Novel Heparin Degradation Products

ISOLATION AND CHARACTERIZATION OF NOVEL DISACCHARIDES AND OLIGOSACCHARIDES PRODUCED FROM HEPARIN BY BACTERIAL DEGRADATION

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1. Heparin was degraded by enzymes of adapted *Flavobacterium heparinum*. Several degradation products were separated by combined Sephadex-gel filtration and paper chromatography, and chemically analysed. 2. These products were identified as glucosamine 2,6-disulphate, saturated disaccharides constituted of uronic acid and glucosamine and containing two and three sulphate residues, and tetra- and hexa-saccharides with the same basic disaccharide units. 3. The implications of these findings with respect to the present knowledge of heparin structure and its enzymic degradation are discussed.

Degradation of heparin by induced enzymes isolated from *Flavobacterium heparinum* was first described by Korn & Payza (1956). Some products of this reaction were subsequently characterized as monosaccharides, with two of these tentatively identified as glucosamine *N*-sulphate and glucosamine *N*-sulphate *O*-sulphate (Linker & Sampson, 1960). Unsaturated oligosaccharides were also obtained when purified enzyme preparations were used, but accumulation of detectable intermediate products was not observed with crude enzyme systems (Linker & Hovingh, 1965); this lack of intermediates can be interpreted as a characteristic of the enzymic systems, as the authors suggested, or as being due to an incapacity of the analytical systems employed to detect the presence of oligosaccharides.

In the present paper, use was made of a solvent system that proved to have a high degree of resolution for sulphated disaccharides and oligosaccharides. Under these conditions several sulphated saturated di- and oligo-saccharides were detected as products of the degradation of heparin by crude enzymes of *Flavobacterium heparinum*. Data are presented on the structure and properties of these disaccharides and oligosaccharides as well as those of the disulphated glucosamine obtained. It is concluded that positions 2 and 6 are the positions for the *N*-sulphate and *O*-sulphate respectively in the disulphated glucosamine molecule.

MATERIALS AND METHODS

Heparin. Preparations of heparin kindly supplied by Lederle Laboratories, Pearl River, N.Y., U.S.A., The

Upjohn Company, Kalamazoo, Mich., U.S.A., and Abbott Laboratories, North Chicago, Ill., U.S.A., were used. Their anticoagulant activity (U.S.P. assay) and chemical composition were as follows: Lederle, 160 i.u./mg. (Found: C, 21.6; H, 4.7; N, 4.5; S, 9.6; Na, 10.2; sulphated ash 32.5%); Abbott, 168 i.u./mg. (Found: C, 19.4; H, 3.7; N, 1.9; S, 10.6; Na, 12.2; sulphated ash, 29.2%); Upjohn, 167 i.u./mg. (Found: C, 20.4; H, 4.1; N, 2.2; S, 11.9; Na, 12.3; sulphated ash, 34.7%). The high value obtained for nitrogen in the Lederle heparin probably represented a mixed sodium-ammonium salt.

Preparation of enzymes. *Flavobacterium heparinum* (kindly supplied by Dr A. Linker) was grown in Trypticase Soy Broth Without Dextrose (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.), containing 200 mg. of heparin/l. After growth for 24 hr. at room temperature the cells were harvested at 10000g for 15 min., washed with 0.02 M-potassium phosphate buffer, pH 7.0, and freeze-dried. In one group of experiments the freeze-dried cells were resuspended in small amounts of phosphate buffer and treated ultrasonically for 5 min. in a Bronwill Sonicator at 3°. The cell debris was removed by centrifuging and the supernatant freeze-dried, but some enzyme activity was lost by this procedure. By using the freeze-dried cells without ultrasonic treatment a higher yield of products was obtained when compared with the activity of a mixture of supernatant and cell debris. The freeze-dried cells were then used to prepare large amounts of degradation products.

Preparation of degradation products. For the isolation of mono- and oligo-saccharides, 1 g. of heparin (Lederle Laboratories) was incubated with 250 mg. of freeze-dried cells in 1 mM-MgCl₂ in 0.02 M-potassium phosphate buffer, pH 7.0, in a total volume of 100 ml. After 15 hr. incubation at 25° the cells were separated by centrifugation at 10000g for 10 min. and the supernatant was shaken with 100 ml. of 80% (w/v) phenol for 30 min. After centrifugation at 3000g for 30 min. the water phase was separated and extracted with three 200 ml. portions of ether to remove the dissolved phenol. The ether was discarded and the water

phase freeze-dried. The yield was 0.72 g. The whole product was dissolved in 5 ml. of 0.01 M-acetic acid and applied to a column of Sephadex G-25 (coarse grade).

Periodate oxidation. A microscale modification (Suzuki & Strominger, 1960) of the technique of Jeanloz & Forchielli (1951) for glucosamine and its derivatives was used.

Paper chromatography and electrophoresis. Solvent *A* (isobutyric acid-aq. m-NH_3 soln., 5:3, v/v) and solvent *B* (butanol-acetic acid-water, 3:1:1, by vol.) were used in descending chromatography on Whatman no. 1 paper sheets (46 cm. $\times$ 57 cm.) and in ascending thin-layer chromatography on Eastman Chromagram sheets (Distillation Products Industries, Rochester, N.Y., U.S.A.). For large-scale preparations, Whatman no. 3MM filter paper was used. After the chromatograms had been dried at room temperature guide strips were cut and the fractions located with AgNO_3 reagent. Toluidine Blue dye was also used to locate oligosaccharides that reacted poorly with AgNO_3 reagent. The following procedure was used: the dried thin-layer chromatograms and chromatography papers were immersed in 0.1% solution of Toluidine Blue in aq. 95% (v/v) ethanol for 5–10 min. The blue background was then removed by immersing the sheets in 95% (v/v) ethanol containing 0.1% of acetic acid for 10 min. with two changes of solution. The spots appeared dark blue to purple in colour against a pale-blue background. Paper electrophoresis was carried out on Whatman no. 3MM filter paper in 0.3 M-pyridine-acetate buffer, pH 4.5, at a potential gradient of about 10 v/cm. for 3 hr. Compounds were detected by the above-mentioned methods.

Infrared spectroscopy. Spectra were obtained with KBr pellets in a Perkin-Elmer model 237B instrument with a NaCl prism. About 1 mg. of sample was ground with 100 mg. of KBr.

Borohydride reduction. To identify the reducing end of disaccharides, 0.2 μmole of the disaccharides was incubated

with 5 μmoles of NaBH_4 for 18 hr. at room temperature. After incubation, uronic acid and glucosamine were determined by the methods described below.

Other analytical procedures. Amino sugars were measured after acid hydrolysis by a modified Elson-Morgan reaction (Glick, 1958). For acid hydrolysis the compounds were heated at 100° in 4 N-HCl for 4 hr. in sealed tubes. The HCl was then removed by evaporation over NaOH and H_2SO_4 . Under these hydrolytic conditions 90% of added glucosamine could be recovered. A correction was applied to the amino sugar content of the compounds.

Reducing value was measured by the Somogyi-Nelson procedure (Nelson, 1944). Sulphate was measured after hydrolysis in 8 N-HCl for 6 hr. at 100° by a micromodification of the benzidine procedure (Dodgson & Spencer, 1953). Uronic acid was measured by the Dische carbazole reaction (Dische, 1947). Metachromatic activity was measured by the method of Jaques & Wollin (1967). Anticoagulant activity was measured by a modified Howell assay (Jaques & Bell, 1959).

RESULTS

Plate 1 shows the degradation products of three commercial heparin preparations obtained by digestion with ultrasonically treated enzymes from heparin-adapted *Flavobacterium*. No differences were found among the three heparins. The degradation products had the same chromatographic mobilities in solvents *A* and *B* as well as on electrophoresis. Essentially the same results were obtained with freeze-dried cells. Freeze-dried non-adapted cells (grown in glucose medium) were unable to degrade heparin in the conditions used. At least five different compounds could be detected on

Fig. 1. Fractionation of the degradation products of heparin in Sephadex G-25. A 0.75 g. sample of degradation products dissolved in 5 ml. of 0.01 M-acetic acid was applied to a column (120 cm. $\times$ 2 cm.) of Sephadex G-25 (coarse grade). The compounds were eluted from the column with 0.01 M-acetic acid at a flow rate of 0.5 ml./min.; fractions (6 ml.) were collected. Uronic acid (▲) and reducing-sugar (●) determinations were performed with samples (20 $\mu\text{l.}$) from each fraction. A 50 mg. sample of heparin was run through the same column in a separate experiment as a control and 100 $\mu\text{l.}$ was taken for determinations of uronic acid (△). Samples of the fractions were also run in thin-layer chromatography and located with AgNO_3 reagent or Toluidine Blue or both. The R_F of the spots obtained in each fraction is schematically represented (■).

EXPLANATION OF PLATE 1

Enzymic degradation of three commercial heparin preparations. The incubation mixture was: 50 μ g. of ultra-sonically treated enzyme and 100 μ g. of heparin in 1 mM-MgCl₂-0.02 M-phosphate buffer, pH 7.0, in a total volume of 20 μ l. The reaction was stopped by heating at 100° for 1 min. after incubation for 3 hr. or after zero time. The samples were centrifuged and the supernatants spotted in duplicate in two thin-layer sheets. After being developed for 4½ hr. in solvent A the chromatograms were dried and stained with Toluidine Blue (a) or AgNO₃ reagent (b).

EXPLANATION OF PLATE 2

Thin-layer chromatography of the purified degradation products in solvent *A*. (a) Toluidine Blue stain; (b) AgNO_3 reagent stain. M, Mixture of degradation products applied. Only the lower-molecular-weight components stained with AgNO_3 reagent.

Fig. 2. Paper-chromatographic separation in solvent A of combined fractions from the Sephadex G-25 column. Group 1 (fractions 56-60), group 2 (fractions 53-55) and group 3 (fractions 44-52) were developed for 24, 48 and 72 hr. respectively.

chromatography and electrophoresis. These compounds were partially resolved in Sephadex G-25, as shown in Fig. 1. The fractions containing small-molecular-weight components that exhibited chromatographic mobility were combined into three different groups: group 1, fractions 56-60; group 2, fractions 53-55; group 3, fractions 44-52. After concentration *in vacuo* each of the three groups was applied as a band on one 40cm.-wide sheet of Whatman no. 3MM filter paper and developed with solvent A for 24 hr., 48 hr. and 72 hr. for groups 1, 2 and 3 respectively. Fig. 2 shows the compounds as they were located in the guide strips with silver nitrate reagent or Toluidine Blue or both. These compounds were eluted from the remainder of the chromatograms with water. After the eluates had been dried *in vacuo* over sodium hydroxide and sulphuric acid, further purifications were carried out by precipitating the compounds with acetone from aq. methanol solution three times. They were then dried *in vacuo* and weighed. From 0.75g. of starting material, the following yields were obtained: fraction A, 75mg.; fraction B₁, 37mg.; fraction B₂, 16mg.; fraction C, 67mg.; fraction D₁, 57mg.; fraction D₂, 59mg. Plate 2 shows the chromatographic mobilities of the purified fractions in thin-layer chromatography. The chromatographically immobile compounds (fractions 30-43; Fig. 1) were combined and samples were treated with different concentrations of enzyme. Again the same degradation products were obtained, e.g. fractions A-D₂ plus some chromatographically immobile material. When an excess of enzyme was added heparin was completely degraded to fraction A and smaller degradation products.

Chromatographic and electrophoretic mobilities. The chromatographic mobilities of the compounds decreased from fraction A to fraction D₂ (Table 1). These mobilities suggested an increasing molecular weight or an increasing number of charged groups from fraction A to fraction D₂. The electrophoretic mobilities of the compounds indicated that the first assumption was correct. Electrophoretic mobility decreases slightly with increasing molecular weight but increases markedly with increasing number of charged groups. With the exception of fraction B₁ the isolated compounds had almost the same mobility. Fraction B₁ migrated about one-third further than the rest of the compounds, suggesting one extra negative group. On the other hand, glucosamine N-sulphate had about one-half of the mobility of most of the compounds. This indicated that most of the compounds had at least two negative charges and fraction B₁ at least three negative charges. After mild acid hydrolysis (0.04N-hydrochloric acid at 100° for 2 hr. in sealed tubes) fractions A, B₁ and B₂ were degraded to an immobile compound detected with silver nitrate reagent, probably glucosamine O-sulphate. Fractions B₁ and B₂ also give a second electrophoretically mobile compound not identified. After strong acid hydrolysis (4N-hydrochloric acid at 100° for 4 hr.) only a single compound could be detected from fractions A, B₁ and B₂, with the same electrophoretic mobility as glucosamine. Similar results were obtained by chromatography in solvent A, where glucosamine was the only sugar present after acid degradation of these three fractions. Fractions C, D₁ and D₂ gave glucosamine after strong acid hydrolysis and other unidentified compounds.

These results indicated that fraction A was a compound similar to that described by Linker & Hovingh (1965), having two sulphate groups, one of which was acid-labile, and releasing glucosamine as the only sugar after strong acid hydrolysis. This compound was described as glucosamine N-sulphate O-sulphate. Fractions B₁ and B₂ seemed to be sulphated saturated disaccharides by their properties on electrophoresis and chromatography. The chemical analysis presented in Table 1 confirmed these hypotheses. Fraction A contained 2 moles of sulphate (one of which was acid-labile)/mole of glucosamine. Fraction B₂ had 2 moles of sulphate and 1 mole of uronic acid/mole of glucosamine. Fraction B₁ was essentially the same as fraction B₂ but with 3 moles of sulphate/mole of glucosamine. The determinations of reducing sugar again indicated that fraction A was a monosaccharide and that fractions B₁ and B₂ were disaccharides. The molar ratios of glucosamine to reducing sugar in these compounds was somewhat lower than 1.0. Nevertheless, the chemically synthesized glucosamine N-sulphate gave similar values. Fractions C

Table 1. Analytical data for fractions obtained by bacterial degradation of heparin and reference compounds

Chromatographic mobilities are expressed as follows: system 1, R_F of the compounds in thin-layer chromatography with solvent A [isobutyric acid–aq. 1 <i>M</i> - NH_3 (5:3, v/v)]; system 2, $R_{\text{glucuronic acid}}$ of the compounds in Whatman no. 1 filter paper with solvent A ; system 3, $R_{\text{glucuronic acid}}$ of the compounds in Whatman no. 1 filter paper with solvent B [butanol–acetic acid–water (3:1:1, by vol.).]													
	Chromatographic mobilities			Electro-phoretic mobility (cm.)	Molar proportions			Periodate oxidation (moles/mole of glucosamine)		Reducing sugar (moles/mole of glucosamine)	Borohydride reduction (% of sugars remaining after reduction)		
	System 1	System 2	System 3		Glucuronic acid	Glucosamine	Sulphate	Formaldehyde formed	Periodate reduced		Glucuronic acid	Glucosamine	
Fraction A	0.25	0.33	0.07	19.5	0.00	1.05	2.0	0.05	3.85	0.61	—	8	
Fraction B ₂	0.22	0.16	0.014	18.9	1.04	1.10	2.0	0.08	2.04	0.79	114	18	
Fraction B ₁	0.19	0.13	0.02	24.0	0.94	0.97	3.0	0.00	0.00	0.68	110	13	
Fraction C	0.10	0.02	0.00	19.5	1.18	1.01	3.0	—	—	0.22	105	65	
Fraction D ₁	0.05	0.01	0.00	18.0	1.25	0.88	3.0	—	—	0.11	—	—	
Fraction D ₂	0.02	0.00	0.00	17.4	1.31	0.92	3.0	—	—	—	—	—	
Heparin	0.00	0.00	0.00	16.5	1.77	1.11	3.0	—	—	—	—	—	
Glucosamine	0.56	> 2.00	1.08	—	—	1.00	0.0	1.03	4.75	1.00	—	0	
Glucuronic acid	0.37	1.00	1.00	10.5	1.00	0.00	0.0	—	5.00	1.05	2	—	
Glucosamine	0.35	0.76	0.23	10.5	—	1.02	1.0	1.08	5.04	0.80	—	—	
<i>N</i> -sulphate													

Chromatographic mobilities are expressed as follows: system 1, R_F of the compounds in thin-layer chromatography with solvent A [isobutyric acid-aq. 1 M-NH₃ (5:3, v/v)]; system 2, $R_{glucuronic}$ acid of the compounds in Whatman no. 1 filter paper with solvent A; system 3, $R_{glucuronic}$ acid of the compounds in Whatman no. 1 filter paper with solvent B [butanol-acetic acid-water (3:1:1, by vol.)].

Fig. 3. Rate of reduction of periodate during periodate oxidation of glucosamine (▲), glucosamine N-sulphate (Δ), fraction A (×), fraction B₁ (○) and fraction B₂ (●).

and D gave decreasing values for reducing sugar/mole of glucosamine, suggesting higher-molecular-weight compounds, probably tetra- and hexa-saccharides, with the same basic structure of the disaccharide.

One feature of these analyses was that, though with heparin the value for glucuronic acid by the carbazole reaction was much higher than the glucosamine value (as is usually found), the values found with di- and oligo-saccharides gave 1:1 ratios for glucuronic acid and glucosamine and the values with the D fractions gave ratios between these. At present no explanation for these results is available.

Periodate oxidation. Fig. 3 and Table 1 show the results of periodate oxidation of fractions A, B₁ and B₂ and glucosamine and glucosamine N-sulphate. Both standards (glucosamine and glucosamine N-sulphate) reduced 5 moles of periodate/mole of sugar with the formation of 1 mole of formaldehyde. Fraction A reduced 4 moles of periodate with no formaldehyde formation up to 22hr. This fact strongly suggested that the second sulphate residue of fraction A was in the 6-position of the sugar, thus identifying the compound as glucosamine 2,6-disulphate.

Fractions B₁ and B₂ when further digested with ultrasonically treated enzyme from heparin-adapted cells were completely converted into glucosamine 2,6-disulphate (fraction A). This provided evidence that the glucosamine moiety of the disaccharides contained two sulphate groups. Fraction B₂, which contained only two sulphate groups, would therefore be glucuronyl-glucosamine-2,6-disulphate. Periodate oxidation provided further data in support for the proposed structure. Only 2 moles of periodate were reduced from fraction B₂ up to 22hr. of reaction.

Fraction B₁ contained three sulphate residues. It did not reduce periodate and did not release

formaldehyde. Further, it maintained the same chromatographic mobility after the reaction period,

again indicating that the compound was totally resistant to periodate oxidation. Also, fraction B₁ could be converted into glucosamine 2,6-disulphate by the enzymes of *Flavobacterium*. This result suggested that the third sulphate residue should be in the glucuronic acid moiety. The total resistance to periodate oxidation indicated that the third sulphate group must be in the 3-position of glucuronic acid.

Table 2. *Metachromatic and anticoagulant activity of the several fractions compared with heparin activity*

	Metachromatic activity (units/mg.)	Anticoagulant activity (i.u./mg.)
Fraction A	0.0	<0.1
Fraction B ₁	0.4	<0.1
Fraction B ₂	0.0	<0.1
Fraction C	8.0	<0.1
Fraction D ₁	130.0	2.5
Fraction D ₂	136.0	3.0
Heparin	114.0	160.0

Nature of the reducing end. Table 1 shows the results of uronic acid and glucosamine determinations after borohydride reduction in fractions A, B₁, B₂ and C. Only 10–20% of glucosamine was recovered after reduction of fractions B₁ and B₂, whereas the glucuronic acid value remained unchanged. These results strongly indicated that

Fig. 4. Infrared spectra of glucosamine *N*-sulphate (a), fraction A (b), fraction B₂ (c), fraction B₁ (d) and heparin (e). The spectra of fractions C and D₁ were identical with that of fraction B₁.

glucosamine was the reducing end of the disaccharides. In fraction C 65% of the glucosamine was resistant to borohydride reduction. This again indicated that glucosamine was the reducing end in fraction C and further that the compound was probably a tetrasaccharide.

Activities of the fractions. Table 2 shows the results of metachromatic and anticoagulant activities of the fractions. Only fractions D₁ and D₂ showed some anticoagulant activity, although it was very low when compared with that of heparin. On the other hand, fractions D₁ and D₂ retained full metachromatic activity, whereas fraction C produced only a minor colour change of the dye. Fractions A, B₁ and B₂ were devoid of these two properties.

Infrared spectroscopy. This is shown in Fig. 4. The sulphate absorption at 1230 cm.⁻¹ was seen in all the sulphate compounds. This signal was absent from glucosamine and glucuronic acid but present in glucosamine *N*-sulphate. New bands in the sulphated fractions A, B₁ and B₂ and heparin were shown in the region from 700 to 1000 cm.⁻¹. The bands were located at 800, 875 and 925 cm.⁻¹, and

were absent from glucuronic acid and glucosamine spectra. The infrared spectra of glucosamine *N*-sulphate showed that the bands at 800 and 925 cm.⁻¹ were absent. The band at 875 cm.⁻¹ was assigned to *N*-sulphate-substituted glucosamine and the other bands were assigned to the *O*-sulphate substitutions.

When fractions A and B₂ were compared with all the other fractions a difference of relative intensity was noticed between absorption bands at 800 and 925 cm.⁻¹. About the same intensity of absorption was noticed in fractions A and B₂ in both bands, whereas the other fractions showed a much higher intensity at 800 cm.⁻¹ than at 925 cm.⁻¹. The chemical data showed that a third sulphate residue was present in fractions B₁, C and D and heparin that was absent from fractions A and B₂. These data, when considered with the above-mentioned observation, indicated that the third sulphate residue (in the uronic acid moiety) was partially responsible for the absorption band at 800 cm.⁻¹. Nevertheless, model compounds would be of paramount importance to prove this conclusion.

Other differences in the relative intensity in

Fig. 5. Proposed structures of fractions A, B₂ and B₁. The broken lines represent the possible bonds susceptible to periodate oxidation.

absorption bands were also noticed, e.g. in the absorption at 1410 cm^{-1} , which was much decreased in the heparin spectrum when compared with the spectra of the other fractions. At present, no explanation is available for these quantitative differences.

DISCUSSION

The results presented in this paper provide strong presumptive evidence that sulphate is substituted on to the 6-position of the disulphated glucosamine, and the disaccharide fractions are glucuronyl-glucosamine 2,6-disulphate (fraction B₂) and 3-sulphatoglucuronylglucosamine 2,6-disulphate (fraction B₁) (Fig. 5).

Isolation of these disaccharides containing two and three sulphate residues confirms and extends the previous chemical data obtained for the structure of heparin (Wolfson, Vercellotti & Horton, 1962; Hoffman & Meyer, 1962). It also gives some evidence for the sulphate substitution on the 3-position of the uronic acid residue of heparin. The yields obtained for the trisulphated disaccharide units in the tetra- and hexa-saccharides and the ability of the enzymes from *Flavobacterium* to digest these compounds further to sulphated monosaccharides suggest that the trisulphated disaccharide is a fundamental unit in the heparin molecule. Nevertheless, clarification is still needed for the unexpected high ratio of uronic acid to glucosamine for heparin when compared with the ratio obtained from the small oligosaccharides.

Another observation derived from these data is the metachromatic and anticoagulant activity of the fractions when compared with heparin. Full metachromatic activity could be obtained with a hexasaccharide (fraction D₁) in contrast with the almost complete absence of activity in the tetra-saccharide (fraction C). However, anticoagulant activity was practically absent from all fractions tested. These results confirm the previous findings of Payza & Korn (1956) that the anticoagulant activity of heparin is lost faster than its metachromatic activity when heparin is degraded by *Flavobacterium* enzymes.

The enzymes involved in the formation of the oligosaccharides are not known. The isolation of an eliminase and a hydrolase from heparin-adapted *Flavobacterium* was reported by Linker & Hovingh (1965). With the eliminase preparation these authors were able to isolate unsaturated oligosaccharides from heparin, thus suggesting that the eliminase would act as an endoenzyme. A possibility then exists that the hydrolase would subsequently remove the $\Delta^{4,5}$ -uronic acid residue from the unsaturated oligosaccharides, forming saturated ones. Nevertheless, if the compounds described in

this paper had come from this hypothetical reaction only tri-, penta- and odd-numbered saccharides should be obtained (if it is assumed that heparin is constituted of disaccharide repeating units). Since only even-numbered oligosaccharides were obtained it can be concluded that either the structure of heparin is not that of a repeating disaccharide unit or the degradation by *Flavobacterium* involves other enzymes not yet identified.

Although only two alternating sugars are generally accepted as present in a molecule of heparin, there has been some evidence to the contrary. Lindahl & Rodén (1965) showed that heparin contains galactose and xylose; Cifonelli & Dorfman (1962) and Radhakrishnamurthy & Berenson (1963) identified iduronic acid in heparin, a finding that was confirmed in degradation products by Lindahl (1966). Recently in studies on the structure of heparin by nuclear magnetic resonance, Jaques, Kavanagh, Mazurek & Perlin (1966) obtained signals for three anomeric protons with the same intensity and assigned these to glucuronic acid, glucosamine and to a third sugar, possibly iduronic acid. This last sugar was subsequently isolated from heparin in high yield (Perlin, Mazurek, Jaques & Kavanagh, 1968). Studies on the mechanism of the enzymic degradation of heparin will be of paramount importance to the clarification of these apparent contradictions with respect to the structure of heparin.

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