactone (peak I) than for the free acid (peak III) fraction.

of sulfation of newly-synthesized polysaccharide was quantitated by subtracting the amount of sulfate incorporated in the absence of sugar nucleotide (experiment 5) from that incorporated in the presence of both UDP-GlcNAc and UDP-^{14}C-GlcUA (experiment 2). The difference was related to the amount of uronic acid transferred (experiment 2), yielding a sulfate/uronic acid molar ratio of 1.7 (see also Silbert (16)). A similar ratio, 1.5, was obtained in a separate series of experiments. It should be noted that in some experiments (3 and 4) UDP-^{14}C-GlcUA and ^{35}S-PAPS were incubated under different conditions and for different periods of time; in these cases the amounts of sulfate and uronic acid incorporated cannot be directly compared.

The uronic acid composition of the labelled glycosaminoglycan samples is shown in Fig. 2 and in Table 1. Polysaccharide synthesized in the absence of PAPS (experiment 1) invariably contained ^{14}C-GlcUA as the only labelled uronic acid present in detectable amounts. In contrast, analysis of polysaccharide synthesized in the presence of PAPS showed a second labelled uronic acid component, which migrated like IdUA on paper chromatography in two solvent systems.

Experiments 3 and 4 (Table 1) represent pulse-chase incubations, in which

sulfation was carried out subsequent to completed incorporation of ^{14}C-uronic acid. This was accomplished by diluting the UDP-^{14}C-GlcUA, after a 30-min. pulse period, with unlabelled nucleotide sugar, either simultaneously with (experiment 3) or 15 min. before the addition of PAPS (experiment 4). Although the rate of ^{14}C-incorporation was decreased more than twenty-fold during the chase period[3], the observed ratios of ^{14}C-IdUA/^{14}C-GlcUA did not differ to any significant extent from that recorded after concurrent sulfation and ^{14}C-labelling (experiment 2).

DISCUSSION

The results obtained in the present investigation show for the first time the cell-free biosynthesis of a glycosaminoglycan containing IdUA residues. UDP-GlcUA was the precursor to both the GlcUA and the IdUA units of the completed polysaccharide.

Radioactivity from UDP-^{14}C-GlcUA could be incorporated into microsomal polysaccharide, both in the absence (experiment 1) and in the presence (experiment 2) of PAPS. Polysaccharide formed in the absence of PAPS contained ^{14}C-GlcUA as the only radioactive uronic acid present in significant amounts (experiment 1). On the other hand, the formation of IdUA was found to be strongly dependent on the presence of PAPS during the incubation (experiment 2). Pulse-chase experiments revealed that ^{14}C incorporated in the absence of PAPS could be recovered as ^{14}C-IdUA subsequent to sulfation (experiments 3 and 4). Hence, the ^{14}C-IdUA residues must have been formed by epimerization on the polymer level of ^{14}C-GlcUA units. It is concluded that UDP-IdUA is not required for the biosynthesis of IdUA in heparin.

The detailed mechanism of this epimerization process can not be formulated at present, nor is the relationship between sulfation and epimerization clearly understood. Nevertheless, an observation pertinent to the latter problem may be considered. The majority, but not all, of the IdUA units of heparin are sulfated, whereas the GlcUA residues are nonsulfated (8). In view of these facts the hypothesis illustrated in Fig. 3 should merit some attention. According to this scheme, epimerization of GlcUA to yield IdUA residues occurs prior to sulfation, but the equilibrium of the reaction favors retention of the D-gluco configuration. By subsequent sulfation of the IdUA residues formed (and/or of neighbouring sugars) these units are removed from the equilibrium, thus promoting further epimerization. This hypothesis unifies the structural and biosynthetic findings into one rational picture; however, its validity remains to be established.

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3) Determined in a separate experiment, in which UDP-^{14}C-GlcUA and unlabelled UDP-GlcUA (amounts as indicated in Table 1) were both present from the start of the incubation.

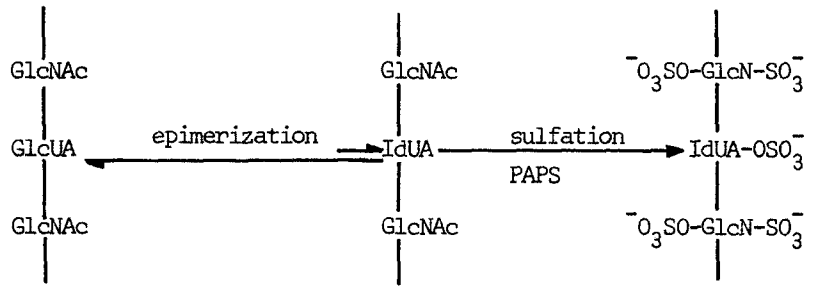

Fig. 3. Proposed scheme for the formation of sulfated IdUA units in heparin.

Acknowledgement. The present work was supported by grants from the Swedish Medical Research Council (B72-13X-2309-05A; B70-13X-139-06B), the Swedish Cancer Society (53-B71-04XC), Gustaf V:s 80-årsfond, and the Medical Faculty, University of Lund.

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