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Distribution of Sulphate and Iduronic Acid Residues in Heparin and Heparan Sulphate

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1. A method was developed for determination of the uronic acid composition of heparin-like glycosaminoglycans. Polymers or oligosaccharides are degraded to monosaccharides by a combination of acid hydrolysis and deamination with HNO_2. The resulting uronic acid monosaccharides (accounting for about 70% of the uronic acid contents of the starting materials) are isolated and converted into the corresponding aldono-1,4-lactones, which are separated by g.l.c. The calculated ratios of glucuronic acid/iduronic acid are reproducible within 5%. 2. Samples of heparin from pig intestinal mucosa (molar ratio of sulphate/disaccharide unit, 2.40) and heparan sulphate from human aorta (sulphate/disaccharide ratio, 0.46) were subjected to uronic acid analysis. L-Iduronic acid constituted 77% and 19% respectively of the total uronic acid contents. 3. The correlation between the contents of sulphate and iduronic acid indicated by this finding also applied to the fractionated deamination products of the two polymers. The sulphated fragments varied in size from disaccharide to octasaccharide (or larger) and showed sulphate/disaccharide molar ratios in the range of 0.05–2.0. The proportion of iduronic acid increased with increasing ester sulphate contents of the oligosaccharides. 4. Previous studies on the biosynthesis of heparin in a cell-free system have shown that L-iduronic acid residues are formed by C-5 epimerization of D-glucuronic acid units at the polymer level; the process requires concomitant sulphation of the polymer. The results obtained in the present structural study conform to these findings, and suggest further that similar mechanisms may operate in the biosynthesis of heparan sulphate. The epimerization reaction appears to be linked to the sulphation of hydroxyl groups but does not seem to require sulphation of the target uronic acid residues. The significance of sulphamino groups in relation to the formation of iduronic acid is unknown.

Heparin and heparan sulphate are both sulphated polymers consisting of alternating residues of uronic acid and glucosamine. At the reducing end, the polysaccharide chains are linked to polypeptide via galactosylgalactosylxylose trisaccharide sequences, extending to serine residues (Lindahl, 1966; Knecht *et al.*, 1967). The glucosamine units of heparin are believed to have the α-anomeric configuration (Wolfrom *et al.*, 1964). Both polysaccharides contain two kinds of uronic acid, D-glucuronic acid and L-iduronic acid (Cifonelli & Dorfman, 1962; Wolfrom *et al.*, 1969; Lindahl & Axelsson, 1971). The iduronic acid component of heparin appears to be of the α-L-type (Perlin *et al.*, 1970), whereas most, and possibly all, of the glucuronic acid residues show the β-D configuration (Helting & Lindahl, 1971).

The relationship between heparin and heparan sulphate is expressed by certain structural features unique to these polysaccharides. Both polymers thus contain *N*-sulphated as well as *N*-acetylated glucosamine residues, which in the case of heparan sulphate appear to be distributed in a blockwise fashion through the molecule (Cifonelli, 1968). Heparin generally contains less *N*-acetyl groups and more *N*-and *O*-sulphate groups than does heparan sulphate; however, it is notable that the section located immediately adjacent to the carbohydrate–protein linkage region is exclusively *N*-acetylated in both polysaccharides (Lindahl, 1966; Cifonelli, 1968).

Recent studies on the biosynthesis of heparin have demonstrated a close connexion between sulphation of the molecule and the formation of iduronic acid. In the presence of adenosine 3′-phosphate 5′-sulphatophosphate, a microsomal fraction from mouse mastocytoma was found to catalyse the C-5 epimerization of glucuronic acid units, previously incorporated into a heparin precursor polymer, to yield iduronic acid residues (Lindahl *et al.*, 1972). Structural characterization of a partially sulphated ^{14}C-labelled product indicated that the epimerization

process depends on the formation of ester sulphate groups (Lindahl *et al.*, 1973*b*; M. Höök, U. Lindahl, G. Bäckström, A. Malmström & L.-Å. Fransson, unpublished work).

The present structural study was undertaken in order to gain further insight into the relationship between polymer sulphation and uronic acid epimerization. Preparations of heparin and heparan sulphate, differing widely in sulphate contents, were analysed with regard to the distribution of sulphate and iduronic acid residues. The results support the previous conclusions about the biosynthesis of heparin, and suggest further that similar mechanisms may apply to the formation of the iduronic acid component of heparan sulphate. Results consistent with those presented here have been obtained by Dr. H. E. Conrad (personal communication).

Experimental

Materials

Heparin (stage 14) from pig intestinal mucosa was purchased from Wilson Laboratories, Chicago, Ill., U.S.A., and purified by repeated precipitation with cetylpyridinium chloride (Lindahl *et al.*, 1965) from 1.2M-NaCl. The heparan sulphate preparation used (HSII, from human aorta) has been described previously (Iverius, 1971). Analytical data of the heparin and heparan sulphate preparations are shown in Table 1. Both polysaccharide preparations yielded a spot with the migration properties of heparin (or heparan sulphate, which migrates similarly to heparin in the system used) on cellulose acetate electrophoresis in 0.1M-barium acetate (Wessler, 1968); in addition, the heparin preparation appeared to contain trace amounts of dermatan sulphate. On electrophoresis in 0.1M-HCl (Wessler, 1971) the heparin sample migrated well ahead of a chondroitin 4-sulphate reference, containing one sulphate residue per repeating disaccharide unit, whereas the heparan sulphate showed a homogeneous spot with a mobility intermediate between those of hyaluronic acid and chondroitin 4-sulphate. The glycosaminoglycans used as reference standards were as described by Lindahl (1970). The low migration rate of the heparan sulphate preparation with 0.1M-HCl was consistent with the sulphate contents indicated by chemical analysis (Table 1).

N-Acetylheparin was prepared as described previously (Lindahl *et al.*, 1973*a*) by hydrolytic *N*-desulphation of heparin followed by treatment with acetic anhydride.

Mono- and di-sulphated uronosylanhydromannose disaccharides were isolated after treatment of heparin with HNO_2 (Lindahl & Axelsson, 1971). ^{14}C-Labelled non-sulphated uronosylanhydromannose was obtained after similar degradation of a polysaccharide prepared by incubating a microsomal fraction from a transplantable mast-cell tumour with UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylglucosamine (Lindahl *et al.*, 1973*a*).

D-Glucuronic acid was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-Iduronic acid was isolated by preparative paper chromatography (see below) of hydrolysed (2M-trifluoroacetic acid; 100°C; 4h) pig skin dermatan sulphate.

[U-^{14}C]Glucose (288μCi/μmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of [^{14}C]heparin

The transplantable mast-cell tumour used has been described previously (Helting *et al.*, 1972).

Freshly dissected tumour tissue (about 5g, after removal of necrotic areas) was cut into small pieces with scissors and was then incubated with 400μCi of [^{14}C]glucose in 30ml of Krebs–Ringer phosphate solution (Herbai & Lindahl, 1970). After 3h at 37°C the mixture was heated at 100°C for 5min and mixed with 7.2ml of 0.5M-sodium acetate buffer, pH5.5, containing 1M-NaCl, 0.05M-cysteine–HCl and 0.05M-EDTA. The mixture was digested at 65°C for 20h with 20mg of papain [Sigma; purified by the procedure of Kimmel & Smith (1954)] and was then centrifuged; the precipitate was extracted with 3ml of 2M-KCl. To the combined clear supernatant and extract were added 10ml of 2% (w/v) cetylpyridinium chloride in water. The precipitate formed after 30min at 30°C was collected by centrifugation, suspended in 0.15M-NaCl, re-centrifuged and finally dissolved in 4ml of 2M-KCl containing 0.1% glucose. The solution was left at

Table 1. *Analytical data of heparin and heparan sulphate preparations*

For details see the text. Uronic acid and hexosamine contents are expressed as percentages of dry weight. The hexosamine values were not corrected for losses during hydrolysis.

	Uronic acid (%)	Hexosamine (%)	Sulphate/uronic acid molar ratio	Glucosamine/total hexosamine (%)	Anticoagulant activity (British Pharmacopoeia units/mg)
Heparin	35	28	2.4	96	146
Heparan sulphate	40	34	0.46	>99%	<10

30°C for 30min and polysaccharide was precipitated by the addition of 2ml of 2% (w/v) cetylpyridinium chloride and 4ml of water. Treatment with 2M-KCl–0.1% glucose was repeated once. The polysaccharide was finally precipitated with 3vol. of ethanol from 2ml of 2M-NaCl in 10% (v/v) ethanol, washed once with ethanol and dissolved in 1ml of water. The product (total yield, 1.7mg of uronic acid) showed a specific radioactivity of 990c.p.m. of $^{14}C/\mu g$ of uronic acid and migrated like commercial heparin on cellulose acetate electrophoresis in 0.1M-HCl.

Methods

Analytical methods. Uronic acid was determined by the method of Bitter & Muir (1962), with D-glucuronolactone as standard. Anhydromannose was determined by the indole method described by Dische & Borenfreund (1950). Hexosamine analyses were performed by a modification (Gardell, 1953) of the Elson–Morgan reaction, after hydrolysis of saccharides in 4M-HCl at 100°C for 14h. Glucosamine/galactosamine ratios were measured by chromatography on a column of Aminex A-5 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), as described by Lohmander (1972). Sulphate was determined by the method of Terho & Hartiala (1971).

Radioactivity was measured with a Beckman model LS 250 liquid-scintillation counter, with Insta-Gel (Packard Instrument Co., La Grange, Ill., U.S.A.) as scintillation medium. Labelled compounds on paper chromatograms or electrophoretograms were localized with a Packard model 7201 strip scanner and were then measured quantitatively, after elution, by liquid-scintillation counting.

High-voltage paper electrophoresis was performed in 1.6M-formic acid, pH1.7 (40V/cm for 2h), or in 0.08M-pyridine–0.05M-acetic acid, pH5.3 (70V/cm for 40min), on Whatman 3 MM paper. Papers were stained by the silver-dip method (Smith, 1960).

Paper chromatography was carried out with ethyl acetate–acetic acid–water (3:1:1, by vol.) on Whatman 3MM paper.

Ion-exchange chromatography was carried out on a column (2.2cm×20cm) of ECTEOLA-cellulose (Serva Entwicklungslabor, Heidelberg, West Germany), eluted with a linear gradient of NH_4HCO_3 (Balasubramanian *et al.*, 1967). The mixing vessel and reservoir contained 200ml of 0.02M-NH_4HCO_3 and 200ml of 1M-NH_4HCO_3 respectively; the elution rate was 10ml/h. In preparative experiments (10mg or less of uronic acid/separation) the NH_4HCO_3 was eliminated from effluent fractions by freeze-drying. The recovery of uronic acid-containing material generally exceeded 95% of the amounts applied to the column.

Determination of uronic acid composition. Heparin or heparan sulphate (or oligosaccharides of these polymers) (0.1–0.5mg) was dissolved in 0.5ml of 2M-trifluoroacetic acid and the solutions were heated at 100°C in sealed glass tubes for 3h. The hydrolysates were evaporated to dryness and were then treated with 100μl of a freshly prepared solution of 3.9M-$NaNO_2$ in 0.28M-acetic acid. After 10min at room temperature, 1ml of 1M-acetic acid was added and the deamination mixtures were passed through columns (1cm×3cm) of Dowex 50 (×8; H^+ form), which had been equilibrated with 1M-acetic acid. The effluents were repeatedly evaporated to dryness in the presence of methanol and were then hydrolysed with 2ml of 2M-trifluoroacetic acid at 100°C for 4h.

The relative amounts of glucuronic acid and iduronic acid in the hydrolysates were determined by g.l.c. of the corresponding aldonolactones. After evaporation to dryness the hydrolysates were dissolved in 1ml of 0.1M-Tris–HCl buffer, pH8.0, and were then left at room temperature overnight for conversion of lactones into free acids (Blake & Richards, 1968). The uronic acids were absorbed on a column (1cm×3cm) of Dowex 1 (X8; acetate form) and eluted with 15 bed volumes of 1M-trifluoroacetic acid. The samples were evaporated to dryness and treatment with Tris–HCl buffer, pH8.0, was repeated as described above. The uronic acids were then reduced with 10mg of $NaBH_4$ in a final volume of 1ml of 0.1M-Tris–HCl buffer, pH8.0. After 30min at room temperature the excess of $NaBH_4$ was destroyed by the addition of 4M-acetic acid, and the reaction mixtures were passed through columns (1cm×3cm) of Dowex 50 (X8; H^+ form). After repeated evaporation of the effluents to dryness in the presence of methanol, the resulting samples were transferred to small glass tubes and freeze-dried. After the addition of 100μl of hexamethyldisilazane in dimethylformamide (2:5, v/v), silylation was carried out by immersing the sealed tubes in a boiling-water bath for 2min. The trimethylsilyl derivatives were extracted from the reaction mixtures with hexane. The clear supernatants of the centrifuged extracts were concentrated under reduced pressure and were then analysed by g.l.c. by the method of Perry & Hulyalkar (1965). Samples (1–2μl) were separated at 170°C in a Perkin–Elmer model 881 gas chromatograph, equipped with a flame-ionization detector, on glass columns (2m×2mm internal diam.) packed with 10% (w/w) neopentyl glycol sebacate polyester on Chromosorb W (120–140 mesh). N_2 was used as carrier gas, at a flow rate of approx. 30ml/min. Peak areas were measured by planimetry.

Degradation of polysaccharides with HNO_2. Heparin and heparan sulphate (0.5g of each) were degraded with HNO_2 in aqueous 1,2-dimethoxyethane at −15°C, by the method of Cifonelli (1968).

The procedure adopted has been described in detail previously (Lindahl, 1966). The degradation products were separated on a column (2cm×200cm) of Sephadex G-25 (superfine grade) eluted with 0.2M-NaCl at a rate of 12ml/h. Effluent fractions of about 4ml were collected, analysed for uronic acid and for hexosamine and combined as indicated in Fig. 6. The pooled fractions were desalted by passage through a column (1.5cm×180cm) of Sephadex G-15, which was eluted with aq. 10% (v/v) ethanol.

Results

Determination of uronic acid composition of heparin-like polysaccharides

Previous attempts to determine the uronic acid composition of heparins have been largely hampered by the resistance of these polysaccharides towards acid hydrolysis. The hydrolytic conditions required for quantitative conversion of the polymers into monosaccharides have thus resulted in undue destruction of the uronic acids. Recently, a method based on the reduction of uronic acid carboxyl groups has been developed, which enables the essentially complete non-destructive depolymerization of glycosaminoglycans, including heparin (Taylor & Conrad, 1972).

The procedure used in the present study effects the liberation in high yield of intact uronic acids as monosaccharides. Heparin-like polysaccharides are degraded by deamination with HNO_2, in conjunction with acid hydrolysis, as illustrated in Fig. 1. In the deamination reaction used (Shively & Conrad, 1970) glucosamine residues with unsubstituted amino groups are converted into 2,5-anhydromannose units, with concomitant cleavage of the corresponding

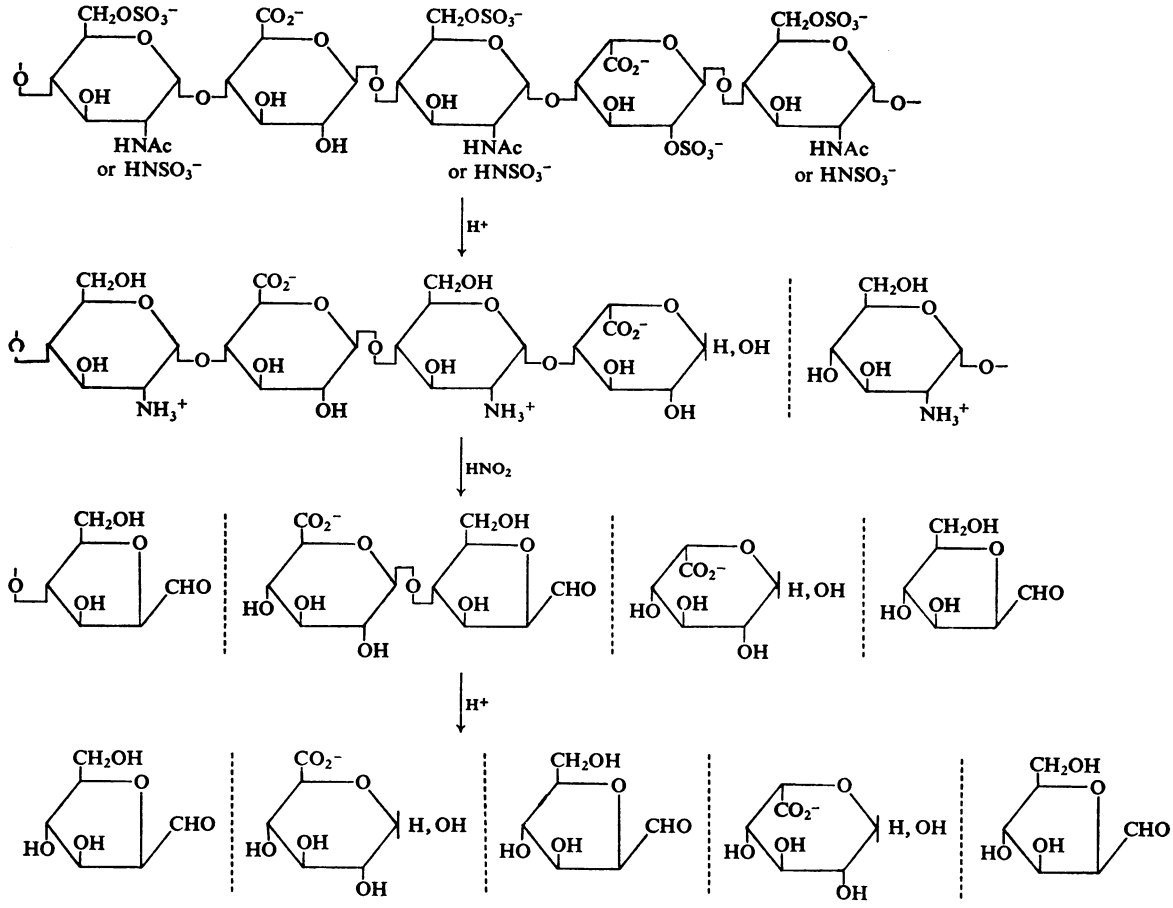

Fig. 1. *Procedures used in the degradation of heparin (or heparan sulphate) to monosaccharides*

The initial hydrolysis results in *N*-deacetylation and desulphation, but also in cleavage of some of the uronidic linkages (arbitrarily indicated as an iduronidic bond). For details see the Experimental section.

glucosaminidic linkage, whereas *N*-sulphated or *N*-acetylated glucosamine residues remain intact (Lindahl *et al.*, 1973*a*). To render the polysaccharides susceptible to deamination, the *N*-substituents have to be removed in a preliminary hydrolytic step. Since the *N*-acetyl group is more resistant towards acid hydrolysis than the *N*-sulphate group (Cifonelli, 1966), a heparin preparation with exclusively acetylated amino groups (denoted *N*-acetylheparin under 'Materials') was used as model substance. Under the conditions used, 3h of hydrolysis were required for complete *N*-deacetylation, as measured by the formation of 2,5-anhydromannose on treatment with HNO_2 (Fig. 2).

After hydrolysis and deamination the samples occurred largely as mixtures of uronosylanhydromannose disaccharides and uronic acid monosaccharides, the latter components being formed on deamination of oligosaccharides with uronic acid in the reducing terminal position (Fig. 1). Optimum hydrolysis conditions for cleavage of the disaccharides were determined as follows. A sample of ^{14}C-labelled heparin was taken through the initial hydrolysis and deamination steps, as described above, and was then subjected to a second period of hydrolysis in 2M-trifluoroacetic acid. After various periods of time samples were withdrawn and analysed by paper chromatography (Fig. 3). The radioactive components found corresponded to standards of uronosylanhydromannose disaccharide, glucuronic acid, iduronic acid and the corresponding lactones respectively. In addition a minor unidentified peak was located between iduronic acid and glucuronolactone. Quantitative determination of the various eluted components showed that maximal yields of uronic acid monosaccharides were obtained after about 4h of hydrolysis (Fig. 4).

The standard degradation procedure described under 'Methods' is based on optimum hydrolysis conditions as determined by the experiments illustrated in Figs. 2–4. When applied to a microsomal sulphated heparin preparation containing ^{14}C-labelled uronic acid and unlabelled hexosamine (Lindahl *et al.*, 1972) the degradation method yielded more than 70% of the radioactivity as free uronic acid monosaccharide.

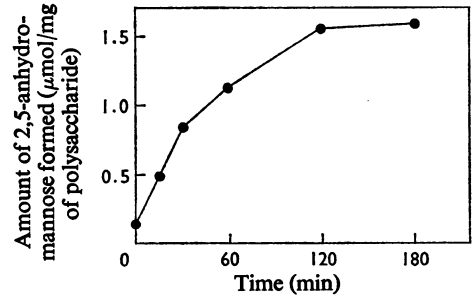

Fig. 2. *Time-course of N-deacetylation during hydrolysis of N-acetylheparin in* 2M-*trifluoroacetic acid* (0.05 *mg of polysaccharide/ml*)

After various periods of time hydrolysates were evaporated to dryness, deaminated (Dische & Borenfreund, 1950; Lagunoff & Warren, 1962) and analysed for 2,5-anhydromannose.

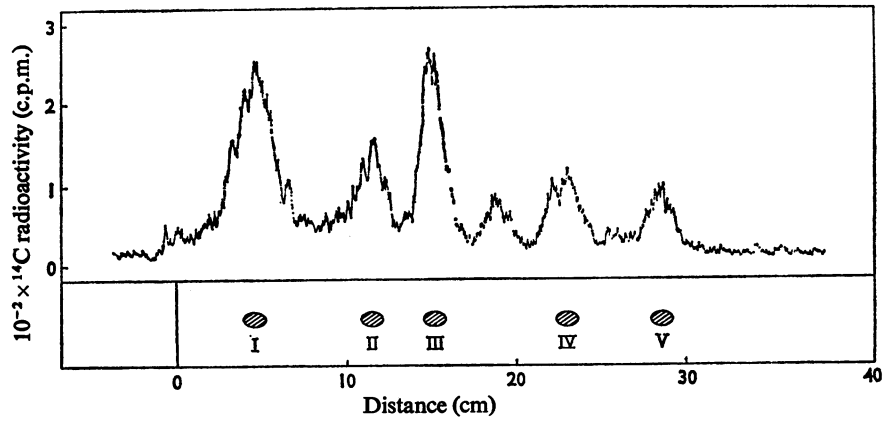

Fig. 3. *Separation by paper chromatography of labelled components obtained on degradation of* [^{14}*C*]*heparin*

Degradation was done by the procedures outlined in Fig. 1 and described in the Experimental section, except that the second hydrolysis was for 2 rather than 4h. The standards indicated below the tracing are: I, non-sulphated uronosylanhydromannose; II, D-glucuronic acid; III, L-iduronic acid; IV, D-glucuronolactone; V, L-iduronolactone. 2,5-Anhydromannose migrated off the paper.

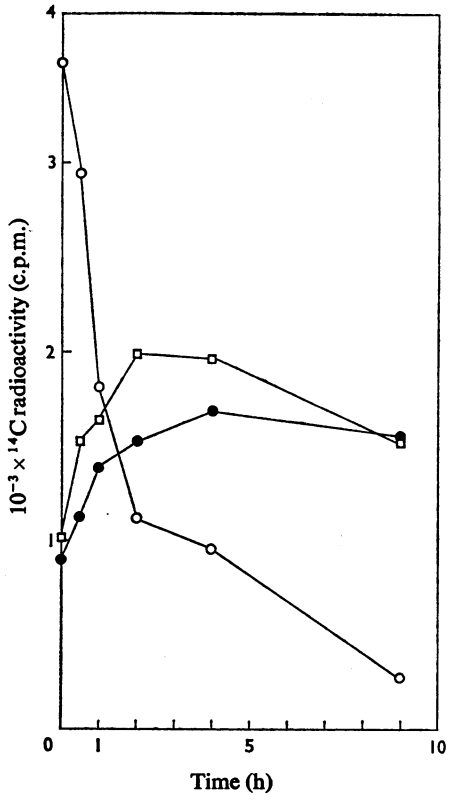

Fig. 4. *Liberation of uronic acid monosaccharides during hydrolysis in* 2M-*trifluoroacetic acid of deamination products from* [^{14}C]*heparin*

Degradation was carried out by the combined hydrolysis–deamination procedure described under 'Methods'; the time of the second hydrolysis step was varied as indicated. The hydrolysates (990c.p.m./ml) were fractionated as shown in Fig. 3 and the separated components were eluted and determined by liquid-scintillation counting. ○, Uronosylanhydromannose disaccharides; ●, D-glucuronic acid (free acid and lactone combined); □, L-iduronic acid (free acid and lactone combined).

The separation by g.l.c. of uronic acid derivatives from heparin and heparan sulphate is shown in Fig. 5. The relative retention times of the two major peaks were identical with those of L-gulono-1,4-lactone and L-idono-1,4-lactone (Perry & Hulyalkar, 1965). Ratios of glucuronic acid/iduronic acid were calculated by use of a calibration curve obtained with standards of the two uronic acids; the plot of idonolactone peak area/(idonolactone+gulonolactone) peak areas versus iduronic acid/(iduronic acid+glucuronic acid) showed excellent linearity. All samples analysed were subjected to the degradation procedure in duplicate; the results of such analyses invariably differed by less than 5%.

Characterization of deamination products

Analysis of the intact polysaccharide preparations demonstrated large differences with regard to uronic acid composition, iduronic acid constituting 77% of the total uronic acid in heparin but only 19% in heparan sulphate (Table 2). The distribution of sulphate and iduronic acid residues in the two polymers was examined by analysis of fragments isolated after deaminative cleavage with HNO_2. In the deamination procedure used glucosamine residues with sulphated amino groups are selectively attacked, with cleavage of the glucosaminidic bond (Cifonelli, 1968). The presence of *N*-acetylglucosamine units thus results in the formation of oligosaccharides, with 2,5-anhydromannose residues in the reducing-terminal position; the size of such fragments varies with the number of consecutive *N*-acetylated disaccharide repeating units.

Gel chromatograms of the heparin and heparan sulphate deamination products respectively are shown in Fig. 6. The largest, and most retarded, uronic acid-containing peak of the heparin products had the same elution position as the uronosylanhydromannose disaccharide standards. Assuming that two consecutive peaks differ by a disaccharide unit, the less retarded peaks would represent tetra-, hexa- and octa-saccharides respectively, as indicated in Fig. 6; these structural assignments are in fair agreement with the corresponding hexosamine/uronic acid molar ratios (Table 2). The heparan sulphate fragments showed a reverse elution pattern with a large proportion of excluded material and a small disaccharide peak. These findings agree with the concept that the *N*-acetylglucosamine-containing sections of heparan sulphate are generally more extended than those of heparin (Cifonelli & King, 1972).

The various oligosaccharide fractions obtained on deamination of heparin and heparan sulphate differed considerably with regard to ester sulphate contents (Table 2). The uronic composition reflected the degree of sulphation, the amounts of iduronic acid increasing with the sulphate contents. This relationship apparently applied also to the components within a single size class of oligosaccharides, as demonstrated by ion-exchange chromatography of the heparin tetrasaccharide fraction. The resulting elution pattern indicated pronounced charge heterogeneity, which was verified by direct sulphate analysis (Fig. 7). The variation in sulphate contents was closely matched by the uronic acid composition, subfractions of higher sulphate content showing larger amounts of iduronic acid (Fig. 7).

The sulphate contents of the disaccharide fractions

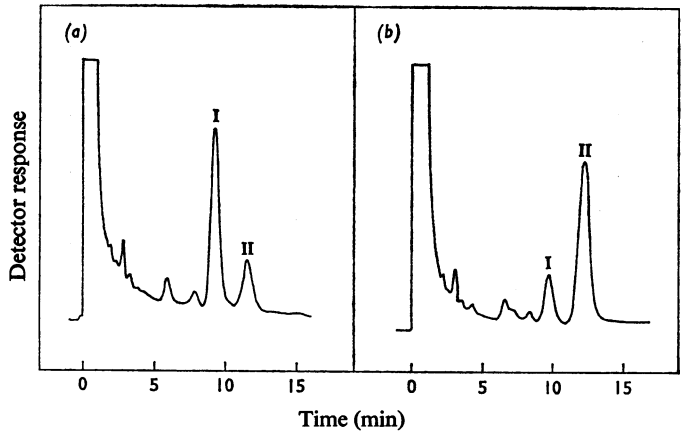

Fig. 5. *Gas–liquid chromatography of uronic acid derivatives from (a) heparin and (b) heparan sulphate*

I, L-idono-1,4-lactone; II, L-gulono-1,4-lactone.

Table 2. *Analysis of heparin and heparan sulphate and of their deamination products*

For details see the text.

Preparation	Yield (mg of uronic acid)	Hexosamine/uronic acid (molar ratio)	Sulphate/uronic acid (molar ratio)	Iduronic acid/total uronic acid (%)
Heparin	—	0.80	2.40	77
Disaccharide	46.1	0.06	—*	86†
Tetrasaccharide	31.8	0.47	0.92	60
Hexasaccharide	18.1	0.64	0.96	62
Octasaccharide	16.2	0.85	1.14	68
Heparan sulphate	—	0.88	0.46	19
Disaccharide	8.6	0.01	—*	77†
Tetrasaccharide	26.4	0.36	0.23	20
Hexasaccharide	26.1	0.69	0.17	13
Octasaccharide	63.7	0.75	<0.05	6

* Not determined owing to contamination of the disaccharide fractions with inorganic sulphate released from sulphamino groups during deamination.

† Determined after hydrolysis with 2M-trifluoroacetic acid at 100°C for 4h.

could not be directly measured owing to contamination with the inorganic sulphate released from sulphamino groups during deamination. Instead, these fractions were characterized further by ion-exchange chromatography on ECTEOLA-cellulose, as shown in Fig. 8. The heparin disaccharide fraction was thus separated into three distinct peaks, corresponding, in the order of elution, to free uronic acid, monosulphated uronosylanhydromannose and disulphated uronosylanhydromannose respectively. The identities of the three subfractions were confirmed by paper electrophoresis at pH5.3 (Fig. 9*a*) and at pH1.7 (Fig. 9*b*), against the appropriate standards. The heparan sulphate disaccharide fraction differed from that of heparin in lacking disulphated uronosylanhydromannose (Fig. 8*b*); however, the disulphated disaccharide might have escaped detection by ending up in the 'tetrasaccharide' fraction during gel chromatography, as the latter fraction included products of smaller molecular size than the corresponding heparin fraction (Fig. 6). Conversely, a component (HS 1) which appeared early in the ion-exchange chromatogram (Fig. 8) was unique to the heparan sulphate deamination products. This component was non-sulphated, as indicated by electrophoresis at pH1.7 (Fig. 9*b*), and migrated like the disaccharide, glucuronosylanhydromannose, at pH5.3 (Fig. 9*a*).

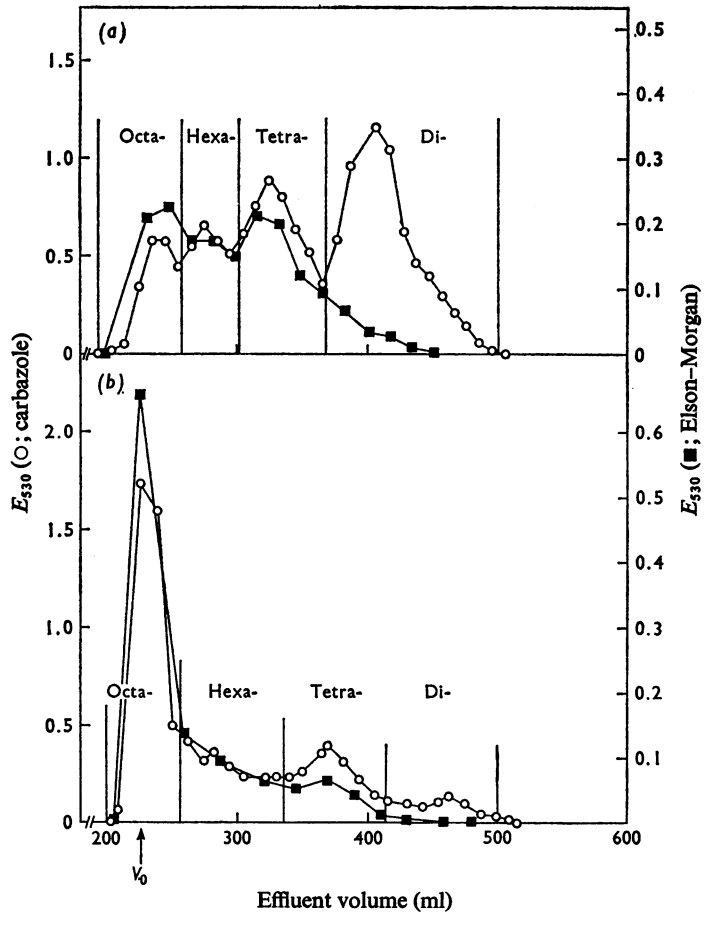

Fig. 6. *Gel chromatography on Sephadex G-25 of products obtained on deamination of (a) heparin and (b) heparan sulphate*

Effluent fractions were analysed for uronic acid (○) and hexosamine (■), and were then combined as indicated by the vertical lines. The pooled fractions are referred to in the text as indicated in the figures. The 'octasaccharide' fractions may contain larger components, emerging at the excluded volume (V_0) of the column.

The uronic acid composition of the subfractions obtained by ion-exchange chromatography is given in Fig. 8. Analysis of the uronosylanhydromannose disaccharides from heparin conformed to previous findings (Lindahl & Axelsson, 1971), as the disulphated species (H3) contained iduronic acid as the only significant uronic acid component, whereas the monosulphated disaccharide (H2) showed both uronic acids. The uronic acid monosaccharide (H1) appeared to be almost exclusively glucuronic acid. It is notable that the monosulphated uronosylanhydromannose (HS2) from heparan sulphate contained more iduronic acid than the corresponding heparin component. The non-sulphated heparan sulphate disaccharide fraction [HS1, completely free of monosulphated disaccharide (HS2), as indicated by paper electrophoresis (Fig. 9)] showed equal amounts of the two uronic acids.

Discussion

Heparin and heparan sulphate have a number of structural features in common, including the presence of two types of uronic acid (D-glucuronic acid and L-iduronic acid) and the occurrence of *N*-acetylated along with *N*-sulphated glucosamine residues. Recent studies on the X-ray-crystallographic properties of heparan sulphate suggest that the similarity with heparin may be extended to include the occurrence of β-glucuronidic linkages (Atkins & Laurent, 1973). In view of the structural

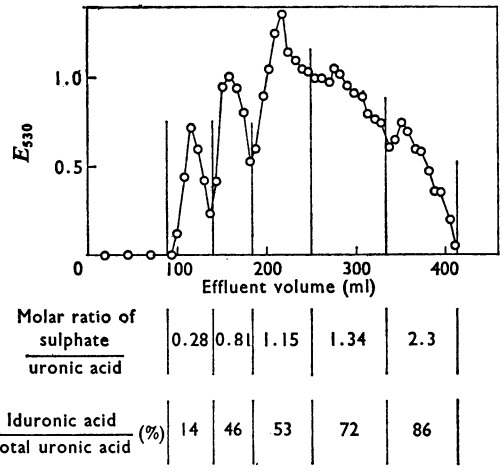

Fig. 7. *Ion-exchange chromatography on ECTEOLA-cellulose of the tetrasaccharide fraction from the deamination products of heparin (see Fig. 6)*

Gradient elution with NH_4HCO_3 was carried out as described under 'Methods'. Effluent fractions were analysed for uronic acid and were then combined, as indicated by the vertical lines. The uronic acid composition and sulphate contents of the pooled fractions are given below the chromatogram.

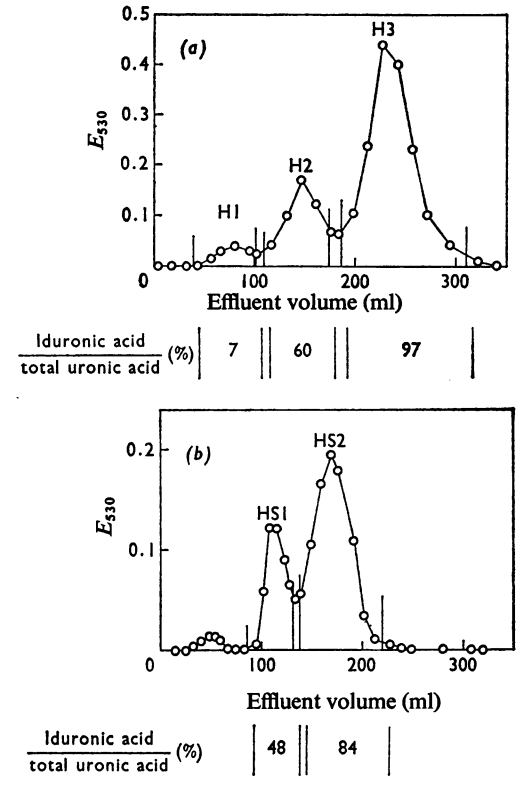

Fig. 8. *Ion-exchange chromatography on ECTEOLA-cellulose of the disaccharide fractions from the deamination products of (a) heparin and (b) heparan sulphate (see Fig. 6)*

Gradient elution with NH_4HCO_3 was carried out as described under 'Methods'. Effluent fractions were analysed for uronic acid and were then combined, as indicated by the vertical lines. The uronic acid composition of the pooled fractions is given below the chromatograms. The slight retardation of fraction HS2 in relation to fraction H2 cannot be evaluated, as different batches of ECTEOLA-cellulose powder were used in the two separations.

similarities it might be expected that the two polysaccharides are synthesized in an analogous fashion. The results of the present structural study point to a common mechanism for the formation of L-iduronic acid residues.

Previous studies on the biosynthesis of heparin have shown that iduronic acid residues are formed by C-5 epimerization, on the polymer level, of glucuronic acid units. The epimerization process requires concomitant sulphation of the polymer (Lindahl *et al.*, 1972). The results of the present investigation clearly conform to this scheme, as the iduronic acid contents of two preparations of heparin and heparan sulphate, respectively, were found to reflect the degree of sulphation of the polymers. Similar results, based on a larger number of polysaccharide preparations of widely different sulphate contents, have been obtained recently by Dr. H. E. Conrad, University of Illinois (personal communication). Further, the correlation between sulphate and iduronic acid contents shown by the two parent polysaccharide preparations was maintained through the entire range of deamination products [except the disaccharide HS1 (Fig. 8*b*) from heparan sulphate; see below], thus suggesting that sulphate and iduronic acid residues are concentrated in the same sections within the polysaccharide chain.

As sulphamino groups are eliminated during the deamination reaction, the observed correlation between sulphate and iduronic acid contents in the deamination products of heparin and heparan sulphate involves ester sulphate substituents only. The formation of iduronic acid thus appears to be linked to the sulphation of hydroxyl groups. The location of these hydroxyl groups in relation to the target uronic acid residues has not been established; it may be noted that sulphation of

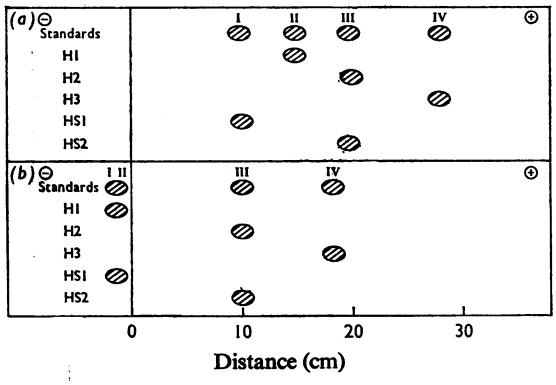

Fig. 9. *High-voltage paper electrophoresis at (a) pH 5.3 or (b) pH 1.7 of deamination products of low molecular weight from heparin and heparan sulphate*

The designation of the various samples is given in Fig. 8. Standards: I, non-sulphated uronosylanhydromannose; II, D-glucuronic acid; III, monosulphated uronosylanhydromannose; IV, disulphated uronosylanhydromannose. A minor portion of the samples applied was adsorbed to the paper at the spot of application (not indicated in the Figure).

the uronic acid units is apparently not a prerequisite for their epimerization, as the non-sulphated uronosylanhydromannose disaccharide (HS1 in Fig. 8*b*) from heparan sulphate was found to contain appreciable amounts of iduronic acid.

The significance of *N*-sulphation in this context is less clear. The results available do not exclude the possibility that formation of sulphamino groups may promote epimerization of uronic acids. However, the isolation of tetra- and hexa-saccharide fractions with molar ratios of iduronic acid/total uronic acid exceeding 1:2 and 1:3 respectively (see Table 2 and Fig. 7) shows that iduronic acid may occupy more than one position in these fragments. The occurrence, thus demonstrated, of iduronic acid residues linked both at C-1 and at C-4 to *N*-acetylglucosamine units suggests that C-5 epimerization of a uronic acid does not require *N*-sulphation of the adjacent amino sugars. Conversely, *N*-sulphation of two consecutive glucosamine residues does not necessitate epimerization of the interjacent uronic acid, as shown by the glucuronic acid-containing disaccharide fractions HS1, HS2 and H2 (Fig. 8). These conclusions conform to recent results obtained with ^{14}C-labelled microsomal heparin from mouse mastocytoma (Lindahl *et al.*, 1973*b*; M. Höök, U. Lindahl, G. Bäckström, A. Malmström & L.-Å. Fransson, unpublished work).

The nature of the relationship between sulphation and uronic acid epimerization remains obscure, with regard to both the mechanism and the sites of sulphation involved in the process. It is obvious that the biosynthesis of iduronic acid requires further study.

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References

Atkins, E. D. T. & Laurent, T. C. (1973) *Biochem. J.* **133**, 605–606
Balasubramanian, A. S., Spolter, L., Rice, L. I., Sharon, J. B. & Marx, W. (1967) *Anal. Biochem.* **21**, 22–23
Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
Blake, J. D. & Richards, G. N. (1968) *Carbohyd. Res.* **8**, 257–281
Cifonelli, J. A. (1966) *Carbohyd. Res.* **2**, 150–161
Cifonelli, J. A. (1968) *Carbohyd. Res.* **8**, 233–242
Cifonelli, J. A. & Dorfman, A. (1962) *Biochem. Biophys. Res. Commun.* **7**, 41–45
Cifonelli, J. A. & King, J. (1972) *Carbohyd. Res.* **21**, 173–186
Dische, Z. & Borenfreund, E. (1950) *J. Biol. Chem.* **184**, 517–522
Gardell, S. (1953) *Acta Chem. Scand.* **7**, 207–215
Helting, T. & Lindahl, U. (1971) *J. Biol. Chem.* **246**, 5442–5447
Helting, T., Ögren, S., Lindahl, U., Pertoft, H. & Laurent, T. C. (1972) *Biochem. J.* **126**, 587–592
Herbai, G. & Lindahl, U. (1970) *Acta Physiol. Scand.* **80**, 502–509
Iverius, P.-H. (1971) *Biochem. J.* **124**, 677–683
Kimmel, J. R. & Smith, E. L. (1954) *J. Biol. Chem.* **207**, 515–531
Knecht, J., Cifonelli, J. A. & Dorfman, A. (1967) *J. Biol. Chem.* **242**, 4652–4661
Lagunoff, D. & Warren, G. (1962) *Arch. Biochem. Biophys.* **99**, 396–400
Lindahl, U. (1966) *Biochim. Biophys. Acta* **130**, 368–382
Lindahl, U. (1970) *Biochem. J.* **116**, 27–34
Lindahl, U. & Axelsson, O. (1971) *J. Biol. Chem.* **246**, 74–82
Lindahl, U., Cifonelli, J. A., Lindahl, B. & Rodén, L. (1965) *J. Biol. Chem.* **240**, 2817–2820
Lindahl, U., Bäckström, G., Malmström, A. & Fransson, L.-Å. (1972) *Biochem. Biophys. Res. Commun.* **46**, 985–991
Lindahl, U., Bäckström, G., Jansson, L. & Hallén, A. (1973*a*) *J. Biol. Chem.* in the press
Lindahl, U., Höök, M., Malmström, A. & Fransson, L.-Å. (1973*b*) in *The Biology of Fibroblasts* (Kulonen, E., ed.), Academic Press, London, in the press

Lohmander, S. (1972) *Biochim. Biophys. Acta* **264**, 411–417
Perlin, A. S., Casu, B., Sanderson, G. R. & Johnson, L. F. (1970) *Can. J. Chem.* **48**, 2260–2268
Perry, M. B. & Hulyalkar, R. K. (1965) *Can. J. Chem.* **43**, 573–584
Shively, J. E. & Conrad, H. E. (1970) *Biochemistry* **9**, 33–43
Smith, I. (1960) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), vol. 1, pp. 246–260, Interscience Publishers, New York
Taylor, R. L. & Conrad, H. E. (1972) *Biochemistry* **11**, 1383–1388
Terho, T. & Hartiala, K. (1971) *Anal. Biochem.* **41**, 471–476
Wessler, E. (1968) *Anal. Biochem.* **26**, 439–444
Wessler, E. (1971) *Anal. Biochem.* **41**, 67–69
Wolfrom, M. L., Vercelotti, J. R. & Horton, D. (1964) *J. Org. Chem.* **29**, 540–547
Wolfrom, M. L., Honda, S. & Wang, P. Y. (1969) *Carbohyd. Res.* **10**, 259–265