Isolation and Characterization of Oligosaccharides Obtained from Heparin by the Action of Heparinase*

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ABSTRACT: The oligosaccharides obtained in an 85% yield by the action of purified heparinase on heparin were isolated and characterized. They all contained an α,β-unsaturated uronic acid on the nonreducing end showing that enzymatic degradation proceeds by an elimination reaction. The data also indicate that more than 30% of the glucosamine units are disulfated, with the second sulfate most likely on the 6 position. At least 30% and probably more of the uronic acid units appear to be nonsulfated. In agreement with earlier data, both glycosidic linkages of heparin are $1 \rightarrow 4$.

Heparin differs from the other known acid mucopolysaccharides (with one exception) mainly in containing α linkages, *N*-sulfate, and high total sulfate. Although the overall structure of heparin has been determined by chemical means (Wolfrom *et al.*, 1964; Danishefsky and Steiner, 1965; Wolfrom *et al.*, 1966), the position of *O*-sulfate groups and the distribution of iduronic acid remain somewhat uncertain. Some heterogeneity in composition (Jaques *et al.*, 1967; Cifonelli and King, 1970) and biological activity (Jaques *et al.*, 1967; Walton *et al.*, 1966) for heparins obtained from different sources has been demonstrated. A crude enzyme mixture obtained from induced Flavobacteria has been shown to degrade heparin to monosaccharides (Linker and Hovingh, 1965). Recently, the fractionation of the crude enzyme into a heparinase and a heparitinase has been described (Hovingh and Linker, 1970). Evidence presented showed that both enzymes appear to act as eliminases rather than hydrolases. However, some controversy exists about the mechanism of the overall breakdown of heparin by the Flavobacteria system (Dietrich, 1969). The isolation, in excellent yield, and characterization of the oligosaccharide products formed by the action of the purified heparinase, are described here. The results are pertinent to the degradative mechanism as well as to the structure of heparin. The succeeding paper (Warnick and Linker, 1972) is concerned with the further breakdown of the heparin oligosaccharides to monosaccharides.

Materials and Methods

Analysis. The following were analyzed by the procedures indicated: uronic acid colorimetrically by the carbazole procedure (Dische, 1947), reducing sugar by hypoiodite titration (Macleod and Robison, 1929) and by a ferricyanide method (Rapport *et al.*, 1951), total hexosamine and hexosamine derivatives by a modified Elson–Morgan method (Swann and Balazs, 1966), *N*-acetylhexosamine and its derivatives by a modified Morgan–Elson procedure (Reissig *et al.*, 1955), N-sulfated hexosamine and free hexosamine by the indole method (Dische and Borenfreund, 1950), free amino by the dinitrofluorobenzene method (Foster *et al.*, 1953), and sulfate by titration (Meyer and Rapport, 1950). Galactosamine and glucosamine were determined as lyxose and arabinose after ninhydrin oxidation by paper chromatography in butanol–ethanol–water (4:1:1, v/v) (Stoffyn and Jeanloz, 1954). Optical rotations were obtained with a Zeiss polarimeter with a light path of 1 dm, in water at the sodium D-line wavelength.

Chromatography. Products of enzyme digestion were chromatographed on Whatman No. 1 paper in butanol–acetic acid–water (50:15:35, v/v), system I; butanol–acetic acid–NH_4OH (2:3:1, v/v), system II; and *n*-butyric acid–0.5 N NH_4OH (5:3, v/v), system III. Compounds were located by ultraviolet absorption using a short-wavelength ultraviolet lamp or by spraying with alkali–silver nitrate reagent (Trevelyan *et al.*, 1950).

Cellulose acetate electrophoresis was performed in a Brinkman low-voltage electrophoresis apparatus on cellulose acetate at a constant current of 1 mA/cm for 2 hr in 0.2 M calcium acetate (Seno *et al.*, 1970). Compounds were located by ultraviolet absorption or by staining with alcian blue (Seno *et al.*, 1970).

Column Chromatography. Oligosaccharides from enzyme digestion were placed on columns of Sephadex G-50 superfine (190 × 3 cm) and eluted with 0.2 M NaCl in 10% ethanol (Fransson and Rodén, 1967). The flow rate was approximately 12 ml/hr and 6-ml samples were collected. An ion-exchange column (DEAE-Sephadex A-25), 33 × 2.1 cm, was used for isolation of monosaccharides and disaccharide from heparin. The column was eluted in a stepwise fashion with 100 ml of 0.1, 0.5, 1.0, 1.3, 1.5, and 2.0 M NaCl. All compounds were desalted on Sephadex G-10 (120 × 2.1 cm) and precipitated with ethanol in the presence of 2.5% sodium acetate and 0.25 N acetic acid.

Materials. Heparin was a commercial bovine liver preparation obtained from the Nutritional Biochemical Co. Analytical data are as follows: uronic acid (carbazole), 39.5%; hexosamine (indole), 27.5%; hexosamine (Elson–Morgan), 19%; sulfate, 35%; $[\alpha]_D^{21°}$ +51.5° (H_2O, 1%). Crude Flavobacteria enzyme was prepared as previously described (Linker and Hovingh, 1965). Purified heparinase (specific activity 775 units/mg of protein) was described previously (Hovingh and Linker, 1970) and was free of heparitinase, chondroitinase, sulfatase (as measured on H-I), and glucuronidase activities. α-Glucuronidase was prepared as shown in the next paper (Warnick and Linker, 1972). A β-glucuronidase preparation from chondroitin sulfate C induced Flavobacteria was in the crude form, and contained a small amount of α-glucuronidase.

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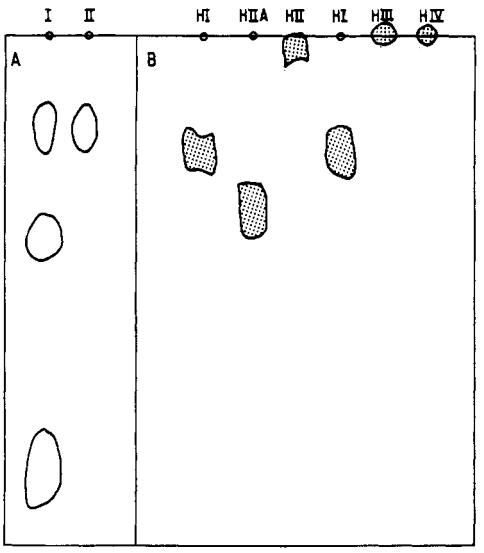

FIGURE 1: Tracing of paper chromatograms of enzyme digests. (A) Solvent system I; I, crude heparinase digest of heparin; II, isolated disulfated glucosamine. (B) Solvent system II; isolated oligosaccharides, see text for meaning of lettering. Hatching indicates ultraviolet absorption by the spots otherwise detected by the silver nitrate reagent.

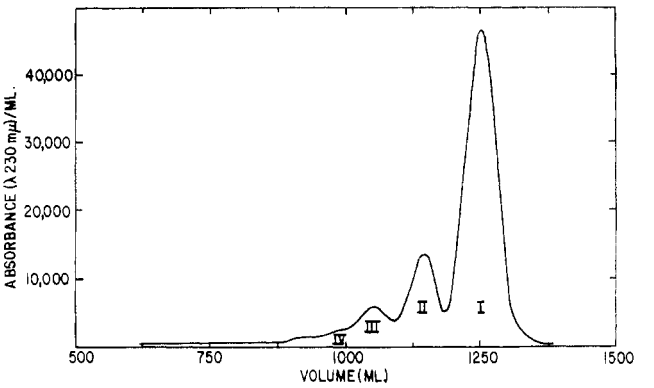

FIGURE 2: Elution pattern of purified heparinase digest from a Sephadex G-50 column. Six milliliters was collected per tube.

Chemical Methods. Sodium borohydride reduction was performed under the following conditions. Oligosaccharide (10 mg) was dissolved in 1 ml of water and 3 ml of 0.1 M sodium borate (pH 8.2). To this solution was added 25 mg of $NaBH_4$ in several steps and left at room temperature for 3 hr. The sample was refrigerated for 3 hr, neutralized to pH 5.0 with acetic acid, filtered through approximately 2.5 ml of Dowex 50 (H^+), and evaporated *in vacuo*. The sample was washed several times with methanol, taken up in 0.5 M sodium acetate, and precipitated with ethanol. This method resulted in a small loss of *N*-sulfate groups and the free amino group value increased, but less than 10%.

Hydrogenation of the α,β-unsaturated uronic acid was performed with the Brown Micro-Hydro Analyzer (Delmar Instruments). Hydrogen was generated by the addition of alkaline $NaBH_4$ to acetic acid. The catalyst was 100 mg of 10% palladium on charcoal (K & K Laboratory) in 3 ml of water. After the system was in equilibrium the sample (30 mg) was introduced in stepwise fashion. After determining the amount of H_2 that was consumed, the sample was filtered from the charcoal and precipitated with ethanol.

N-Desulfation was accomplished with 10 mg of sample dissolved in 1 ml of 0.04 M HCl and heated at 100° for 2 hr. Results were checked by paper chromatography. N-Acetylation was carried out according to a previously described method (Roseman and Ludowieg, 1954).

TABLE I: Analysis of Disulfated Hexosamine from Heparin.

Optical rotation, $[\alpha]_D^{25°}$ (deg)	+44
Hypoiodite reduction (equiv/mole)	1.4
Sulfate (%)	43
Morgan–Elson hexosamine	0
Total hexosamine[a] (%)	51
Galactosamine (%)	<5
Total hexosamine[a] after borohydride reduction (%)	1

[a] Elson–Morgan reaction (maximum at 568 mμ).

Results

Disulfated Monosaccharide from Heparin. The disulfated glucosamine obtained by crude enzyme digestion of heparin serves as a useful reference compound in oligosaccharide analysis. It was therefore isolated and characterized more fully than previously (Linker and Sampson, 1960). Heparin (500 mg) was digested in 50 ml of 0.1 M sodium acetate (pH 7.0) with 100 mg of crude Flavobacterium enzyme at 31°. After 10 hr the digest was lyophilized and extracted with 65% ethanol and centrifuged. The supernatant fluid was flash evaporated and added to a DEAE-Sephadex A-25 column and eluted with varying salt concentrations. The eluates were analyzed for reducing sugar, desalted, lyophilized, and checked by paper chromatography. The fractions containing the disulfated hexosamine were combined (73 mg) and analyzed (Table I). Paper chromatography is shown in Figure 1A. The disulfated hexosamine gave no color in the Morgan–Elson reaction (Reissig *et al.*, 1955). There was no detectable galactosamine. As can be seen (Table I), borohydride reduction resulted in loss of all the glucosamine color value and hypoiodite reduction resulted in the consumption of 1.4 equiv of iodine/mole. The data presented here and earlier (Linker and Sampson, 1960) show this compound to be N,O-disulfated glucosamine.

When the disulfated glucosamine was N-desulfated and then N-acetylated the resulting *N*-acetyl derivative (still containing *O*-sulfate) gave a Morgan–Elson color reaction of 52% based on total hexosamine content. This indicates that the *O*-sulfate group is not on the 4 position (Horton, 1969) in agreement with Wolfrom's data (Wolfrom *et al.*, 1966) which places the glycosidic linkage in this position.

Oligosaccharides from Heparin Digestion. Heparin (1 g) was digested in 50 ml of 0.1 M sodium acetate (pH 7.0) at 31° with purified heparinase (3.0 mg as protein) for 10 hr. The digest was lyophilized and extracted with 65% ethanol (50 ml). The residue was lyophilized and digested under the same conditions with heparinase (0.78 mg as protein) for an additional 5 hr. The ethanol extract was evaporated to dryness on a flash evaporator and the material redissolved in a small volume of water. This and the redigested residue were combined and placed on a 190-cm Sephadex G-50 column. Figure 2 shows

TABLE II: Analysis of Heparin Oligosaccharides.

Fraction	Yield[a] (mg)	Uronic Acid (%)[b]	Hexosamine Indole (%)	Hexosamine E + M[c] (%)	Sulfate (%)	Absorbance		H_2 Uptake (Moles/ Mole)	$[\alpha]_D$
						Per mg	Molar[d]		
H-I	300	28 (1.0)[e]	27 (1.0)[e]	24 (0.9)[e]	31 (2.1)[e]	9.1	5.1×10^3	1.0	2
H-II	215	34 (1.0)	30 (1.0)	23 (0.8)	30 (1.9)	4.5	5.4×10^3	0.9	21
H-IIA	90	28 (1.0)	26 (1.0)	27 (1.0)	24 (1.7)	5.9	5.4×10^3	0.9	20
H-III	120	34 (1.2)	27 (1.0)	23 (0.9)	29 (2.0)	3.5	5.9×10^3	0.8	41
H-IV	125	32 (1.2)	25 (1.0)	25 (1.0)	25 (1.9)	1.9			42

[a] One gram of starting material. [b] Carbazole method. [c] Modified Elson–Morgan method. [d] Assuming that H-I is a disaccharide, H-II a tetra-, and H-III hexosaccharide (all with two sulfate groups per repeating unit). A molar absorbance of 5.6×10^3–5.9×10^3 for α,β-unsaturated disaccharides has been reported previously (Linker and Hovingh, 1968). [e] Ratios to hexosamine based on the indole reaction. This reaction appears to give better values than the Elson–Morgan reaction which tends to be low for heparin.

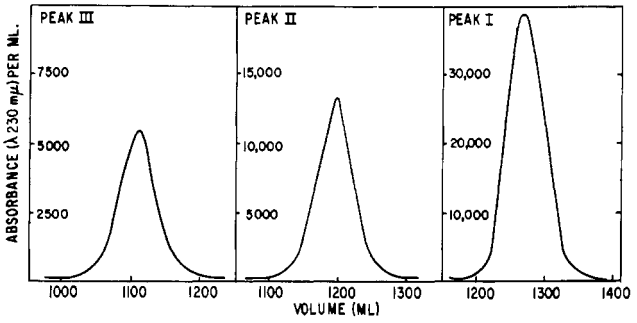

FIGURE 3: Elution pattern of reapplied peak material of Figure 2. Peaks I, II, and III have been reapplied individually to the Sephadex G-50 column and eluted as before.

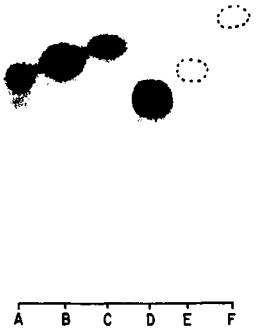

FIGURE 4: Electrophoresis of isolated oligosaccharides on cellulose acetate. (A) H-IV, (B) H-III, (C) H-II, (D) heparin standard, (E) H-IIA, and (F) H-I. The oligosaccharides were visualized by staining with alcian blue except for H-IIA and H-I which had to be detected with ultraviolet light and traced, as they were not fixed by the dye.

the elution pattern as followed by ultraviolet absorption at 230 mμ where there is strong absorption by α,β-unsaturated uronides (Linker and Hovingh, 1965). Four peaks were distinguished and three of these (I, II, and III) were isolated and reapplied to this same column. Figure 3 shows the elution pattern of the isolated fractions. As can be seen, the material was eluted in single peaks.

Cellulose acetate electrophoresis and paper chromatography showed the presence of two components in peak I. It was therefore added to a DEAE-Sephadex A-25 column. A main fraction (H-I) was eluted with 1.3 M NaCl and a minor fraction with 1.0 M NaCl (H-II A). Electrophoresis of the material in peaks II, III, and IV indicated that each contained essentially a single component. The material contained in the peaks was, therefore, desalted and isolated. Total recovery of all the oligosaccharides from Sephadex and DEAE-Sephadex was 850 mg or 85%. Table II shows the analysis of all the oligosaccharides.

Disulfated Disaccharide (H-I). The yield was 30%. Paper chromatography is shown in Figure 1B and electrophoresis in Figure 4. This compound was obtained originally from peak I of the Sephadex G-50 column and by refractionation in the 1.3 M NaCl eluate of the DEAE-Sephadex column. The molar extinction coefficient at 235 mμ (maximum absorbancy in 0.03 M HCl) was 5.1×10^3, which agrees with previous data (Linker and Hovingh, 1968). On hydrogenation this disaccharide took up 1.0 mole of hydrogen/mole of disaccharide and the product of this hydrogenation gave no ultraviolet absorbance or carbazole value (Table III). The reducing values were unusually low with Schales ferricyanide reagent (2.7%) and relatively low by hypoiodite titration of the hydrogenated disaccharide[1] (1 equiv/mole). With borohydride reduction, hexosamine was lost substantially, although not completely while no change in the carbazole reaction occurred (*i.e.*, the amino sugar is on the reducing end). When the hydrogenated disaccharide[2] was N-desulfated and N-acetylated, it gave no color with the Morgan–Elson reaction.

H-I was resistant to hydrolysis with β-glucuronidase, but was readily hydrolyzed by α-glucuronidase (Warnick and Linker, 1972). The products of this hydrolysis formed two spots on paper chromatography that had reducing properties but no ultraviolet absorption. One spot had the same mobility as disulfated glucosamine and the other the same mobility as α-keto acid previously described (Linker and Hovingh, 1965). No other spots could be detected.

Tetrasulfated Tetrasaccharide (H-II). The yield was 21.5%. Paper chromatography shown in Figure 1B and electrophoresis in Figure 4. This compound was isolated from peak II of

[1] The hypoiodite method cannot be used on the original disaccharide containing the α,β-unsaturated uronic acid, as the double bond takes up iodine under the conditions of the reaction.

[2] The disaccharide itself cannot be N desulfated without degradation as the glycosidic linkage is labile due to the presence of the unsaturation in the uronic acid moiety.

TABLE III: Analysis of Reduced Heparin Oligosaccharides.

Fraction	Uronic Acid[c] (%)	Hexosamine E + M[d] (%)	Reducing Sugar[e] μequiv/mg	Reducing Sugar[e] equiv/Mole
H-I-BO[a]	27	7		
H-I-H_2[b]	3	24	1.7	1.0
H-II-BO	37	18		
H-II-H_2	23	27	1.3	1.5
H-III-BO	31	18		
H-III-H_2	27	28	0.9	1.5

[a] Borohydride reduced. [b] Reduced with H_2 and Pd. [c] Carbazole method. [d] Modified Elson–Morgan method. [e] Hypoiodite method. Theory is 2.0 equiv/mole (oligosaccharides obtained from other mucopolysaccharides usually take up considerably more than the theoretical amount.)

the Sephadex G-50 column. The molar extinction coefficient at 235 mμ in 0.03 M HCl was 5.1×10^3. Upon hydrogenation this compound took up 0.9 mole of hydrogen/mole calculated for a tetrasaccharide, while losing about one-third of its carbazole value and all the absorbance. H-II gave better reducing value with the hypoiodite titration (1.5 equiv/mole) than the disulfated disaccharide. When H-II was reduced with sodium borohydride it lost about one-fourth the hexosamine value as determined by the Elson–Morgan method with no change in uronic acid analysis. The optical rotation was halfway between the disaccharide and that of the hexa- and larger oligosaccharide.

Low-Sulfated Tetrasaccharide (H-IIA). The yield was 9%. For paper chromatography studies, see Figure 1B. Electrophoresis is shown in Figure 4. This compound was also eluted in peak I of the Sephadex G-50 column and separated from H-I in the 1.0 M NaCl fraction of the DEAE-Sephadex column. It had a faster mobility than H-I on paper chromatography (Figure 1A) and a slower mobility than H-I or H-II on cellulose acetate electrophoresis (Figure 4). There was a small contamination of H-I present. The molar extinction coefficient at 235 mμ in 0.03 M HCl was 5.4×10^3, based on a tetrasaccharide with two sulfates. Upon hydrogenation H-II A took up 0.9 mole of hydrogen/mole of tetrasaccharide with concomitant loss of absorbancy and loss of half the carbazole value. There was essentially no free amino or *N*-acetyl, therefore, the amino groups must be sulfated and the lowered sulfate content should be due to lack of *O*-sulfate.

Hexasulfated Hexasaccharide (H-III). The yield was 12.5%. The compound did not move on paper chromatography. Electrophoresis is showin in Figure 4. This compound was isolated from peak III of the Sephadex G-50 column. The molar extinction coefficient at 235 mμ in 0.03 M HCl was 5.9×10^3. H-III took up 0.8 mole of hydrogen/mole of hexasaccharide. It gave the same reducing value on a molar basis on treatment with hypoiodite as H-II (1.5 equiv/mole). Two sulfate groups per repeating unit are indicated by analysis.

Larger Oligosaccharides. The yield was 12.5%. This fraction was obtained from peak IV of the Sephadex G-50 column. From the absorbancy this fraction consists of an average of five disaccharide units. Electrophoresis (Figure 4) shows that this fraction is heterogeneous.

Action of Enzymes upon Oligosaccharides. The oligosaccharides isolated and described above were subjected to further purified heparinase treatment. In all cases no detectable amount of disaccharide was formed as determined by paper chromatography or by an increase in ultraviolet absorption, although a heparin control run at the same time was digested at a high rate.

H-II, H-IIA, H-III, and H-IV, however, were digested readily with the crude flavobacterium enzymes; paper chromatography showed only the expected monosaccharides and no spots with ultraviolet absorption.

Discussion

Heparin is a highly sulfated polysaccharide containing alternating units of glucosamine and uronic acid. Both glycosidic linkages have been shown to be α-1 → 4 by Wolfrom and coworkers (Wolfrom *et al.*, 1964, 1966) with preparations from two major biological sources. Helbert and Marini (1963, 1964) have shown that heparin contains only two sulfates per disaccharide unit. The extra amounts reported by some laboratories could be due to inorganic sulfate contamination. Sulfate position, aside from the *N*-sulfate, is not definitely known. Some of the glucosamine units are certainly disulfated (Linker and Hovingh, 1965; Wolfrom *et al.*, 1969b) and evidence has been presented that the second sulfate group is on the six position (Wolfrom *et al.*, 1969b; Dietrich, 1968; Danishefsky *et al.*, 1969). Data indicating a sulfate group on the 2 position of some of the uronic acids has also been reported (Lindahl and Axelsson, 1971; Danishefsky *et al.*, 1969; Foster *et al.*, 1963). Considerable evidence for the presence of iduronic acid in addition to glucuronic acid has accumulated (Cifonelli and Dorfman, 1962; Wolfrom *et al.*, 1969a; Perlin and Sanderson, 1970) although the possibility of epimerization during hydrolysis or neutralization is a serious problem (Yamauchi *et al.*, 1968; Carter *et al.*, 1969). The relative amounts of the two uronic acids are still controversial.

Heparin is of considerable biological as well as pharmacological importance. Since preparations from different sources appear to vary in activity (Jaques *et al.*, 1967; Walton *et al.*, 1966) better knowledge of the detailed structure of this polymer is essential. The data presented here and in the next paper contribute to this end as well as to a better understanding of the mechanism of action of the enzyme complex of Flavobacteria. This is particularly true as a highly specific heparinase was used and oligosaccharides were obtained in excellent yield.

Analysis of the disulfated glucosamine, obtained in 50% yield, in particular the Morgan–Elson reaction, support other eivdence (Wolfrom *et al.*, 1969b; Dietrich, 1968; Danishefsky *et al.*, 1969) that the sulfate group is on the 6 position of a substantial portion of glucosamine units.

The isolation of the disulfated disaccharide (H-I) in a 30% yield allows several observations to be made. The compound has an unusually low reducing value by the standard methods probably due to the *N*-sulfate group. That it is a disaccharide, however, is shown by the ultraviolet absorption (Table I), the complete loss of this absorption and of the carbazole reaction on hydrogenation and the loss of glucosamine on borohydride reduction. The compound seems somewhat resistant to borohydride reduction under standard conditions perhaps for the same reason which accounts for the low reducing value. When the disaccharide (H-I) was N-acetylated it gave no color with the Morgan–Elson reaction. As the disulfated monosaccharide did, this agrees with other data (Wolfrom *et al.*, 1964;

Danishefsky and Steiner, 1964; Wolfrom *et al.*, 1966) that the linkage is in the 4 position of the amino sugar (Horton, 1969). When hydrolyzed by α-glucuronidase, H-I gave only two products: disulfated glucosamine and α-keto acid. As the compound contains only two sulfate groups, shown by analysis, the uronic acid must be nonsulfated. Based on the yield of H-I at least 30% of the uronic acid of heparin is therefore nonsulfated. This is in agreement with other data (Lindahl and Axelsson, 1971; Wolfrom *et al.*, 1969b; Danishefsky *et al.*, 1969). Since the asymmetry on C-5 is lost by eliminase action (Linker *et al.*, 1956), it cannot be determined whether the uronic acid in H-I may be derived from glucuronic or iduronic acid.

H-II appears to be a single compound as shown by chromatography and electrophoresis. The molar ultraviolet absorption, the hydrogen uptake and the loss of more than one-third of the carbazole value on reduction show it to be a tetrasaccharide. On borohydride reduction one-fourth of the hexosamine color value was lost with no change in the uronic acid content indicating that the hexosamine is on the reducing end. Some resistance to borohydride reduction (a 50% loss of hexosamine would be expected) is again indicated. Analysis (Table II) shows the presence of two sulfate groups per repeating disaccharide unit.

The available data do not allow us to determine whether one of the uronic acid groups is sulfated (Lindahl and Axelsson, 1971; Danishefsky *et al.*, 1969; Foster *et al.*, 1963) since the tetrasaccharide is resistant to further degradation by purified heparinase. Although the crude enzyme does digest H-II to monosaccharides and no sulfated uronic acid can be detected, the presence of sulfatases of yet unknown specificity prevents a definite interpretation of the results in this respect.

The data presented for H-II A indicate that it is also a tetrasaccharide. Analysis, paper chromatography and electrophoresis show that it contains one less sulfate group than H-II.

Compound H-III appears to be a hexasaccharide consisting of repeating units of H-I. It is quite significant that it or H-II are not degraded further in the original digest. Even the isolated compounds are resistant to further degradation by the purified heparinase, although they are digested by crude enzyme. Size specificity as in testicular hyaluronidase (Weissman, 1955) may play a role. However, the large yield of disaccharide H-I (30%) makes this unlikely. Positions of sulfate groups, particularly on the uronic acid, resolved here only for the disaccharide, or the presence of iduronic acid could be a determining factor in resistance.

The good yield of degradation products (85%) allows certain conclusions about structure to be drawn. The data presented support the presence of 1 → 4 linkages for the major repeating structure of heparin. They also indicate that a substantial portion of the glucosamine is disulfated. In view of the fact that there appear to be only two sulfates per repeating unit (Helbert and Marini, 1963), although curiously enough a large variety of sulfate values are still being reported without taking the precautions suggested by Helbert *et al.* (Helbert and Marini, 1964),[3] it follows that only a limited number of uronic acid units can be sulfated (Lindahl and Axelsson, 1971).

As far as the breakdown mechanism is concerned it is quite clear that the purified heparinase acts as an eliminase as reported previously (Linker and Hovingh, 1965; Hovingh and Linker, 1970). Oligosaccharides containing α,β-unsaturated uronic acid were obtained in an 85% yield by *actual isolation* and were characterized. No compounds containing saturated uronic acid on the nonreducing end could be detected (Dietrich, 1969). It is apparent, therefore, that elimination is the main degradative mechanism of the Flavobacteria complex. This is in agreement with the action of other mucopolysaccharidases of bacterial origin (Linker *et al.*, 1956; Yamagata *et al.*, 1968).

It is difficult to evaluate the specificity of the heparinase with regard to iduronic and glucuronic acid, since the elimination mechanism yields the same product from both. In view of the action of other mucopolysaccharidases (Linker *et al.*, 1956; Weissman, 1955; Yamagata *et al.*, 1968), it would be surprising if the enzyme were nonspecific in this respect.

[3] Ratios of sulfate to glucosamine are also misleading as hexosamine values obtained for heparin, particularly by the Elson–Morgan reaction, tend to be low.

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Purification of an Unusual α-Glycuronidase from Flavobacteria*

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ABSTRACT: An unusual α-glycuronidase was isolated from *Flavobacterium heparinum* and purified. The enzyme acts on degradation products of heparin or heparitin sulfate containing α,β-unsaturated uronic acid. It appears to be specific for α-linked unsaturated uronides derived from glucuronic or iduronic acid. Saturated uronides are not hydrolyzed. Activity is highest with disaccharides and falls off rapidly with increasing molecular weight of oligosaccharides. The glycuronidase in conjunction with eliminases present in the same organism degrades heparin and heparitin sulfate to monosaccharides.

The enzymatic degradation of mucopolysaccharides has been shown to proceed by two different pathways: (1) a hydrolytic pathway characteristic of enzymes found in animal tissues (Mathews, 1966) and (2) an elimination pathway characteristic of enzymes found in bacteria (Linker *et al.*, 1956; Nakada and Wolfe, 1961; Yamagata *et al.*, 1968). Oligosaccharides produced by hydrolysis can be further degraded by glucuronidases which are found in a variety of sources (Levvy and Conchie, 1966).

Oligosaccharides produced by elimination contain a 4,5-unsaturated uronic acid at the nonreducing end (Linker *et al.*, 1956). It has been found that the β-linked oligosaccharides produced from the chondroitin sulfates and hyaluronic acid can be further degraded by β-glucuronidases (Linker *et al.*, 1960) which appear to be specific for the unsaturated uronic acid (Yamagata *et al.*, 1968).

Adapted cultures of *Flavobacterium heparinum* produce an enzyme complex which will degrade the α-linked mucopolysaccharides, heparin and heparitin sulfate (Korn and Payza, 1956; Linker and Hovingh, 1965). These enzymes have been separated into eliminases which degrade heparin and heparitin sulfate to a series of $\Delta^{4,5}$-unsaturated oligosaccharides and an α-glycuronidase which has the unusual property of hydrolyzing the uronic acid from unsaturated α-linked oligosaccharides, the only reported enzyme with this specificity (Linker and Hovingh, 1965, 1968).

As this α-glycuronidase[1] has a specificity which is unique (namely, the hydrolysis of unsaturated α-linked uronic acids), it was thought to be worthwhile to investigate this enzyme and some of its properties further.

Materials and Methods

Paper Chromatography and Electrophoresis. Paper chromatography was carried out on Whatman No. 1 paper in a descending system with butanol–acetic acid–water (50:15:35, v/v) as solvent. Compounds were located by ultraviolet absorption using a short-wavelength ultraviolet light, or by spraying with alkaline silver nitrate to locate reducing sugars (Trevelyan *et al.*, 1950) or *o*-phenylenediamine to locate α-keto acids (Lanning and Cohen, 1951).

Electrophoresis was carried out on cellulose acetate using a Savant flat-plate apparatus, at a potential of 35 V/cm in a buffer system of pyridine–formic acid (pH 3.0) (Mathews, 1961). Reducing sugars were located by spraying with alkaline silver nitrate with the substitution of 95% ethanol for the acetone in the spray.

Chemical Methods. Sugars were reduced with sodium borohydride or hydrogenated as in the previous paper (Linker and Hovingh, 1972). Phenyl α-D-glucopyranoside was oxidized to (phenyl α-D-glucopyranosido)uronic acid by the method of

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[1] The enzyme has been called a glucuronidase previously. As it acts on 4,5-unsaturated uronides this is not strictly correct as it would also act on unsaturated uronides derived from L-iduronic acid. We are, therefore, using the term α-glycuronidase in this paper.