Changes in disaccharide composition of heparan sulphate fractions with increasing degrees of sulphation

Stephen R. DELANEY and H. Edward CONRAD

Department of Biochemistry, University of Illinois, Urbana, IL 61801, U.S.A.

(Received 25 May 1982/Accepted 5 October 1982)

Heparan sulphate by-products from the commercial manufacture of pig mucosal heparin were freed of chondroitin sulphate and fractionated according to anionic density. The fractions were treated with HNO_2 at pH 1.5, and the resulting mixtures of oligosaccharides were reduced with NaB^3H_4 and analysed for their disaccharide composition by paper chromatography and by high-pressure liquid chromatography. The results show that the molar ratio of 2-*O*-sulpho- α -L-iduronosylanhydromannose to 6-*O*-sulpho-(2-*O*-sulpho- α -L-iduronosyl)anhydromannose decreased from 2.5 to 0.04 as the degree of sulphation of the fractions increased. In contrast, the molar ratio of 6-*O*-sulpho-(β -D-glucuronosyl)anhydromannose to 6-*O*-sulpho-(α -L-iduronosyl)anhydromannose was approx. 2.4 in all heparan sulphate fractions and decreased to only half of this value in the most highly sulphated heparin fractions. These results are consistent with biosynthetic studies, which have shown that the *N*-sulpho-(2-*O*-sulpho- α -L-iduronosyl)-D-glucosamine disaccharide is the metabolic precursor of the *NO*-disulpho-(2-*O*-sulpho- α -L-iduronosyl)-D-glucosamine disaccharide in heparin biosynthesis. The high-pressure liquid chromatography of the heparan sulphate oligosaccharides also revealed a number of unidentified oligosaccharides in the deamination mixtures.

The repeating disaccharide sequence of heparin is synthesized by alternating transfers of *N*-acetyl-D-glucosamine and D-glucuronic acid from their UDP derivatives to the non-reducing ends of the growing polysaccharide chains (Silbert, 1963). The chain then undergoes an ordered sequence of enzyme-catalysed maturation reactions, which proceed via *N*-deacetylation followed by *N*-sulphation, C-5 epimerization of some of the D-glucuronic acid residues to L-iduronic acid residues and *O*-sulphation of some of the L-iduronic residues at C-2 and of some of the D-glucosamine residues at C-6 and at C-3 (Silbert, 1967; Lindahl *et al.*, 1973, 1980; Höök *et al.*, 1975; Forsee & Rodén, 1981; Riesenfeld *et al.*, 1982a). Although the *N*-deacetylation of the *N*-acetyl-D-glucosamine residues in the polymer appears to occur randomly along the chain, the *N*-sulpho-D-glucosamine residues formed by *N*-sulphation of the freed amino groups in a reaction that is coupled to the *N*-deacetylation (Riesenfeld *et al.*, 1982a) stimulate further *N*-deacetylation/*N*-sulphation at adjacent disaccharides (Riesenfeld

et al., 1980). The subsequent epimerization and *O*-sulphation reactions both occur preferentially or exclusively in the vicinity of the *N*-sulpho-D-glucosamine residues (Jacobsson & Lindahl, 1980).

As isolated from natural sources, the product of these reactions is a mixture of polymer chains containing sequences of disaccharides in all stages of maturation. The fraction of the mixture that has undergone the most extensive metabolic maturation (high L-iduronic acid content, almost quantitative *N*-sulphation and high degree of *O*-sulphation) is a potent inhibitor of blood clotting (Taylor *et al.*, 1973) and is referred to as heparin. In the commercial preparation of heparin from bovine lung or pig mucosa, the heparin is separated from by-products composed of a mixture of heparin-like polymers that contain relatively low contents of L-iduronic acid, *N*-sulphate and *O*-sulphate and higher contents of D-glucuronic acid and *N*-acetyl-D-glucosamine. This latter fraction, referred to as heparan sulphate, lacks anticoagulant activity and, on the basis of structural correlations, appears to be a mixture of intermediates formed either in the direct pathway for the biosynthesis of heparin or as side products of this pathway. Heparin sulphate has been shown to contain blocks of *N*-sulphated di-

Abbreviations used: AMan, 2,5-anhydro-D-mannose; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; HS, heparan sulphate starting material; LAH, low-activity heparin starting material.

saccharides interspersed with blocks of *N*-acetylated disaccharides (Cifonelli & King, 1975, 1977; Linker & Hovingh, 1975; Linker, 1979), a finding that is consistent with the observation that all of the heparin maturation enzymes exhibit a specificity for regions of the polymer chain in which the *N*-sulpho-D-glucosamine residues are formed initially (Jacobsson & Lindahl, 1980).

When heparin or heparan sulphate is treated with HNO_2 at low pH, the polymer is cleaved selectively at the *N*-sulpho-D-glucosamine residues (Cifonelli, 1968; Shively & Conrad, 1976). In this reaction the *N*-sulphate groups are released as SO_4^{2-} and the D-glucosamine residues are deaminated and converted into 2,5-anhydromannose residues. Since the *N*-sulphated disaccharides occur in blocks in heparin and heparan sulphate, and since most of the *O*-sulphate substituents are found in these blocks, HNO_2 treatment releases most of the sulphated sequences as disaccharides that have anhydromannose at their reducing terminals. The major disaccharide that is released by HNO_2 treatment of heparin is $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}(6\text{-SO}_4)$ with smaller amounts of $\text{Glc}\rightarrow\text{AMan}$, $\text{IdoA}\rightarrow\text{AMan}$, $\text{GlcA}\rightarrow\text{AMan}(6\text{-SO}_4)$, $\text{Ido}\rightarrow\text{AMan}(6\text{-SO}_4)$ and $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}$ (Kosakii *et al.*, 1978; Jacobsson *et al.*, 1979; Delaney *et al.*, 1980a; Weissmann *et al.*, 1982). The present paper describes the contents of these disaccharides in heparin and heparan sulphate fractions as a function of increasing sulphate substitution. The results show that $\text{Glc}\rightarrow\text{AMan}(6\text{-SO}_4)$ and $\text{IdoA}\rightarrow\text{AMan}(6\text{-SO}_4)$ are always present in approximately a 2:1 molar ratio and together constitute only 10–15% of the total disaccharides released in the HNO_2 reaction. In contrast, the ratio of $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}$ to $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}(6\text{-SO}_4)$ decreases from 2.5:1 in the least sulphated fractions to 0.04:1 in the most sulphated fractions. A number of unidentified oligosaccharides are also observed in the deamination mixtures.

Experimental

Materials

Chondroitinase ABC was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Two fractions of by-products from the commercial manufacture of pig mucosa heparin, one with no anticoagulant activity, and one with 76 i.u./mg, were kindly supplied by Mr. Erwin Coyne, Cohelfred Laboratories, Chicago, IL, U.S.A. These are referred to below as HS (heparan sulphate) and LAH (low-activity heparin) samples. Chondroitin sulphate and dermatan sulphate, present in these samples at a concentration of about 35% of the dry weight, were removed by digesting the samples with chondroitinase and removing the resulting oligo-

saccharides by dialysis. Each sample (625 mg) was dissolved in 62.5 ml of water, and 7.5 ml of pH 8.2 enriched Tris buffer (Saito *et al.*, 1968) was added. Then 25 units of chondroitinase ABC in 5 ml of a 1:10 dilution of pH 7.3 enriched Tris buffer (Yamagata *et al.*, 1968) was added, and the mixture was incubated for 15 h at 37°C. The reaction mixture was heated in a boiling-water bath for 2 min to inactivate the enzyme. The solution was then freeze-dried, redissolved in 10–20 ml of water, placed in a 10.2 mm Spectra/Por 6 dialysis bag (Spectrum Medical Industries, Queens, NY, U.S.A.) and dialysed exhaustively at 4°C against 0.1 M-NaCl and then against distilled water. The bovine serum albumin and the chondroitinase were removed from the non-diffusible material by ultrafiltration through an XM-50 membrane (50 000-mol.wt. cut-off) in an Amicon model 52 stirred cell (Amicon Corp., Lexington, MA, U.S.A.). Ultrafiltration was conducted at 4°C under 350 kPa (50 lbf/in²) above atmospheric pressure of N_2 , and the filtrate was freeze-dried and used as the starting material for the studies described below.

Analytical procedures

D-Glucuronic acid, L-iduronic acid and D-glucosamine (as anhydromannose) were quantified by the hydrolysis/deamination procedure described previously (Conrad, 1980). To determine the disaccharide compositions of heparin and heparan sulphate, fractions were treated with HNO_2 at pH 1.5 (Shively & Conrad, 1976) and reduced with NaBH_4 , and then analysed by high-pressure liquid chromatography (Delaney *et al.*, 1980a) or by paper chromatography (Conrad, 1980). Total carbohydrate was measured by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956) and expressed as equivalent of glucose read from a D-glucose standard curve. Chondroitinase-susceptible material was quantified by high-pressure-liquid-chromatographic analysis of the unsaturated disaccharides obtained in chondroitinase-digested samples (Delaney *et al.*, 1980b). The molecular weights of the HS and LAH samples were determined for us by Dr. N. Sugisaka and Dr. F. J. Petrcek of Riker Laboratories, Northridge, CA, U.S.A., by the high-pressure-liquid-chromatography procedure that they have described (Sugisaka & Petrcek, 1977).

Anion-exchange chromatography

Each of the starting materials was fractionated on a 2.5 cm × 30 cm column of Dowex AG1-X2 (200–400 mesh; Cl^- form) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) prepared in 0.5 M-NaCl. The sample (340 mg) was dissolved in 10 ml of 0.5 M-NaCl and, after removal of a small amount of insoluble material by centrifugation, was loaded on to the column and eluted at a flow rate of 2 ml/min

with a linear salt gradient generated from 750 ml each of 0.5 M-NaCl and 3.0 M-NaCl. Fractions (15 ml) were collected and their NaCl concentrations were determined by comparison of their conductivities with a standard curve prepared with NaCl solutions with known concentrations. Portions (250–500 μ l) of each fraction were taken for analysis of total carbohydrate. The eluate fractions from each column were combined to obtain a series of combined fractions, each of which contained approximately equal amounts of total carbohydrate. Each combined fraction was analysed for its NaCl concentration and then desalted by dialysis against water with Spectra/Por 6 dialysis membranes with a 2000-mol.wt. cut-off. The non-diffusible fraction was freeze-dried, and stock solutions containing 10 mg of sample/ml of water were prepared for further analyses. Portions of each stock solution were treated with HNO_2 at pH 1.5, reduced with NaB^3H_4 and analysed for the disaccharide composition of the deamination mixtures. The yield of each disaccharide is expressed as mol of disaccharide/mol of total anhydromannose determined by the hydrolysis/deamination procedure.

Anticoagulant activity

The anticoagulant activities of the combined fractions were determined by the method of Lam *et al.* (1976), in which the inhibition of the thrombin-catalysed hydrolysis of *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide (S-2160; Sigma Chemical Co.) in the presence of antithrombin III and a portion of each heparin stock solution was measured. This same assay was used to prepare a standard curve with serial dilutions of a standard heparin (183 i.u./mg) obtained from Dr. M. B. Mathews and Dr. J. A. Cifonelli, University of Chicago, Chicago, IL, U.S.A. Anticoagulant activities of the HS and LAH fractions were determined by comparison with the reference standard. All measurements were performed in triplicate. Samples of pure thrombin and antithrombin III were generously given by Dr. R. D. Rosenberg, Massachusetts Institute of Technology, Boston, MA, U.S.A.

Results

Fractionation of starting materials

Two side fractions that are usually discarded in the manufacture of commercial heparin were studied. The first, designated as HS (heparan sulphate), was devoid of anticoagulant activity. The second, designated as LAH (low-activity heparin), had an anticoagulant activity of 76 i.u./mg. All chondroitinase-susceptible materials were removed from these samples before analysis and fractionation. The properties of the two starting materials are shown in Table 1.

Table 1. *Properties of HS and LAH samples*
For full details see the text.

Property	HS	LAH
Molecular weight		
$\bar{M}_w$	4000	10400
$\bar{M}_n$	3000	6700
Uronic acid composition		
mol/mol of AMan	1.26	1.32
IdoA/GlcA ratio	64:36	70:30
Chondroitinase-digestion products		
mol of disaccharide/mol of AMan	0.003	0.001
Deamination products		
(mol/mol of AMan)		
Ido(2-SO ₄)→AMan(6-SO ₄)	0.52	0.58
Monosulphated disaccharides*	0.45	0.32
Unsulphated disaccharides†	0.17	0.08

* IdoA(2-SO₄)→AMan plus IdoA→AMan(6-SO₄) plus GlcA→AMan(6-SO₄).

† IdoA→AMan plus Glc→AMan.

Fig. 1. *Fractionation of HS and LAH samples by anion-exchange chromatography*

Each purified sample (350 mg) was dissolved in 10 ml of 0.5 M-NaCl and applied to a 2.5 cm $\times$ 30 cm column of Dowex AG1-X2 equilibrated in 0.5 M-NaCl. Carbohydrate was eluted with a linear NaCl gradient from 0.5 M-NaCl to 3.0 M-NaCl (750 ml of each). Fractions (15 ml) were analysed for carbohydrate by the phenol/ H_2SO_4 reaction (Dubois *et al.*, 1956) and combined as shown. O, Conc. of NaCl; $\bullet$, carbohydrate. (a) HS sample. (b) LAH fraction.

Table 2. Properties of combined fractions from Dowex columns

For full details see the text.

Fraction	Concn. of NaCl (M)	Dry wt. (mg)	Composition ($\mu\text{mol/mg dry wt.}$)		
			AMan	GlcA	IdoA
HS-1	0.90	12.2	0.27	0.19	0.18
2	1.05	9.6	0.36	0.25	0.30
3	1.15	39.4	0.46	0.28	0.29
4	1.25	24.9	0.58	0.25	0.40
5	1.35	16.2	0.66	0.31	0.46
6	1.45	21.9	0.71	0.28	0.53
7	1.53	23.7	0.80	0.29	0.55
8	1.65	23.1	0.82	0.27	0.65
9	1.75	19.4	0.75	0.19	0.58
10	1.88	10.2	0.84	0.18	0.54
11	2.08	21.7	0.73	0.17	0.54
LAH-1	1.46	24.2	0.74	0.35	0.67
2	1.58	11.2	0.71	0.32	0.63
3	1.63	19.4	0.72	0.35	0.74
4	1.68	24.6	0.72	0.34	0.80
5	1.74	28.4	0.75	0.30	0.76
6	1.75	29.3	0.69	0.28	0.71
7	1.86	30.0	0.73	0.29	0.67
8	1.89	24.8	0.80	0.32	0.79
9	1.93	19.7	0.70	0.25	0.76
10	2.00	23.1	0.61	0.26	0.79

Each of the samples was fractionated according to anionic charge density by chromatography on a Dowex 1 anion-exchange column in an NaCl gradient. The elution profiles in Fig. 1 show that the carbohydrate from the HS sample was eluted in a very broad peak whereas that from the LAH sample was recovered in a sharper peak. Groups of tubes collected from each column were combined to obtain a series of fractions, each of which contained a similar amount of carbohydrate. Each combined fraction was assayed for NaCl concentration (position in the gradient), total dry weight after dialysis, total carbohydrate and content of D-glucosamine (as anhydromannose), D-glucuronic acid and L-iduronic acid. These data, presented in Table 2, show that the percentage of total uronic acid obtained as L-iduronic acid increased with the increasing NaCl concentration required for the elution of the fraction.

Disaccharide composition of the HS and LAH fractions

Each of the combined fractions from the Dowex columns was treated with HNO_2 at pH 1.5, and the resulting mixture of oligosaccharides was reduced with NaB^3H_4 and analysed for its disaccharide composition. The high-pressure-liquid-chromatography profiles of HS fractions that were eluted early, midway and late in the Dowex fractionation

Fig. 2. High-pressure liquid chromatography of deamination products of HS fractions

Portions of each fraction were treated with HNO_2 at pH 1.5, reduced with NaB^3H_4 and then chromatographed on an anion-exchange column in a KH_2PO_4 gradient to separate GlcA $\rightarrow$ AMan(6- SO_4) (1), IdoA $\rightarrow$ AMan(6- SO_4) (2), IdoA(2- SO_4) $\rightarrow$ AMan (3) and IdoA(2- SO_4) $\rightarrow$ AMan(6- SO_4) (4). Note the 10-fold change in scale for peaks of very high radioactivity. (a) Fraction 1. (b) Fraction 5. (c) Fraction 11.

are shown in Fig. 2. Peaks labelled 1, 2, 3 and 4 in these profiles were identified previously (Delaney *et al.*, 1980a); the other peaks in these profiles represent unidentified oligosaccharides. Striking differences are observed in the oligosaccharide compositions of the three fractions. In the early fraction (Fig. 2a) the only members of the previously identified sulphated disaccharides that are present in significant amounts are IdoA(2- SO_4) $\rightarrow$ AMan and IdoA(2- SO_4) $\rightarrow$ AMan(6- SO_4). The late fraction (Fig.

Table 3. Disaccharides formed by HNO_2 treatment of combined fractions from Dowex columns
For full details see the text.

Fraction	Composition (mmol/mol of AMan)					
	IdoA→ AMan	GlcA→ AMan	GlcA→ AMan(-SO ₄)	IdoA→ AMan(-SO ₄)	IdoA(-SO ₄)→ AMan	IdoA(-SO ₄)→ AMan(-SO ₄)
HS-1	17	80	8	7	182	83
2	24	82	28	11	345	140
3	21	73	46	16	350	144
4	21	48	80	32	330	238
5	14	54	91	34	249	292
6	11	58	87	35	175	395
7	12	61	86	36	118	480
8	9	46	74	34	90	571
9	8	60	68	29	60	591
10	9	51	69	31	38	600
11	9	75	78	33	33	757
LAH-1	5	78	72	35	127	425
2	3	79	71	30	104	587
3	10	69	65	26	94	571
4	12	68	65	37	93	578
5	8	60	88	56	88	773
6	10	79	79	58	65	711
7	2	86	94	129	80	868
8	13	83	70	62	44	669
9	6	89	93	80	70	918
10	8	99	79	68	51	918

2c) contains all four of the sulphated disaccharides, with IdoA(2-SO₄)→AMan(6-SO₄) as the predominant peak in the profile, just as it is in a typical heparin. Marked differences are also seen in the concentrations of the unidentified oligosaccharides in these fractions.

Table 3 shows the amount of all of the disaccharides found in the deamination mixture of the combined column fractions. If the sum of the amounts of the six disaccharides is taken as 100%, then the percentage of this total can be calculated for each disaccharide in the mixture. In Fig. 3 the percentage of each of the sulphated disaccharides in each fraction is plotted against the NaCl concentration at which the fraction was eluted from the Dowex column. The biggest changes are found for the HS fractions, where the early fractions are high in IdoA(2-SO₄)→AMan but low in IdoA(2-SO₄)→AMan(6-SO₄), whereas the reverse is true for the late fractions. In contrast, the relative concentrations of GlcA→AMan(6-SO₄) and IdoA→AMan(6-SO₄) remain quite constant, as do the concentrations of GlcA→AMan and IdoA→AMan (not plotted; see Table 3). Although the LAH fractions are eluted at a higher point in the NaCl gradient, the changes in the percentages of the different disaccharides with increasing NaCl concentration follow those observed for those LAH fractions that were eluted over the same range of salt concentrations.

Anticoagulant activities

Fig. 4 shows a plot of the anticoagulant activities of the LAH fractions against their position of elution from the Dowex column. These results show a progressive rise in anticoagulant activity from the early to the late fractions. No anticoagulant activity was found in any of the HS fractions, presumably because of the low molecular weights of these polymers (Laurent *et al.*, 1978; Thunberg *et al.*, 1979).

Discussion

Heparin sulphate was originally isolated from the by-products of commercial preparations of heparin and was recognized as a polymer that was structurally related to heparin even though it lacked anticoagulant activity (Jorpes & Gardell, 1948; Cifonelli & Dorfman, 1960). Virtually all knowledge of the structure of heparan sulphate has been obtained from studies conducted on material isolated from heparin by-products. Since the pig mucosa and the bovine lung tissues usually used for preparation of heparin (Coyne, 1981) are heterogeneous with respect to cell types, it is uncertain whether the heparin and heparin by-products are derived from the same cell types. Thus the metabolic relationships of the heparin and heparan sulphate fractions have remained uncertain.

Fig. 3. Yield of sulphated disaccharides formed by HNO_2 deamination of HS and LAH fractions

The yield of each disaccharide, expressed as the percentage of the total disaccharides released from the fraction by HNO_2 treatment, is plotted versus the NaCl concentration of the fraction. (a) HS fractions. (b) LAH fractions. The disaccharides are $\text{GlcA} \rightarrow \text{AMan}(6\text{-SO}_4)$ (■), $\text{IdoA} \rightarrow \text{AMan}(6\text{-SO}_4)$ (□), $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}$ (▲) and $\text{IdoA}(2\text{-SO}_4) \rightarrow \text{AMan}(6\text{-SO}_4)$ (△).

As a result of cell-culture studies in recent years, biological distinctions between heparin and heparan sulphate have become apparent (Rodén, 1980). Heparan sulphate is produced as an extracellular proteoglycan in most cell types (Kramer, 1971*a,b*). Heparin has also been shown to occur as a proteoglycan in rat skin (Horner, 1971) and in mast cells (Metcalf *et al.*, 1980), where it appears primarily as an intracellular polymer. A further difference between these two proteoglycans is seen in the amino acid compositions of their core proteins (Robinson *et al.*, 1978; Oldberg *et al.*, 1979; Metcalf *et al.*, 1980). Thus, as a result of these studies, several rather basic distinctions between heparin and heparan sulphate have been shown that suggest that the different core proteins might be involved in the regulation of both the proteoglycan secretion and the degree of uronic acid epimerization and polymer sulphation. However, Riesenfeld *et al.* (1982*b*) have compared the heparin-like proteo-

Fig. 4. Anticoagulant activities of the LAH fractions. The inhibition of thrombin activity by a mixture of antithrombin III and 1:1000 dilutions of each of the LAH fraction stock solutions was measured. The results were used to obtain the international units (i.u.) of activity of each LAH fraction from a curve prepared with a standard heparin sample in the same assay. The mean values $\pm$ s.d. for triplicate determinations are plotted versus the NaCl concentrations of the combined fractions from the Dowex column.

glycans synthesized by intact hepatocytes with those synthesized by microsomal preparations from the same cells, and have found that the cell-free system yielded a heparin product whereas the intact cells yielded a heparan sulphate. These authors concluded that the production of heparan sulphate depended, not only on the core-protein structure, but also on the regulatory mechanisms for the polymer-modification reactions, which were lost when the cells were disrupted. Thus the distinction between heparin and heparan sulphate may be one of cellular regulation of the polymer-modification reactions.

In the present study heparan sulphate was purified from heparin by-products. Because of commercial considerations the details of preparation of the by-products are not available. However, the heparan sulphate preparation purified from the heparin by-product was devoid of other glycosaminoglycans and was completely depolymerized by HNO_2 treatment. The heparan sulphate fractions were separated by ion-exchange chromatography into a continuum of fractions with increasing degrees of sulphation, as indicated by several observations. Fractions eluted later in the gradient gave increased

yields of total disaccharides (mol/mol of anhydromannose) when treated with HNO_2 , an indication that an increasing proportion of the D-glucosamine residues were *N*-sulphated. Increasing degree of sulphation was also reflected in the higher concentrations of $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}(6\text{-SO}_4)$, the most heavily sulphated of the disaccharides, in the deamination products of the later fractions from the column. If it is assumed that these fractions represent intermediates or side reactions in the pathway for heparin biosynthesis, the increased degrees of sulphation from the early column fractions to the late column fractions suggest that the fractions emerge from the Dowex column in the order of the metabolic sequence of polymer maturation. This suggestion is strengthened by the observation that L-iduronic acid represents an increasing fraction of the total uronic acid in the later fractions.

The disaccharide content of the deamination products may be examined in the light of the possibility that the early Dowex fractions represent early intermediates in the maturation of heparin whereas late Dowex fractions represent late intermediates. The HS fractions cover a broader range of polymer structures and therefore would represent a better illustration of the overall maturation sequence than the LAH fractions, which nevertheless would describe later stages of maturation that are identical with those described by the later HS fractions. In the HS fractions the yield of $\text{GlcA}\rightarrow\text{AMan}$ per mol of total anhydromannose is very similar for all fractions. However, this unsulphated deamination product represents more than 20% of the total disaccharides in the earliest fraction but only 6–7% of the total disaccharides in the later fractions. The $\text{IdoA}\rightarrow\text{AMan}$ yield is also relatively high in the early fractions but lower in the late fractions, in spite of the increasing percentages of L-iduronic acid in the later fractions. The most striking changes in disaccharide composition are found for $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}$ and $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}(6\text{-SO}_4)$. Together these two disaccharides represent 70–75% of the total disaccharides in all fractions, but the $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}$ content, which is quite high in the early fractions, falls in the later fractions as the amount of $\text{IdoA}(2\text{-SO}_4)\rightarrow\text{AMan}(6\text{-SO}_4)$ rises. This is consistent with the metabolic precursor-product relationship suggested for these two disaccharides (Jacobsson & Lindahl, 1980). The $\text{GlcA}\rightarrow\text{AMan}(6\text{-SO}_4)$ and $\text{IdoA}\rightarrow\text{AMan}(6\text{-SO}_4)$ are present in a 2.4:1 molar ratio in all but the late LAH fractions, and together represent 10–15% of the total disaccharides in all of the fractions. Several explanations may be advanced for the constant amounts and ratios of the 6-*O*-monosulphated disaccharides. One is that immediately after the *N*-sulphation step a constant fraction of the $\rightarrow\text{GlcA}\rightarrow\text{Glc}(\text{NSO}_3^-)\rightarrow$ sequences undergo 6-*O*-

sulphation on the D-glucosamine residue and that 6-*O*-sulphation precludes 2-*O*-sulphation (Jacobsson & Lindahl, 1980) but not epimerization of the uronic acid. Thus the C-5 epimerase that interconverts the D-glucuronic acid residues and the L-iduronic acid residues in these polymers (Malmström *et al.*, 1980) may bring these two disaccharides to their point of thermodynamic equilibrium. The ratio in which $\text{GlcA}\rightarrow\text{AMan}(6\text{-SO}_4)$ and $\text{IdoA}\rightarrow\text{AMan}(6\text{-SO}_4)$ are found is a reasonable value to expect for the equilibrated epimerization reaction, and is one that is consistent with the ratios of D-glucuronic acid and L-iduronic acid found after exhaustive epimerization of a heparin substrate with the purified mouse mastocytoma epimerase (Prihar *et al.*, 1980). However, the biosynthesis studies in the mouse mastocytoma system show that a disaccharide that contains an *O*-sulphated D-glucosamine residue cannot serve as a substrate for the epimerase (D. S. Feingold, U. Lindahl & L. Rodén, personal communication). If this observation is also true for the epimerase in the pig mucosa system, then the epimerase-equilibrium explanation for the constant ratio of $\text{GlcA}\rightarrow\text{AMan}(6\text{-SO}_4)$ to $\text{IdoA}\rightarrow\text{AMan}(6\text{-SO}_4)$ would not be valid. An alternative explanation is that the constant amounts and ratios are simply a consequence of the steady-state partitioning of the *N*-sulphated disaccharides between the epimerization reaction and the *O*-sulphation reactions.

The high-pressure-liquid-chromatography profiles of the deamination products of the fractions analysed in the present work show a number of peaks in addition to the sulphated disaccharides identified previously (Delaney *et al.*, 1980a). The unsulphated disaccharides that were analysed in the present study by paper chromatography appear in high-pressure-liquid-chromatography profiles together with the reagent blank at about 7 min. Initial work on the characterization of the unidentified peaks has shown that several of the most prominent of these are mixtures of several components. Thus the oligosaccharide mixtures resulting from HNO_2 cleavage of heparin and heparan sulphate are quite complex. However, even though the resolution of these oligosaccharides is incomplete, it is clear that the high-pressure-liquid-chromatography profiles are diagnostic for the main structural features of each heparin and heparan sulphate fraction. It may be noted, however, that the disaccharide compositions of some of the LAH fractions are very similar in spite of quite significant differences in their anticoagulant activities. To date, the oligosaccharide species that are uniquely involved in the binding of heparin to antithrombin III (Rosenberg *et al.*, 1978; Rosenberg & Lam, 1979; Lindahl *et al.*, 1979; Riesenfeld *et al.*, 1981; Ototani & Yosizawa, 1981; Oosta *et al.*, 1981; Thunberg *et al.*, 1982) have not been identified in these profiles.

When such oligosaccharides are identified, it may be possible, by using the methodology described in the present paper, to show a correlation between such peaks and the anticoagulant activities of different heparin samples.

This work was supported by U.S. Public Health Service Grant HL 21763.

References

- Cifonelli, J. A. (1968) *Carbohydr. Res.* **8**, 233–242
- Cifonelli, J. A. & Dorfman, A. (1960) *J. Biol. Chem.* **235**, 3283–3286
- Cifonelli, J. A. & King, J. (1975) *Connect. Tissue Res.* **3**, 97–104
- Cifonelli, J. A. & King, J. (1977) *Biochemistry* **16**, 2137–2141
- Conrad, H. E. (1980) *Biochem. J.* **191**, 355–363
- Coyne, E. (1981) in *Chemistry and Biology of Heparin* (Lundblad, R. L., Brown, W. V., Mann, K. G. & Roberts, H. R., eds.), pp. 9–17, Elsevier/North-Holland, New York
- Delaney, S. R., Leger, M. & Conrad, H. E. (1980a) *Anal. Biochem.* **106**, 253–261
- Delaney, S. R., Conrad, H. E. & Glaser, J. H. (1980b) *Anal. Biochem.* **108**, 25–34
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Forsee, W. T. & Rodén, L. (1981) *J. Biol. Chem.* **256**, 7240–7247
- Höök, M., Lindahl, U., Hallén, A. & Bäckström, G. (1975) *J. Biol. Chem.* **250**, 6055–6071
- Horner, A. A. (1971) *J. Biol. Chem.* **246**, 231–239
- Jacobsson, I. & Lindahl, U. (1980) *J. Biol. Chem.* **255**, 5094–5100
- Jacobsson, I., Höök, M., Pettersson, I., Lindahl, U., Larm, O., Wirén, E. & von Figura, K. (1979) *Biochem. J.* **179**, 77–87
- Jorpes, J. E. & Gardell, S. (1948) *J. Biol. Chem.* **176**, 267–276
- Kosakii, M., Yamauchi, F. & Yosizawa, Z. (1978) *J. Biochem. (Tokyo)* **83**, 1567–1575
- Kramer, P. M. (1971a) *Biochemistry* **10**, 1437–1445
- Kramer, P. M. (1971b) *Biochemistry* **10**, 1446–1451
- Lam, L. H., Silbert, J. E. & Rosenberg, R. D. (1976) *Biochem. Biophys. Res. Commun.* **69**, 570–579
- Laurent, T. C., Tengblad, A., Thunberg, L., Höök, M. & Lindahl, U. (1978) *Biochem. J.* **175**, 691–701
- Lindahl, U., Bäckström, G., Jansson, L. & Hallén, A. (1973) *J. Biol. Chem.* **248**, 7234–7241
- Lindahl, U., Bäckström, G., Höök, M., Thunberg, L., Fransson, L.-Å. & Linker, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3198–3202
- Lindahl, U., Bäckström, G., Thunberg, L. & Leder, I. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551–6555
- Linker, A. (1979) *Biochem. J.* **183**, 711–720
- Linker, A. & Hovingh, P. (1975) *Biochim. Biophys. Acta* **385**, 324–333
- Malmström, A., Rodén, L., Feingold, D. S., Jacobsson, I., Bäckström, G. & Lindahl, U. (1980) *J. Biol. Chem.* **255**, 3878–3883
- Metcalf, D. D., Smith, J. A., Austen, K. F. & Silbert, J. E. (1980) *J. Biol. Chem.* **255**, 11753–11758
- Oldberg, Å., Kjellén, L. & Höök, M. (1979) *J. Biol. Chem.* **254**, 8505–8510
- Oosta, G. M., Gardner, U. T., Beeler, D. L. & Rosenberg, R. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 829–833
- Ototani, N. & Yosizawa, Z. (1981) *J. Biochem. (Tokyo)* **90**, 1553–1556
- Prihar, H. S., Campbell, P., Feingold, D. S., Jacobsson, I., Jensen, J. W., Lindahl, U. & Rodén, L. (1980) *Biochemistry* **19**, 495–500
- Riesenfeld, J., Höök, M. & Lindahl, U. (1980) *J. Biol. Chem.* **255**, 922–928
- Riesenfeld, J., Thunberg, L., Höök, M. & Lindahl, U. (1981) *J. Biol. Chem.* **256**, 2389–2394
- Riesenfeld, J., Höök, M. & Lindahl, U. (1982a) *J. Biol. Chem.* **257**, 421–425
- Riesenfeld, J., Höök, M. & Lindahl, U. (1982b) *J. Biol. Chem.* **257**, 7050–7055
- Robinson, H. C., Horner, A. A., Höök, M., Ögren, S. & Lindahl, U. (1978) *J. Biol. Chem.* **253**, 6687–6693
- Rodén, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
- Rosenberg, R. D. & Lam, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1218–1222
- Rosenberg, R. D., Armand, G. & Lam, L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3065–3069
- Saito, H., Yamagata, T. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536–1542
- Shively, J. E. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
- Silbert, J. E. (1963) *J. Biol. Chem.* **238**, 3542–3546
- Silbert, J. E. (1967) *J. Biol. Chem.* **242**, 5153–5157
- Sugisaka, N. & Petrcek, F. J. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 89–92
- Taylor, R. L., Shively, J. E., Conrad, H. E. & Cifonelli, J. A. (1973) *Biochemistry* **12**, 3633–3637
- Thunberg, L., Lindahl, U., Tengblad, A., Laurent, T. C. & Jackson, C. M. (1979) *Biochem. J.* **181**, 241–243
- Thunberg, L., Bäckström, G. & Lindahl, U. (1982) *Carbohydr. Res.* **100**, 393–410
- Weissmann, B., Chao, H. & Chow, P. (1982) *Carbohydr. Res.* **92**, 239–253
- Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **253**, 1523–1535