Nearest Neighbor Analysis of Heparin: Identification and Quantitation of the Products Formed by Selective Depolymerization Procedures†

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ABSTRACT: Heparin was carboxyl-reduced with sodium boro[^{3}H]hydride and converted to a mixture of oligosaccharides by treatment with nitrous acid at pH 2. The oligosaccharide mixture was aldehyde-reduced with sodium boro[^{3}H]hydride and the mixture of products, labeled both in the hexoses formed in the carboxyl-reduction step and in the reducing sugars formed in the nitrous acid reaction, was separated and analyzed. The major product, L-idosyl 2-sulfate → anhydro-D-mannitol 6-sulfate (I), contained 60% of hexoses derived from the hexuronic acid residues in the original heparin. A second product, which contained 15% of the hexoses derived from the hexuronic acid residues in the original heparin, was identified as a tetrasaccharide composed of two L-idosyl 2-sulfate residues, one anhydro-D-mannitol 6-sulfate residue (the reducing end), and a hydroxymethylpentose sulfate residue formed by deamination of a disulfated D-glucosamine residue without bond cleavage. Several additional disaccharides derived from the regions of the polymer which contained D-glucuronic acid residues and lower degrees of O-sulfation were also identified among the deamination products. The oligosaccharides that were obtained accounted for 100% of the original carboxyl-reduced heparin, and paper chromatographic profiles of the oligosaccharide separations can be used as a fingerprint of the heparin preparation. The properties of I were examined in greater detail. The glycosidic bond of the L-idosyl 2-sulfate residue was found to be extremely labile to 0.1 N HCl at 100 °C, hydrolyzing with a $t_{1/2}$ of 18 min to give high yields of L-idose 2-sulfate and anhydro-D-mannitol 6-sulfate. L-Idofuranose was also identified as an intermediate in the conversion of L-idose 2-sulfate to L-idosan. The acid lability of the L-idosyl 2-sulfate bond in I offers a new route for the selective cleavage of carboxyl-reduced heparin.

Heparin is unique among polysaccharides in that it has a variety of important biological activities, the most prominent of which are its anticoagulant activity and its lipemia clearing capacity. Since the earliest structural studies it has been recognized that heparin is a sulfated polysaccharide made up of a sequence of alternating uronic acid and D-glucosamine residues, but the details of the structure of this polymer have been emerging only slowly over the past 50 years. One of the primary reasons for the difficulties in establishing the fine structure of heparin and the relationship between its structure and function is that it is apparently a mixture of polymers which are in various intermediate stages of metabolic maturation and degradation (Höök et al., 1975; Ögren and Lindahl, 1975). Biosynthesis studies show that heparin is initially synthesized as a polymer of alternating β-D-glucuronosyluronic acid and 2-acetamino-2-deoxy-D-glucosyl residues in 1 → 4 linkage to each other. This polymer then undergoes a series of enzymatic modifications which include N-deacetylation followed by N-sulfation (Silbert, 1967; Lindahl et al., 1973), C5 epimerization of the D-glucuronic acid residues to convert them to L-iduronic acid residues (Höök et al., 1974), and O-sulfation of the D-glucosamine residues at C6 and the L-iduronic acid residues at C2 (Höök et al., 1975). At some advanced point in the maturation, when the polymer contains high percentages of L-iduronic acid and *N*- and *O*-sulfate groups, the product rather abruptly gains its anticoagulant activity (Taylor et al., 1973). When crude heparin is isolated from natural sources such as beef lung or hog mucosa, it is a mixture of these polymeric metabolic intermediates which then must be fractionated to obtain the highly sulfated anticoagulant fractions. The byproducts from the heparin fractionation, designated collectively as heparan sulfate, contain some polymers that are very nearly identical in composition with the heparin fraction but which lack the important anticoagulant activity (Taylor et al., 1973). The present work describes the basic groundwork for a new approach designed ultimately to define the subtle structural differences which result in the very marked differences in biological activities of compositionally similar heparin and heparan sulfate preparations.

The types of residues found in these polysaccharides may include D-glucuronic acid, L-iduronic acid, L-iduronic acid 2-sulfate, 2-acetamino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose, 2-sulfamino-2-deoxy-D-glucose, and 2-sulfamino-2-deoxy-D-glucose 6-sulfate. An analysis of the fine structures of these polymers involves an analysis of the relative amounts of each type of residue and, in addition, a determination of whether each residue is found in a unique molecular environment, i.e., whether there is for each type of residue a characteristic nearest neighbor reducing and nonreducing substituent. The present approach, applied here to a commercial heparin, offers a procedure for addressing these analytical and structural questions. The approach involves reduction of the uronic acid residues in the polymer with sodium boro[^{3}H]hydride to label the resulting hexoses (Taylor and Conrad, 1972; Taylor et al., 1973) and characterization of the products obtained by cleavage of the carboxyl-reduced heparin

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(CR-heparin[1]) by the nitrous acid elimination reaction and by acid hydrolysis. In addition to L-idosan, L-idose, and 2,5-anhydro-D-mannose, described previously (Shively and Conrad, 1976), L-idose 2-sulfate, 2,5-anhydro-D-mannitol 6-sulfate, L-idofuranose, and several oligosaccharides are identified among the CR-heparin depolymerization products, and procedures for quantitation of the relative abundance of these residues in the original polymers are described. The extreme acid lability of the glycosidic bonds of the L-idose 2-sulfate residues described here offers an important new approach to selective cleavage of CR-heparin.

Experimental Procedures

The beef lung heparin, a gift from the Upjohn Company, is the same preparation used in the accompanying study (Shively and Conrad, 1976). The heparin was carboxyl-reduced with sodium boro[^{3}H]hydride or with unlabeled borohydride by procedures described earlier (Taylor and Conrad, 1972; Taylor et al., 1973; Shively and Conrad, 1976) and the product was purified by preparative paper chromatography and paper electrophoresis (Shively and Conrad, 1976). Heparin and CR-heparin were converted to oligosaccharides by deaminative cleavage using the low pH nitrous acid procedure described in the accompanying paper (Shively and Conrad, 1976). Oligosaccharides and monosaccharides were isolated by preparative paper chromatography on Whatman No. 3 paper strips using solvent systems (see below) appropriate for the separation of the components in question. Strips were scanned for radioactivity using a Packard strip scanner, and the sections of the chromatogram containing the separated peaks were excised and eluted with water using spin thimbles (Reeve-Angel). Further purifications were achieved by preparative paper electrophoresis on Whatman No. 3 paper in pyridine–glacial acetic acid–water (1:10:400) buffer.

The quantitative radiochromatographic procedures used in these studies were described earlier (Conrad et al., 1973; Taylor et al., 1973; Shively and Conrad, 1976). For analytical measurements, paper chromatographic separations were carried out using the following systems: system I, Whatman No. 1 paper developed with ethyl acetate–glacial acetic acid–formic acid–water (18:3:1:4); system II, Whatman No. 1 paper developed with 1-butanol–glacial acetic acid–1 N ammonium hydroxide (2:3:1); system III, Whatman No. 3 paper developed with 1-butanol–glacial acetic acid–1 N ammonium hydroxide (4:6:3); system IV, DEAE-cellulose paper sprayed with 0.5 mM $Na_2B_4O_7$ and developed in ethyl acetate–pyridine–5 mM H_3BO_3 (3:2:1). Analytical electrophoretic separations were carried out on 1 × 12 in. cellulose acetate strips (Sepraphore III, Gelman Instrument Company) using pyridine–glacial acetic acid–water (1:10:400) buffer. A potential of 30 V per cm was applied for 1–2 h, depending upon the rates of migration of the materials being separated.

For characterization of purified compounds by gas chromatography–mass spectrometry, the compounds were converted to their trimethylsilyl derivatives by the method of DeJongh et al. (1969), chromatographed on a gas Chrom Q column coated with 3% OV-17, and analyzed by electron-impact mass spectrometry using a Ch-5 mass spectrometer, Model 311-A, coupled to a Varian 1700 gas chromatograph.

[1] Abbreviations used are: CR-heparin, carboxyl-reduced heparin; Me_3Si, trimethylsilyl; GC–MS, gas chromatography–mass spectrometry; HC, hot carboxyl reduced; CC, cold carboxyl reduced; HA, hot aldehyde reduced; CA, cold aldehyde reduced.

Results

Strategies. The overall approach used in these studies for identification of the products obtained from CR-heparin was to label the hexoses and anhydro-D-mannose specifically by selective reduction with sodium boro[^{3}H]hydride. Thus, when the carboxyl reduction of heparin is carried out using sodium boro[^{3}H]hydride, the uronic acid residues are converted to D-glucose and L-idose residues containing at C6 2 equiv of ^{3}H per mol. On the other hand, if carboxyl reduction is carried out with unlabeled borohydride, the CR-heparin bears no label in the hexoses. When the latter is cleaved with nitrous acid, the only reducing groups formed are those of anhydro-D-mannose, and these can be specifically labeled by reduction of the deamination mixture with sodium boro[^{3}H]hydride, thus yielding products labeled with 1 equiv of ^{3}H per mol in the residues derived from D-glucosamine but with no ^{3}H in those derived from the uronic acids.

Acid hydrolysis of CR-heparin yields both free hexoses and oligosaccharides containing hexose reducing terminals. When such hydrolysates are aldehyde reduced with boro[^{3}H]hydride, 1 equiv of ^{3}H is incorporated per mol of hexose reduced. Thus, if the resulting hexitol is derived from a heparin that was carboxyl reduced with sodium boro[^{3}H]hydride, it will contain 3 equiv of ^{3}H per mol; if it is obtained from an unlabeled CR-heparin, it will contain only 1 equiv of ^{3}H per mol. There is one important exception to this stoichiometry which is observed in the ^{3}H labeling of L-idose residues. Under the conditions used for the acid hydrolysis of CR-heparin, the freed L-idose is converted largely to L-idosan, a nonreducing anhydro derivative of L-idose. Since L-idosan cannot be aldehyde reduced, it can only be detected and quantitated in depolymerization mixtures derived from heparin that is carboxyl reduced with sodium boro[^{3}H]hydride; the L-idosan in such products contains 2 equiv of ^{3}H per mol.

It is clear from the above that by judicious choice of the depolymerization methods and the point(s) in the analytical scheme at which sodium boro[^{3}H]hydride is used, hexoses can be labeled with 1, 2, or 3 equiv of ^{3}H per mol under conditions where anhydro-D-mannitol is not labeled, or that anhydro-D-mannitol can be labeled with 1 equiv of ^{3}H per mol under conditions where hexoses or hexitols are not labeled. To calculate micromolar equivalents in mono- or oligosaccharides that have been separated on radiochromatograms, the cpm in each peak are divided by the cpm per μmol of a D-glucose standard which has been aldehyde reduced with the boro[^{3}H]hydride and which therefore contains 1 equiv of ^{3}H per mol. The resulting value is divided by 3 for those hexitols containing 3 equiv of ^{3}H per mol, and by 2 for those hexoses or hexitols containing 2 equiv of ^{3}H per mol.

In the results described below, samples derived from CR-heparin formed by carboxyl reduction with sodium boro[^{3}H]-hydride are designated HC (hot carboxyl reduced) while those derived from CR-heparin formed by reduction with unlabeled borohydride are designated CC (cold carboxyl reduced). Similarly, depolymerization mixtures that were aldehyde reduced with sodium boro[^{3}H]hydride are designated HA (hot aldehyde reduced) while those that were aldehyde reduced with unlabeled borohydride are designated CA (cold aldehyde reduced). For each sample, the nature of both the carboxyl reduction and the aldehyde reduction is designated; thus, mixtures analyzed by radiochromatography may be designated HC-HA, HC-CA, or CC-HA in accord with the abbreviations defined above. The usefulness of these three treatments in identification of ^{3}H peaks on radiochromatograms is illustrated (Figure 1) in the analysis of the products formed from

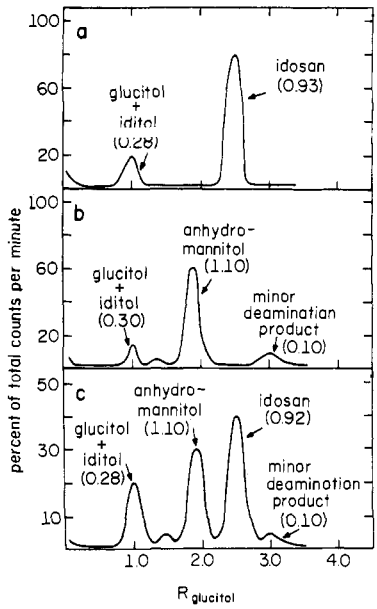

FIGURE 1: Radiochromatographic profiles of products formed when CR-heparin is hydrolyzed for 6 h in 1 N H_2SO_4 at 100 °C and then treated with nitrous acid (Shively and Conrad, 1976). From 20 000 to 50 000 ^3H cpm was applied to each chromatogram along with a D-[^{14}C]glucitol internal standard. Chromatograms were run in system I. Peaks were identified on the basis of their $R_{glucitol}$ values. Numbers in parentheses are μmol of each product per mg of CR-heparin. Panel a shows the profile for a HC-CA sample; panel b, a CC-HA sample; and panel c, a HC-HA sample.

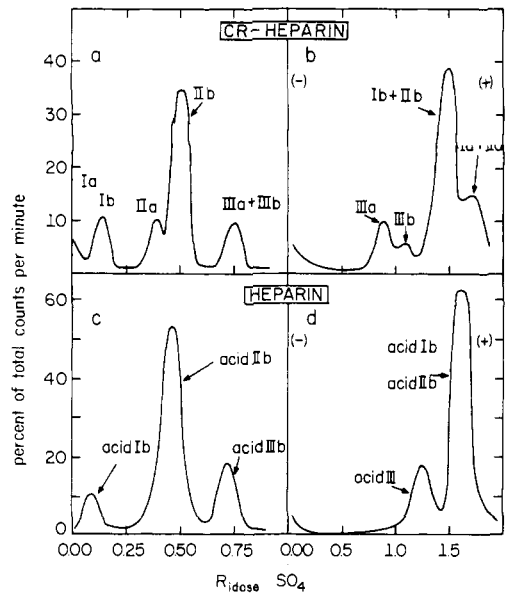

FIGURE 2: Products formed when CR-heparin (panels a and b) and heparin (panels c and d) are treated with low pH nitrous acid (Shively and Conrad, 1976). The deamination mixtures were aldehyde reduced with sodium boro[^{3}H]hydride and chromatographed in system III (panels a and c) or electrophoresed in a pyridine–acetic acid–water (1:10:400) buffer (panels b and d). Rates of migration of oligosaccharides are related to the rate of migration of L-idose sulfate (Table II).

CR-heparin after total acid hydrolysis followed by nitrous acid treatment to cleave the glycosidic bonds of the D-glucosamine residues. Panel a, a radiochromatographic profile of the products from HC-CA CR-heparin, shows a peak for L-[^{3}H]idosan and a peak for D-[^{3}H]glucitol plus L-[^{3}H]iditol (not separated). On the other hand, the radiochromatogram of the products from CC-HA CR-heparin (panel b) shows peaks for the [^{3}H]hexitols and anhydro-D-[^{3}H]mannitol but not for L-idosan, while the HC-HA CR-heparin chromatogram (panel c) shows all of the depolymerization products. The origin of each labeled peak in panel c is readily determined by a comparison of the three panels. Calculations using the cpm in each peak in panel c show that the molar ratio of hexose, i.e., [(D-glucitol cpm + L-iditol cpm) ÷ 3] + (L-idosan cpm ÷ 2), to D-glucosamine (anhydro-D-mannitol cpm + minor deamination peak cpm) is 1.0. Accurate molar ratios are obtained only when the procedures described previously (Shively and Conrad, 1976) for purification of the product formed in the carboxyl reduction reaction are followed, since several unidentified by-products formed from the excess carbodiimide are reduced by the boro[^{3}H]hydride and give artifactual peaks on the radiochromatograms.

Oligosaccharides Formed When CR-Heparin Is Treated with Nitrous Acid. Figure 2 shows the paper chromatographic and paper electrophoretic profiles of the oligosaccharide mixture obtained when unlabeled CR-heparin (panels a and b) and the original heparin (panels c and d) are cleaved by the low pH nitrous acid procedure (Shively and Conrad, 1976) and reduced with sodium boro[^{3}H]hydride. The radiochromatogram of the CR-heparin products (panel a) shows four peaks (Ib, IIa, IIb, and III) with a small amount of material at the origin (Ia). When the same mixture is separated by paper electrophoresis (panel b), the order of migration is reversed, with peaks Ia and IIa migrating most rapidly toward the anode and peaks Ib and IIb migrating together. Peak III is partially separated into two fractions. The identification of the peaks of panel a with those of panel b is based upon the migrations obtained for the individual peaks after their purification by preparative chromatography and electrophoresis.

When heparin itself is cleaved with nitrous acid and the products are analyzed in the same fashion, qualitatively and quantitatively similar profiles are obtained. The major peak, "acid IIb", migrates somewhat more slowly on the radiochromatogram (panel c) than its IIb counterpart from CR-heparin, and faster on the electrophoretogram (panel d), consistent with the presence of an additional charged group in acid IIb. When acid IIb was isolated by preparative paper chromatography and then carboxyl reduced by the carbodiimide–sodium borohydride reaction, the resulting product was identical in all respects (see below) with the IIb obtained from CR-heparin, but no IIa was formed. Similarly, "acid Ib" is reduced to Ib and "acid III" is reduced to yield IIIa and IIIb. It is concluded, therefore, that the major products formed upon nitrous acid treatment of CR-heparin are the carboxyl reduced forms of the oligosaccharides obtained by direct cleavage of heparin, and that many aspects of the structure of the original heparin polymer can be described by the characterization of the products obtained from CR-heparin. In contrast to a previous report (Lindahl and Axelsson, 1971), no monosaccharides were formed from either heparin or CR-heparin by the deamination procedure used here.

Fractions Ib, IIa, IIb, IIIa, and IIIb were prepared in chromatographically and electrophoretically pure forms and each was analyzed for its monosaccharide content after total acid hydrolysis. Table I presents the results of these analyses and also shows the percentages of the total hexoses present in the CR-heparin which are recovered as D-glucose and L-idose in each of these fractions. It can be seen that oligosaccharides Ib and IIb, which contain 75% of the total hexose, do not contain any D-glucose. The D-glucose is recovered along with the remaining L-idose in oligosaccharides IIa, IIIa, and IIIb.

TABLE I: Yield and Composition of Oligosaccharides from CR-Heparin.[a]

Oligosaccharide	% of Original Hexose in CR-Heparin	% of Each Hexose in Fraction		Hexose/AMan
		L-Idose	D-Glucose	
Ib	15	100	0	1.5
IIa	5	60	40	2.5
IIb	60	100	0	1.0
IIIa	14	60	40	1.0
IIIb	6	40	60	0.5

[a] Values given are calculated using the nanomoles of each component recovered on radiochromatograms and paper electrophoretograms of oligosaccharides formed by low pH nitrous acid cleavage of HC-HA CR-heparin. Total hexose is the sum of L-idose, L-idosan, and D-glucose. The L-idose value is the sum of L-idosan and L-idose. The D-glucose value is the nanomoles in the hexose peak after correction for the amount of L-idose in the hexose peak (see text).

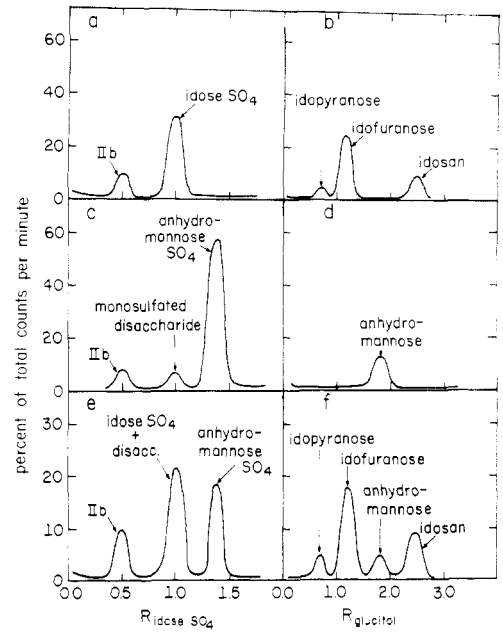

FIGURE 3: Products formed in the partial acid hydrolysis (0.1 N HCl, 100 °C, 60 min) of differently labeled preparations of oligosaccharide IIb. Aliquots containing 25 000 to 50 000 ^3H cpm were taken from hydrolysis mixtures and spotted on Whatman No. 1 paper and developed in system I for 24 h (panels b, d, and f). All but the wick-plus-origin was cut into 0.5-in. segments which were counted to locate and quantitate the unsulfated hydrolysis products. A new strip was then sewed onto the wick-plus-origin and the new strip was developed for 24 h in system II (panels a, c, and e) to separate the sulfated products, which were counted as above. Panels a and b show profiles for a HC-CA sample; panels c and d, a CC-HA sample; and panels e and f, a HC-HA sample.

Oligosaccharide IIa is a minor fraction of the original CR-heparin which contains both D-glucose and L-idose, but which is not formed upon carboxyl reduction of any of the acidic oligosaccharides generated by treatment of heparin with nitrous acid. It is clear from the hexose to anhydro-D-mannose ratios and from the finding of both L-idose and D-glucose in IIa, IIIa, and IIIb that these three preparations are not pure components. Table II summarizes the paper chromatographic and paper electrophoretic behaviors and the hydrolysis properties of each of these components. Further characterization of these fractions is described below.

Structure and Properties of Oligosaccharide IIb. The data in Table I show that total acid hydrolysis of IIb yields equimolar amounts of [L-idose + L-idosan] and anhydro-D-mannitol, but no D-glucose. Its high electrophoretic migration (Figure 2) suggests that it contains one or more sulfate groups. For further characterization, IIb was isolated in three differently labeled forms, HC-HA, HC-CA, and CC-HA, and each form was analyzed by radiochromatography after partial and complete acid hydrolysis. On total hydrolysis the CC-HA preparation yielded anhydro-D-mannitol as the only labeled peak, while the HC-CA preparation yielded L-idose and L-idosan, and the HC-HA form yielded all three labeled products. On partial acid hydrolysis of these preparations a number of previously unidentified products were obtained, as shown in Figure 3. Because the partial hydrolysis products gave such widely different rates of paper chromatographic migration, two different chromatography systems were required to obtain complete separation of all of the products. Each partial hydrolysate was run first in system I to separate the free hexoses and anhydro-D-mannitol from each other and from the sulfated products; then a new strip of Whatman No. 1 paper was sewed onto the wick-plus-origin section of the first chromatogram and this strip was developed in system II in order to separate the sulfated monosaccharides. In Figure 3 panels a and b represent the two stages of the separation of the partial hydrolysis products of HC-CA IIb, while panels c and d represent the CC-HA IIb and panels e and f the HC-HA sample. As before, the differently labeled IIb samples aid in the identification of the new peaks. Thus, since the HC-CA sample is labeled only in the L-idose residue, all of the peaks in panels a and b must be derivatives of L-idose. Similarly, all of the peaks in panels c and d must be derivatives of anhydro-D-mannitol. The HC-CA and the CC-HA chromatograms therefore aid in the identification of the peaks in panels e and f. From the latter chromatograms the relative amounts of each product can be calculated (in the present experiment the anhydro-D-mannitol derivatives contain 1 ^3H per mol while all of the L-idose derivatives contain 2 ^3H per mol). The previously unidentified peaks that appear on these chromatograms are characterized (see below) as L-idose 2-sulfate, anhydro-D-mannitol 6-sulfate, and L-idofuranose. On paper electrophoresis the former two compounds migrated rapidly toward the anode but the latter did not migrate. Each of these peaks was isolated by preparative chromatography and electrophoresis for detailed characterization by chemical and mass spectrometric procedures.

The L-idopyranose was initially identified by its $R_{glucitol}$ value, which is essentially the same for all hexopyranoses in chromatography system I. Aldehyde reduction gave L-iditol which has a unique $R_{glucitol}$ value in system IV (Conrad, et al., 1973). When the Me_3Si derivative of the isolated L-idopyranose was analyzed by GC-MS, two peaks were obtained which gave identical mass spectra identifying them as the α and β anomers of idopyranose (DeJongh et al., 1969).

The appearance of the L-idofuranose among the products of partial hydrolysis of IIb was unexpected at the outset. Its chromatographic migration ahead of the hexopyranoses but behind L-idosan suggested at first that the peak might be L-idosan 2-sulfate. However, this structural assignment was ruled out by the findings that (1) it did not migrate on electrophoresis, and (2) when it was aldehyde reduced with sodium borohydride it gave a product indistinguishable from L-iditol on chromatography in system IV and on gas chromatography of its Me_3Si derivative. Its identity was established by GC-MS

TABLE II: Properties of Products Obtained from CR-Heparin.

Property	Product										
	$Idose_p$	$Idose_f$	Iditol	Idosan	$AMan_R$	$AMan_R$-SO_4	Idose-SO_4	Ib	IIa	IIb	IIIa	IIIb
$R_{glucitol}$												
System I	0.90	1.30	1.00	2.50	1.90							
System IV			0.70	9.50	7.60							
$R_{idose\ SO_4}$												
System II						1.30	1.00		0.20	0.30	0.70	0.80
System III						1.30	1.00	0.12	0.40	0.50	0.75	0.75
Electrophoresis						1.00	1.00	1.50	1.60	1.50	0.90	1.20

Acid Hydrolysis	Products			
	Idosan	$AMan_R$ + SO_4	$Idose_f$ + SO_4	$AMan_RSO_4$ + idose SO_4
k (min^{-1})	0.011	0.00195	0.0092	0.0383

of its Me_3Si derivative. Although the isolated furanose ring form does not show any signs of equilibration with the pyranose form when rechromatographed on paper, the conditions of Me_3Si derivatization yield a mixture of pyranose and furanose forms, all of which were analyzed by mass spectrometry. The L-idofuranose derivative, identified by the mass spectral data shown in Figure 4, was recovered as 55% of the Me_3Si derivatized material, the remainder being the α and β forms of idopyranose. The *m/e* peaks at 335 and 437 and the prominent peak at 319 are characteristic of trimethylsilyl hexofuranosides (DeJongh et al., 1969). The formation of L-idofuranose as an intermediate in the hydrolysis of IIb is consistent with two previous reports: (1) the calculated anomeric composition of L-idose based upon the assumption that it is in the pyranoid form differs significantly from the observed optical rotation (Pigman and Isbell, 1968; Isbell and Pigman, 1969; Durette and Horton, 1971), and (2) the NMR spectrum of 2-fluoro-2-deoxy-L-idofuranose confirms that the furanose tautomer is more stable than the pyranoid form. We have found that L-idofuranose also is formed as a major intermediate in the acid hydrolysis of carboxyl-reduced dermatan sulfate.

When the purified L-idofuranose was subjected to hydrolytic conditions (0.1 N HCl, 100 °C), it was converted to a product identified previously (Taylor et al., 1973) as L-idosan (see also Figures 1 and 3) with a first-order rate constant of 0.011 min^{-1} (Table II). No other intermediates were observed in this conversion. When the isolated L-idosan was converted to its Me_3Si derivative and analyzed by GC–MS, the mass spectrum was that of the 1,6-anhydropyranose structure. Thus, the conversion of L-idofuranose to L-idosan under the acid hydrolysis conditions involves a change in the ring form without the accumulation of detectable stable intermediates. The purified L-idosan, on rechromatography in system I, gives only the expected L-idosan peak; neither L-idofuranose nor L-idopyranose appear on the chromatogram. When it is treated with 1 N H_2SO_4 at 100 °C for 6 h (the conditions used here for total hydrolysis of CR-heparin), an equilibrium mixture containing 10% L-idopyranose and 90% L-idosan, but no L-idofuranose, is obtained without any loss of total L-idose. Consequently, in all work pertaining to the relative amounts of D-glucose and L-idose in hydrolysates containing both of these hexoses, the number of molar equivalents equal to 10% of the L-idosan was subtracted from the hexopyranose peak and added to the L-idosan to obtain the value for total L-idose. The molar equivalents in the hexopyranose peak after subtraction of L-idopyranose was taken as the value for D-glucose.

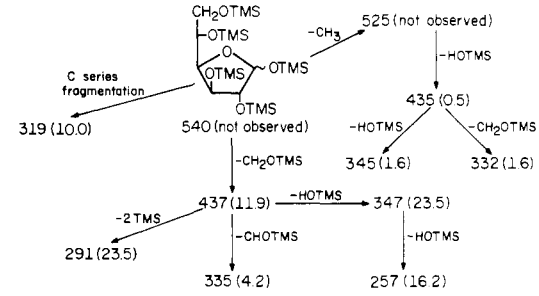

FIGURE 4: Electron impact fragmentation pattern of trimethylsilyl-L-idofuranose isolated from a partial hydrolysate of oligosaccharide IIb. The structure of the molecular ion is given and the identifying *m/e* fragments are indicated. The relative abundance of each fragment is given in parentheses. The C series fragmentation is described by Kochetkov and Chizov (1966).

The purified peak identified as L-idose 2-sulfate was subjected to acid hydrolysis with and without prior aldehyde reduction. In the former instance the product was a mixture of L-idosan and a small amount of L-idopyranose; in the latter, only L-iditol was obtained. The slow chromatographic migration and the rapid anodic electrophoretic migration of this material indicated that it was a sulfated derivative of L-idose. GC–MS analysis of the Me_3Si derivative identified this structure as L-idopyranose 2-sulfate, consistent with previous studies which have located the sulfate on C-2 of L-iduronic acid (Foster et al., 1963; Danishefsky et al., 1969; Perlin et al., 1971; Lindahl and Axelsson, 1971). On graded acid hydrolysis in 0.1 N HCl at 100 °C the L-idose 2-sulfate disappeared with a first-order rate constant of 0.0092 min^{-1} (Table II) yielding first L-idofuranose and, with a time lag, L-idosan.

The anhydro-D-mannitol was characterized previously (Shively and Conrad, 1976), but its 6-O-sulfated derivative, anticipated from previous studies (Danishefsky et al., 1969; Wolfrom et al., 1969), was obtained for the first time in the graded hydrolysis of IIb. Its identification as 2,5-anhydro-D-mannitol 6-sulfate was based upon its acid hydrolysis to yield anhydro-D-mannitol, its migration rates on paper chromatograms and electrophoretograms, its destruction by periodate, and the GC–MS data obtained on its Me_3Si derivative. In 0.1 N HCl at 100 °C anhydro-D-mannitol 6-sulfate was converted

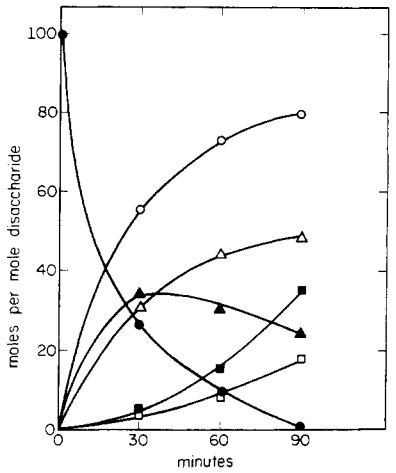

FIGURE 5: Kinetics of hydrolysis of oligosaccharide IIb. Data were taken from radiochromatographic analyses of aliquots withdrawn at 30, 60, and 90 min from a sample of purified HC-HA oligosaccharide IIb heated at 100 °C in 0.1 N HCl (see Figure 3, panels e and f). Hydrolysis products are indicated as follows: (●) IIb; (○) anhydromannose sulfate; (△) idofuranose; (■) idosan; (▲) idose sulfate; and (□) anhydromannose.

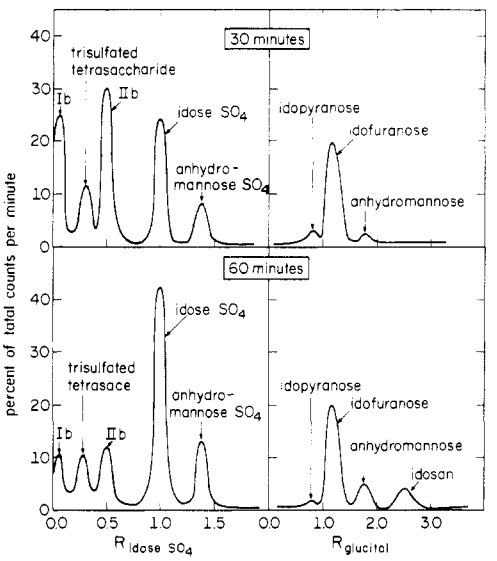

FIGURE 6: Products formed in the partial acid hydrolysis (0.1 N HCl, 100 °C) of HC-HA oligosaccharide Ib. Samples were analyzed as described in the legend to Figure 3.

to anhydro-D-mannitol and free sulfate with a first-order rate constant of 0.00195 min^{-1} (Table II).

Taken together, all of the data on IIb and its hydrolysis products support the conclusion that IIb is L-idopyranosyl 2-sulfate → 2,5-anhydro-D-mannitol 6-sulfate. The α-1,4 linkage between the two residues has been established by the results of others (Perlin et al., 1970, 1971; Helting and Lindahl, 1971, 1972). The kinetics of hydrolysis of IIb in 0.1 N HCl at 100 °C are shown in Figure 5. IIb is converted to products with a first-order rate constant of 0.0383 min^{-1} (Table II). The initial products of the hydrolysis are anhydro-D-mannitol 6-sulfate and L-idopyranose 6-sulfate with a small amount of monosulfated disaccharide (see Figure 3). The sulfated monosaccharides are further hydrolyzed as described above. The remarkable lability of the L-idosyl bond of IIb suggests that hydrolysis of CR-heparin (or CR-heparan sulfate) under these mild conditions should be of value for obtaining selective cleavage of CR-heparin at the L-idosyl bonds.

Structure and Properties of Oligosaccharide Ib. Based upon its paper chromatographic migration at one-half the rate observed for IIb and its electrophoretic migration at the same rate as IIb, Ib may be identified tentatively as a tetrasulfated tetrasaccharide. The only hexose in Ib is L-idose (Table I). Partial acid hydrolysis of Ib yields no 2-acetamino-2-deoxy-D-glucose, and total hydrolysis yields no 2-amino-2-deoxy-D-glucose, a result anticipated from the observations that virtually all of the D-glucosamine residues in this heparin preparation are N-sulfated and that such residues are quantitatively deaminated by nitrous acid under the conditions employed here (Shively and Conrad, 1976). Previous studies have shown that, although the low pH nitrous acid treatment used to generate the oligosaccharides described here removes *N*-sulfate groups quantitatively, glycosidically linked D-glucosamine residues can be converted either to 2,5-anhydro-D-mannitol with glycosidic bond cleavage (the deaminative *cleavage* reaction), *or* to 2-aldehydo-D-pentofuranoside without cleavage of the glycosidic bond (the deaminative *ring contraction* reaction) (Shively and Conrad, 1976). Aldehyde reduction of an oligosaccharide containing the latter deamination product with sodium boro[^{3}H]hydride yields an oligosaccharide containing an internal [^{3}H]hydroxymethylpentofuranosyl residue. If the reaction of some of the N-sulfated D-glucosamine residues in the highly sulfated regions of the CR-heparin chain were to take the latter course, the product that would be formed after aldehyde reduction with boro[^{3}H]hydride would be L-idose 2-sulfate → [^{3}H]hydroxymethylpentofuranose 6-sulfate → L-idose 2-sulfate → [^{3}H]anhydro-D-mannitol 6-sulfate, a product that would exhibit all of the properties observed for Ib. This structure, tentatively assigned to Ib, predicts that partial acid hydrolysis of Ib will generate the same intermediates found in partial hydrolysates of IIb, as well as IIb itself. The radiochromatographic profiles shown in Figure 6 confirm these predictions and add weight to the structural assignment. Thus, after a 30-min hydrolysis period, a large peak that is indistinguishable from IIb is found, and after 60 min, large peaks of anhydro-D-mannitol 6-sulfate, L-idose 2-sulfate, and L-idofuranose are seen. The chromatographic behavior of hydroxymethylpentose 5-sulfate and its stability to the acid hydrolysis conditions have not yet been established but it has been shown that an analogous glycoside, ethyl [^{3}H](2-hydroxymethyl)-α-D-pentofuranoside, formed by deamination of ethyl 2-amino-2-deoxy-α-D-glucoside and boro[^{3}H]hydride reduction (Shively and Conrad, 1976), undergoes a rapid acid hydrolysis that is accompanied by destruction of the hydroxymethylpentose and loss of ^{3}H counts on radiochromatograms of its hydrolysates (Shively, 1975). Consistent with this, it has been observed here that the total recovery of ^{3}H cpm from Ib decreases with increasing hydrolysis time. Uncertainties concerning the migration and recovery of the [^{3}H]hydroxymethylpentofuranose derivative could account for the discrepancy in the hexose/anhydro-D-mannitol ratio seen for Ib in Table I.

Structure and Properties of Oligosaccharides IIIa, IIIb, and IIa. These oligosaccharides contain all of the D-glucose present in CR-heparin (Table I). The rates of chromatographic and electrophoretic migration of IIIa and IIIb (Figure 2) suggest that they are monosulfated disaccharides. IIIa contains equimolar amounts of hexose and anhydro-D-mannitol, also consistent with the assignment of a disaccharide structure. However, the presence of similar amounts of D-glucose and L-idose in IIIa indicates that it is a mixture of at least two disaccharides which are not resolved by the methods used here. Both have anhydro-D-mannitol residues at their reducing

terminals, suggesting that IIIa may be a mixture of L-idose 2-sulfate → anhydro-D-mannitol and D-glucose → anhydro-D-mannitol 6-sulfate. Consistent with this conclusion is the finding that partial hydrolysates of IIIa contain both L-idose 2-sulfate and anhydro-D-mannitol 6-sulfate. However, final proof of structure must await separation of the two disaccharides and analyses of the individual structures.

Oligosaccharide IIIb contains a complement of D-glucose and L-idose similar to that of IIIa but gives an anomalously low hexose/anhydro-D-mannose ratio (Table I). No satisfactory explanation of the latter observation is yet available, and further studies must be conducted to determine the IIIb structure(s). Oligosaccharide IIa, which also gives an anomalous hexose/anhydro-D-mannose ratio, is not found in the product formed by carboxyl reduction of the disulfated disaccharide fraction obtained by deaminative cleavage of heparin, and it contains only 5% of the total hexose recovered from CR-heparin. Its chromatographic and electrophoretic migration rates suggest that it may be a trisulfated tetrasaccharide with a monosaccharide sequence like that of Ib, but with one less sulfate ester. Its partial hydrolysates contain L-idose sulfate, L-idofuranose, anhydro-D-mannitol sulfate, and IIIa but not IIb.

Discussion

The data presented here give a quantitative accounting of the total population of oligosaccharides formed when CR-heparin, or heparin itself, is treated with nitrous acid. The major product, oligosaccharide IIb, contains 60% of the total hexose (hexuronic acid) in the original polymer, and is derived from the L-idosyluronic acid 2-sulfate → 2-sulfamino-2-deoxy-D-glucose 6-sulfate disaccharide sequences in heparin. This disaccharide represents the ultimate stage in the metabolic maturation (uronic acid epimerization, N- and O-sulfation) of heparin. As with model α-linked glycosides of D-glucosamine (Erbing et al., 1973; Shively and Conrad, 1976), the deamination of CR-heparin follows two different pathways. In one of these, the deaminative *cleavage* reaction, the glycosidic bond of the D-glucosamine is cleaved and the D-glucosamine residue is converted to an anhydro-D-mannose residue; in the other, the deaminative *ring contraction* reaction, the amino group is lost and the ring of the D-glucosamine residue is contracted to yield an aldehydopentose (hydroxymethylpentose after borohydride reduction) residue without glycosidic bond cleavage. If only the former path were followed in the deamination reaction, all of the fully matured disaccharide sequences would be converted to IIb. However, the formation of Ib apparently results from the occurrence of the latter deamination pathway. This conclusion is based upon the following observations. The heparin preparation studied contains undetectable amounts of N-acetylated or N-unsubstituted D-glucosamine residues; virtually all of the amino groups are N-sulfated (Shively and Conrad, 1976). In the low pH nitrous acid reaction used to convert CR-heparin to oligosaccharides, N-sulfated D-glucosamines react quantitatively, with release of the sulfate groups as inorganic SO_4 (Shively and Conrad, 1976). If all of the D-glucosamine residues in the polymer reacted via the deaminative cleavage pathway, all of the oligosaccharide products would be disaccharides. Clearly, the chromatographic and electrophoretic properties of Ib are consistent with those anticipated for the tetrasulfated tetrasaccharide that would be formed by the deaminative ring contraction reaction. Furthermore, the rapid conversion of Ib to IIb in dilute acid is consistent with the structural assignment since the glycosidic bond of the hydroxymethylpentose residue has been shown previously to be quite labile to acid (Shively, 1975). The suggestion that Ib and IIb are derived from identical sequences in heparin by divergent pathways of the deamination reaction leads to the conclusion that any heparin preparation that yields IIb in the deamination reaction should also yield Ib, a conclusion that is confirmed in preliminary studies on a series of beef lung heparins and heparan sulfates. The isolation of the anticipated hydroxymethylpentose 5-sulfate will require a better understanding of its stability under the conditions of its hydrolytic release from Ib and of its chromatographic and electrophoretic migration rates. It remains a curious fact that, when CR-heparin is subjected directly to acid hydrolysis to release all of the sulfates and to cleave all of the hexosidic bonds, and *then* treated with nitrous acid, the D-glucosamine residues are converted almost quantitatively to anhydro-D-mannose, with little, if any, aldehydopentose among the deamination products.

The occurrence in the carboxyl-reduced forms of heparin and heparan sulfate of an N-sulfated D-glucosamine residue linked to the nonreducing end of the IIb (or Ib) precursor segments of the chain is a structural prerequisite for release of the disaccharide (or tetrasaccharide) by the low pH nitrous acid treatment. Thus, a high yield of IIb plus Ib depends not only upon the presence of large amounts of the fully matured disaccharide sequences but also upon their occurrence as parts of long contiguous sequences of disaccharides containing N-sulfated D-glucosamine residues. These are expected in heparins, in which the L-iduronic acid and sulfate contents are very high (Taylor et al., 1973), but a quantitative measure of the extent of such contiguous sequences in heparan sulfates, which contain lower levels of L-iduronic acid and sulfate, could not easily be made using previous methodology. A preliminary examination of several carboxyl-reduced beef lung heparan sulfates has shown that those polymers in which the L-iduronic acid is 40% or less of the total uronic acid yield neither IIb nor Ib on nitrous acid treatment, indicating that, if the disaccharide structures which yield these products are present in these polymers, they do not have N-sulfated D-glucosamine residues linked to their nonreducing ends. Conceivably, the biological activities of heparins are dependent upon contiguous sequences of the fully epimerized and sulfated disaccharides, a structural feature which could be strikingly increased during the metabolic maturation of heparan sulfate with relatively small increases in the levels of L-iduronic acid and sulfate. It is also quite possible that polymers from different sources which have the same content of L-iduronic acid and sulfate could have quite different proportions of such contiguous sequences.

These studies provide the background necessary for a new approach to nearest neighbor analysis and mapping of heparins and heparan sulfates. The rates of paper chromatographic and paper electrophoretic migration of all of the possible monosaccharide or disaccharide components of these polymers, described here or elsewhere (Conrad et al., 1973), constitute a new set of criteria for recognition of the structural components of these polymers. The stoichiometries of the boro[^{3}H]-hydride reduction reactions provide a means for convenient and accurate quantitation of nanomole amounts of these products with a minimum of the kinds of experimental manipulations that might alter the quantitation or introduce artifacts. A rationale has been provided for the selective reactions of heparins with different nitrous acid preparations (Shively and Conrad, 1976), and it is shown here that not all N-sulfated D-glucosamine residues in heparin are converted by reaction with nitrous acid to anhydro-D-mannose residues. These observations will permit a more accurate interpretation of data ob-

tained on products formed by reaction of these polymers with nitrous acid. Finally, the results obtained here introduce a new and complementary procedure for selective cleavage of CR-heparins which is based upon the extreme acid lability of the glycosidic bonds of the 2-O-sulfated L-idosyl residues (Lloyd and Forrester, 1971). There remains the complete characterization of the segments of these polymers derived from regions which contain D-glucuronic acid and lower levels of *N*- and *O*-sulfate, but the approaches developed here provide clearly defined directions for the continuing studies.

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Oligosaccharides of Human Milk: Isolation and Characterization of Two New Nonasaccharides, Monofucosyllacto-*N*-octaose and Monofucosyllacto-*N*-neooctaose†

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ABSTRACT: Two new nonasaccharides, fucosyllacto-*N*-octaose and fucosyllacto-*N*-neooctaose, were isolated from human milk. By sequential enzymic degradation and by quantitative methylation analysis, their structures were elucidated as shown in Figure 4 of this article.

A series of higher oligosaccharides derived from lacto-*N*-hexaose and lacto-*N*-neohexaose was found in human milk (Kobata and Ginsburg, 1972b). During the structural study of N-3, a mixture of difucosyllacto-*N*-hexaose and lacto-*N*-neohexaose, we found that this oligosaccharide fraction is contaminated with about 10% of a nonaose. Unlike other major components of N-3, this sugar liberates octaose instead of hexaose upon removal of fucose by mild acid hydrolysis. This paper describes the isolation and structural studies of the nonaose.

Materials and Methods

Enzyme. β-Galactosidase (Arakawa et al., 1974) and β-*N*-acetylhexosaminidase (Li and Li, 1972) were purified from jack bean meal by the method of cited references. α-L-Fucosidase from *Charonia lampas* (Nishigaki et al., 1974) was kindly provided by Dr. T. Okuyama, Seikagaku Kogyo Co. α-L-Fucosidase was also purified from *Bacillus fulminans* (Kochibe, 1973). This enzyme cleaves only Fucα1→2Gal linkage, in contrast to the *Charonia lampas* enzyme which cleaves all fucosyl linkages. The *Bacillus* strain was kindly

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