potheses, but no direct demonstration of conformational changes in responsive tRNAs has yet been secured.

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Formation of Anhydrosugars in the Chemical Depolymerization of Heparin†

John E. Shively‡ and H. Edward Conrad*

ABSTRACT: In the reactions used to break heparin down to mono- and oligosaccharides, anhydrosugars are formed at two stages. The first of these is the well-known cleavage of heparin with nitrous acid to convert the N-sulfated D-glucosamines to anhydro-D-mannose residues; this reaction has been studied in detail. It is demonstrated here that only low pH (<2.5) reaction conditions favor the deamination of N-sulfated D-glucosamine residues; the reaction proceeds very slowly at pH 3.5 or above. On the other hand, N-unsubstituted amino sugars are deaminated at a maximum rate at pH 4 with markedly reduced rates at pH 2 or pH 6. At room temperature solutions of nitrous acid lose one-fourth to one-third of their capacity to deaminate amino sugars in 1 h at all pHs. A low pH nitrous acid reagent which will convert heparin quantitatively to its deamination products in 10 min at room temperature is described, and a comparison of the effectiveness of this reagent with other commonly used nitrous acid reagents is presented. It is also shown that conditions used for acid hydrolysis of heparin convert approximately one-fourth of the L-iduronosyluronic acid 2-sulfate residues to a 2,5-anhydrouronic acid. This product is an artifact of the reaction conditions, and its formation represents one of several pathways followed in the acid-catalyzed cleavage of the glycosidic bond of the sulfated L-idosyluronic acid residues.

Because of the marked resistance of heparin and heparan sulfate to acid hydrolysis, structural studies on these polymers have relied heavily on cleavage of the polymers with nitrous acid to obtain oligosaccharides for further analysis. Nitrous acid cleaves 2-amino-2-deoxy-D-glucosidic bonds via a reaction sequence initiated by nitrosation of the amino group of the sugar followed by loss of N_2 with a ring contraction of the D-glucosamine residue to 2,5-anhydro-D-mannose coupled to elimination of the aglycone (for a review, see Horton and Philips, 1973). A consideration of data in the literature suggests that the yield of 2,5-anhydro-D-mannose may vary depending upon the 2-amino-2-deoxy-D-glucoside treated in the reaction (Shively and Conrad, 1970; Erbing et al., 1973) and the conditions under which the reaction is run (Lagunoff and Warren, 1962). Nitrous acid is usually generated in situ by addition of an inorganic nitrite salt to a solution of acid. A number of different formulations have been used to generate

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the reagent used to cleave heparin and other amino sugar glycosides (Dische and Borenfreund, 1950; Exley, 1957; Lee and Montgomery, 1961; Lagunoff and Warren, 1962; Yosizawa, 1963; Cifonelli, 1968; Yamauchi et al., 1968; Shively and Conrad, 1970; Perlin and Sanderson, 1970; Lindahl et al., 1973). Some preparations of nitrous acid will cleave D-glucosamine glycosides only when the amino group of the D-glucosamine is unsubstituted (Lindahl et al., 1973), while others will cleave the glycosides of N-sulfated D-glucosamine found in heparins and heparan sulfates (Cifonelli, 1968; Lagunoff and Warren, 1962). All such cleavages of amino sugar glycosides convert the D-glucosamine residue primarily to an anhydro-D-mannose residue which becomes the new reducing terminal of the oligosaccharide formed in the deamination reaction.

The process of nitrous acid cleavage of 2-amino-2-deoxy-D-glucosides has most commonly been followed by measurement of the formation of anhydro-D-mannose using the indole color reaction (Dische and Borenfreund, 1950) which is quite specific for anhydrosugars. However, a number of anomalies have been noted in the analysis of the nitrous acid cleavage products obtained from heparin and heparan sulfates. For example, when heparin is treated with nitrous acid using conditions which cleave N-sulfated glucosaminidic bonds, the indole color yield is 15–20% higher than that obtained with a corresponding amount of N-desulfated heparin (Lagunoff and Warren, 1962). Furthermore, the indole color yields for nitrous acid treated heparin and heparan sulfate considerably exceed the theoretical yield, being as much as 60% higher than that for a comparable amount of D-glucosamine (Cifonelli, 1968; Lindahl and Axelsson, 1971).

A further unexplained observation in the studies of the structures of heparin and heparan sulfate is the atypically high color yields obtained in the carbazole assay for the uronic acid content of these polymers (Dische, 1962). For example, heparin gives a 50% higher extinction coefficient than an equivalent amount of D-glucuronic acid (Lindahl and Axelsson, 1971). This anomaly is not explained by the high content of L-iduronic acid in heparin and heparan sulfate (Perlin and Sanderson, 1970; Taylor et al., 1973) since, in the original carbazole reaction of Dische (1947), L-iduronic acid gives only 17% of the color yield obtained from an equivalent amount of D-glucuronic acid. The problem is further complicated by the observation that a much higher carbazole color yield is obtained in the direct analysis of these polymers than in analysis of the same polymers after nitrous acid cleavage, in spite of the fact that the nitrous acid treatment does not chemically modify the uronic acids (Yamauchi et al., 1968; Lindahl and Axelsson, 1971).

One final problem that has arisen in the use of nitrous acid for the cleavage of heparin and that has received less attention is the finding that, when heparin preparations in which the D-glucosamine residues are essentially 100% N-sulfated are cleaved by nitrous acid, the yield of disaccharides is far less than the theoretical yield (Lindahl and Axelsson, 1971; Cifonelli and King, 1972).

Taken together the above observations indicate that quantitative interpretations of structural data based upon nitrous acid cleavage of heparin-like polymers must be viewed with caution until the attainment of a better understanding of the effect of reaction conditions on the stoichiometry of the nitrous acid reaction with different amino sugar glycosides. This paper describes a detailed study of the reactions of carboxyl-reduced heparin (CR-heparin[1]), ethyl 2-amino-2-deoxy-α-D-glucoside (EAG), and D-glucosamine with nitrous acid in citrate buffers over a range of pHs. In addition, data are presented to show that a previously observed but unidentified acid formed in the acid hydrolysis of heparin (Helbert and Brown, 1961; Lindahl and Axelsson, 1971; Shively and Conrad, 1970) is a 2,5-anhydrouronic acid derived from L-iduronic acid 2-sulfate residues. The acid-catalyzed formation of this anhydrouronic acid may be the cause of the anomalous results obtained for heparin in the carbazole and indole assays.

Experimental Procedures

Beef lung heparin, in the form of its sodium salt, was a commercial preparation kindly furnished by Dr. N. D. Jenkins of the Upjohn Company. The sample contained 2.18% N and 10.02% S. Analyses of this preparation by previously described methods (Taylor et al., 1973; Shively and Conrad, 1970) showed a D-glucosamine to uronic acid ratio of 1:1, and an L-iduronic acid to D-glucuronic acid ratio of 84:16. Ethyl 2-amino-2-deoxy-α-D-glucoside (EAG) was prepared by the method of Lloyd et al. (1969). [^{35}S]Sulfur trioxide–triethylamine complex (7.8 mCi/mmol) was obtained from Amersham/Searle Company. Sodium boro[^{3}H]hydride (14 and 177 mCi/mmol) and [^{14}C]-D-glucose (200 mCi/mmol) were purchased from New England Nuclear. Barium nitrite was obtained from A. D. Mackay, Inc., New York, N.Y.

2,5-Anhydro-D-mannose and other reducing sugars were determined by radiochromatography as detailed previously (Shively and Conrad, 1970; Conrad et al., 1973). The procedure involves mixing an aliquot of the sample with a measured quantity of high specific activity [^{14}C]-D-glucose to serve as an internal standard, then hydrolyzing and deaminating the sample, reducing the mixture with sodium boro[^{3}H]hydride of known specific activity, separating the ^{3}H-labeled components of the mixture by paper chromatography, and determining the number of ^{3}H cpm in each separated peak. The micromoles of each component in the sample aliquot analyzed is calculated using the ^{3}H cpm in the peak, the specific radioactivity of the boro[^{3}H]hydride, and the fraction of the total D-[^{14}C]glucitol added to the sample that is recovered on the radiochromatogram (Conrad et al., 1973). The identification of each ^{3}H peak on the chromatogram is based upon its $R_{glycitol}$ value (the ratio of the distance which the ^{3}H peak has moved to that which the [^{14}C]-D-glucitol has moved).

Preparation of N-Desulfated Heparin and [N-$^{35}SO_4$]-CR-Heparin. The *N*-sulfate groups of heparin were specifically removed using the following adaptation of the procedure developed by Nagasawa and Inouye (1974). One hundred milligrams of heparin dissolved in 1 ml of water was converted to the free acid form by passage through a column of Dowex 50 (H^+). The fractions testing acid to pH paper were pooled, neutralized with pyridine, concentrated to dryness under reduced pressure, and washed twice with ethanol, each time evaporating the sample to dryness, until the odor of pyridine was gone. The resulting pyridinium salt of heparin was dissolved in 5 ml of 5% aqueous dimethyl sulfoxide and the solution was heated at 50 °C for 2 h and then dialyzed successively against water, one change of 0.2 M NaCl, and then two more changes of water. The resulting product had virtually 100% of its *N*-sulfate groups removed as judged by the yield of anhydro-D-mannose when it is treated with nitrous acid under conditions which do not deaminate N-sulfated amino sugars (see Table III).

[1] Abbreviations used are: CR-heparin, carboxyl-reduced heparin; Me_3Si, trimethylsilyl; EAG, ethyl 2-amino-2-deoxy-α-D-glucoside; DEAE, diethylaminoethyl.

To re-N-sulfate the N-desulfated product with [^{35}S]sulfate, the procedure of Levy and Petracek (1962) was used. Fifteen milligrams of the N-desulfated heparin was dissolved in 300 μl of water and 15 mg each of Na_2CO_3 and [^{35}S]sulfur trioxide–triethylamine complex was added. The reaction mixture was stirred for 24 h at 50 °C and then cooled and dialyzed successively against water, 0.2 M NaCl, and two changes of water. The dialyzed solution was evaporated at 30 °C in a Buchler Evapo-Mix to 0.2 ml and purified by preparative electrophoresis as follows. The solution was spotted 3.5 in. from one end of a 22-in. strip of Whatman No. 3 paper. The paper was then made wet with pyridine–glacial acetic acid–water (1:10:400) buffer, allowing the buffer to diffuse gradually from both sides into the sample at the origin. The strip was then blotted and placed in a water-cooled electrophoresis apparatus with the origin toward the cathode end. The sample was electrophoresed for 2 h at 30 V/cm, then dried, and scanned on a strip scanner for ^{35}S. The region of the electrophoretogram containing the labeled heparin was cut out of the strip and eluted with water using spin thimbles (Reeve-Angel, Inc.). The residual inorganic $^{35}SO_4$ in the product (about 2% of the total ^{35}S) was removed by preparative paper chromatography on Whatman No. 3 paper in 1-butanol–glacial acetic acid–1 N ammonium hydroxide (2:3:1.5). The strips were scanned and the [^{35}S]heparin was eluted from the origin as above. The product had a specific activity of 2×10^8 dpm/mg; it lost 100% of its $^{35}SO_4$ when N-desulfated as above or upon treatment with low pH nitrous acid (see Table III); at least 98% of the free amino groups were sulfated by this procedure as indicated by the low yield of anhydro-D-mannose when this product was treated with nitrous acid at pH 4.0 (see Table III).

The [^{35}S]heparin was carboxyl reduced by a modification of the procedure described earlier (Taylor and Conrad, 1972) in which the sodium borohydride concentration was brought to 2 M in the reduction step by addition of solid sodium borohydride to the carbodiimide-activated heparin solution (Taylor et al., 1973). Purification of the reduced product by the preparative electrophoresis procedure described above gave a fully reduced product which retained all of the *N*- and *O*-sulfate groups present in the starting material. To prepare the N-desulfated CR-heparin used in this study, a sample of the original heparin was reduced by this same procedure and then N-desulfated by the method of Nagasawa and Inouye (1974) as described above.

Deamination Procedures. A number of different procedures were used in this study for the deamination of the standard substrates. For the heparin deamination reactions a standard CR-heparin solution containing 100 mg of CR-heparin per ml was used. In each of the procedures an aliquot from this standard solution was mixed with an aliquot of a [^{14}C]-D-glucose (200 mCi/mmol, 20 μCi/ml) solution which served as an internal standard. The actual deamination procedures were applied as detailed in the literature with the minor modifications described below that were necessary to adapt the method for radiochromatographic analysis of the products. After deamination the samples were either neutralized by addition of 2 N Na_2CO_3 and analyzed directly by radiochromatography, or they were made 1 N in H_2SO_4 and hydrolyzed at 100 °C for 6 h and then neutralized and analyzed by radiochromatography.

The Procedure of Shively and Conrad (1970). A 30-μl aliquot of the standard CR-heparin solution was mixed with 30 μl of 2.5 N sulfuric acid and 15 μl of [^{14}C]-D-glucose solution. The deamination was carried out by incubating a 2-μl aliquot of this solution with 5 μl of 5.5 M aqueous $NaNO_2$ at room temperature for 10 min.

The Procedure of Lagunoff and Warren (1962). A 30-μl aliquot of the standard CR-heparin solution was mixed with 5 μl of [^{14}C]-D-glucose. To a 5-μl aliquot of this mixture were added 5 μl of 5% aqueous $NaNO_2$ and 5 μl of 33% aqueous acetic acid. The reaction was run for 80 min at room temperature and excess nitrous acid was then destroyed by addition of 10 μl of an aqueous solution of ammonium sulfamate (20 mg/ml).

The Procedure of Cifonelli (1968). A 30-μl aliquot of the standard solution of CR-heparin was mixed with 5 μl of [^{14}C]-D-glucose solution. To 5 μl of this mixture was added 10 μl of glyme (1,2-dimethoxyethane) and the glyme–water mixture was chilled to −20 °C in a dry ice–acetone bath. Fifteen microliters of a nitrous acid solution prepared (see below) and stored at −20 °C was added to the heparin sample and deamination was allowed to proceed for 12 h at −20 °C. The reaction mixture was then evaporated to dryness at 30 °C in vacuo using an Evapo-Mix (Buchler Instruments) and the products were taken up in 20 μl of water for analysis. The nitrous acid solution used in this procedure was prepared by dissolving 114 mg of $Ba(NO_2)_2$ in 1 ml of water and 2 ml of glyme, cooling this solution to −20 °C, and then adding a mixture of 1 ml of 1 N H_2SO_4 and 1 ml of glyme, precooled to −20 °C. The precipitated $BaSO_4$ was removed by centrifugation at 1000 rpm for 2 min at room temperature in a clinical centrifuge. The supernatant containing the nitrous acid was removed with a Pasteur pipet and stored at −20 °C.

The Low pH Nitrous Acid Procedure. This is a new procedure developed as a result of the studies of the pH effects on deamination described in Results. For deamination of CR-heparin by this procedure, 30 μl of the standard solution of CR-heparin was mixed with 5 μl of [^{14}C]-D-glucose, and a 5-μl aliquot of this mixture was treated at room temperature with 20 μl of a nitrous acid solution prepared as follows: 1 ml of 1 N H_2SO_4 (0.5 mmol) at −5 °C was thoroughly mixed with a solution containing 114 mg of $Ba(NO_2)_2$ (0.5 mmol) in 1 ml of water at −5 °C. The precipitated $BaSO_4$ was removed by centrifugation for 2 min at 1000 rpm in a clinical centrifuge and the supernatant solution of nitrous acid was drawn off with a Pasteur pipet. This reagent has a pH of 1.5 and is stable for several hours when stored at −5 °C. When the nitrous acid and CR-heparin solutions are mixed at −5 °C and allowed to warm to room temperature, deamination is complete in 10 min from the time of HNO_2 addition.

Deamination in Buffered Solutions. To determine the effect of pH on the extent of deamination a series of 0.2 M sodium citrate buffers ranging in pH from 2 to 7 was used. The substrate solution for the deamination reaction was prepared by mixing 30 μl of the CR-heparin standard solution with 5 μl of [^{14}C]-D-glucose. A 5-μl aliquot of this sample was mixed with 100 μl of citrate buffer and 5 μl of 1 M $NaNO_2$ was added. The pH of each reaction mixture was measured and recorded. The reaction was allowed to proceed for 60 min at room temperature and then was stopped by addition of 10 μl of an aqueous ammonium sulfamate solution (70 mg/ml). The same procedure was used in a series of reactions in which the citrate buffers were replaced with 0.05 M potassium phthalate buffers.

Isolation and Characterization of the Unidentified Acid. The unidentified acid was released from heparin as the monosaccharide by hydrolysis of heparin in 1 N H_2SO_4 at 100 °C for 6 h followed by nitrous acid cleavage by the procedure of Shively and Conrad (1970). The reaction mixture was reduced with $NaBH_4$ and the glycitol of the unidentified acid was iso-

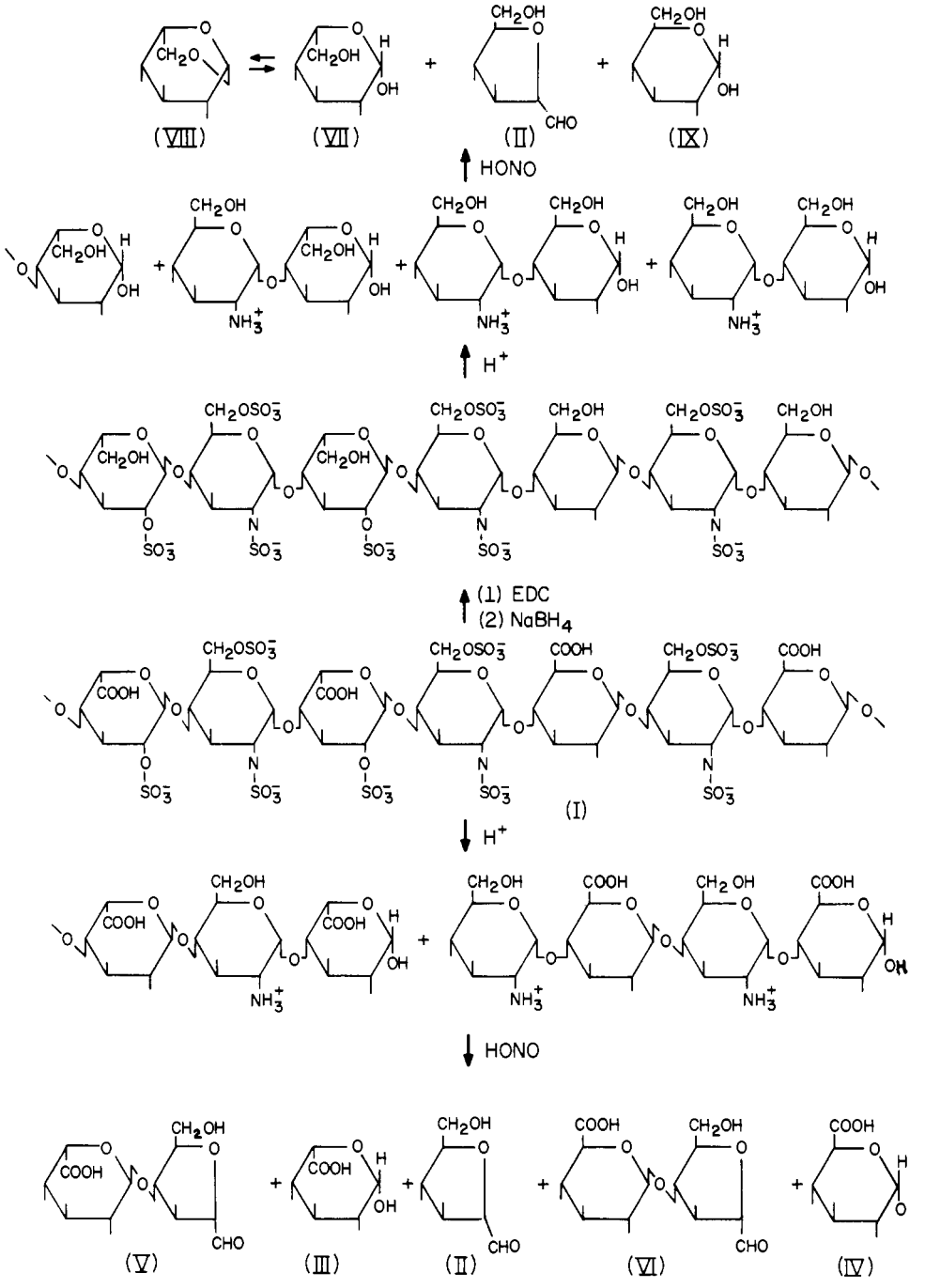

FIGURE 1: Reactions used in the depolymerization of heparin (I).

lated by preparative chromatography on Whatman No. 3 paper using ethyl acetate–glacial acetic acid–formic acid–water (18:3:1:4) as the chromatography solvent. Aliquots containing 1–5 mg of sample were spotted along with an aliquot of [^{14}C]-D-glucitol on several 1 × 22.5 in. strips. From the position of the [^{14}C]-D-glucitol peak the region of the chromatogram containing the unidentified acid was estimated from its previously determined $R_{glucitol}$ value. These segments of the chromatograms were excised from the paper strips and eluted with water using spin thimbles (Reeve-Angel, Inc.). The recovered acid was further purified by rechromatography and the product was evaporated to dryness in vacuo at 30 °C and converted to its trimethylsilyl (Me_3Si) derivative by the method of DeJongh et al. (1969). The Me_3Si derivative was separated by gas chromatography on a gas Chrom Q column coated with 3% OV-17 and analyzed by electron impact mass spectrometry using a Ch-5 spectrometer, Model 311-A, coupled to a Varian 1700 gas chromatograph.

Results

Demonstration of an Unidentified Acid in Heparin Hydrolysates. The reactions in the depolymerization of heparin are shown in Figure 1. The amounts of mono- and oligosaccharides present at each stage of heparin breakdown are determined as shown in Figure 2 by radiochromatography, a procedure in which [^{3}H]glycitols formed by reaction of reducing carbohydrates with sodium boro[^{3}H]hydride are separated by paper chromatography and quantitated by measuring the total ^{3}H cpm in each glycitol peak. When heparin is acid hydrolyzed (reaction 1, Figure 1) and analyzed in this

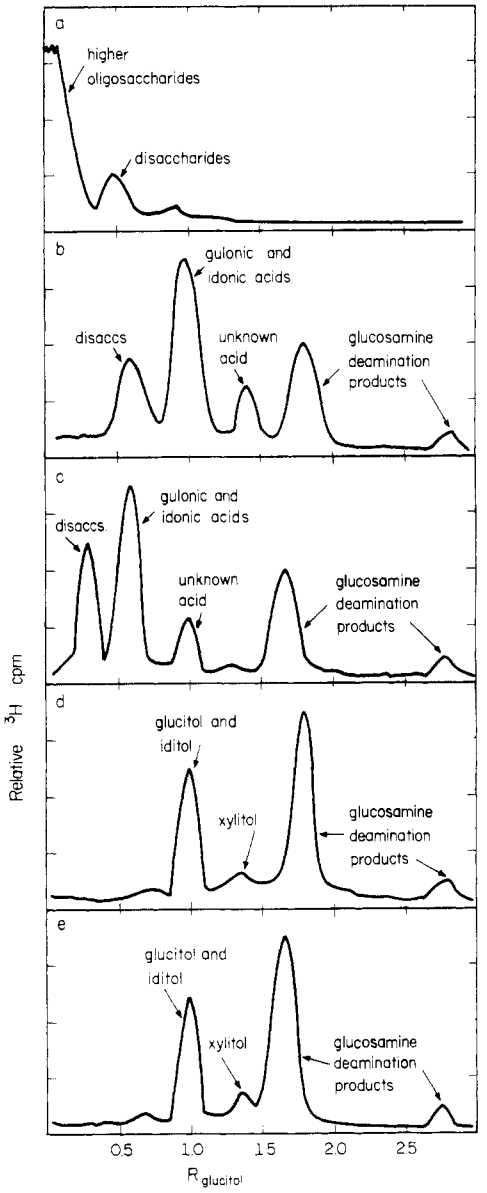

FIGURE 2: Radiochromatographic analysis of the depolymerization products from heparin. All chromatograms were developed in ethyl acetate–acetic acid–formic acid–water (18:3:1:4) on either Whatman No. 1 paper (W1-EAFW system) or DEAE-cellulose paper (DEAE-EAFW system). Reaction products analyzed are those formed in reaction 1 (Figure 1, panel a), in reaction 2 (Figure 1, panels b and c), or in reactions 5 (Figure 1, panels d and e).

manner using chromatography systems designed to separate mono- and disaccharides, virtually no monosaccharides are found, and the largest fraction of reducing equivalents is in oligosaccharides which do not migrate from the origin. This is illustrated in Figure 2a, a radiochromatogram of a 6-h heparin hydrolysate (1 N H_2SO_4, 100 °C). These conditions of hydrolysis are sufficient to cleave the *N*- and *O*-sulfates quantitatively so that all of the amino sugars have free amino groups. When such hydrolysates are treated with nitrous acid (reaction 2, Figure 1), the D-glucosamine glycosides are cleaved and the amino sugar residues are converted into 2,5-anhydro-D-mannose residues some of which are released as the monomer (II) and others of which remain as the glycosyluronic acid-2,5-anhydro-D-mannose disaccharides (V and VI). The nitrous acid reaction also releases some of the uronic acids as monomers (III and IV). A radiochromatogram of the nitrous acid cleaved heparin hydrolysate, Figure 2b, shows the anticipated monomeric deamination products (the reaction of nitrous acid with the D-glucosamine moiety actually gives a mixture of products which includes 2,5-anhydro-D-mannose (II) as the major peak), the uronic acids (III and IV), and the disaccharides (V and VI). In addition to the expected peaks, a fairly prominent and unidentified peak appears in Figure 2b at $R_{glucitol}$ 1.3. If the sample chromatographed in Figure 2b is run in the same solvent system but on strips of DEAE-cellulose, a positively charged chromatographic support, instead of Whatman No. 1 paper strips, the unknown peak is retarded (Figure 2c), as are the peaks of free uronic acids and the acidic disaccharides. This leads to the tentative conclusion that the unknown peak is an acidic monosaccharide.

When all of the uronic acid residues in heparin are first reduced to neutral sugars before acid hydrolysis (reaction 3, Figure 1; Taylor and Conrad, 1972), then the polysaccharide can be broken down completely to monosaccharides by acid hydrolysis (reaction 4) plus deaminative cleavage with nitrous acid (reaction 5). The products formed in reaction 5 are illustrated in Figures 2d and 2e, radiochromatograms run on Whatman No. 1 and on DEAE-cellulose papers, respectively. Comparisons of these radiochromatograms with those in Figures 2b and 2c show that the depolymerized mixture from carboxyl-reduced heparin no longer contains oligosaccharides, uronic acid monomers, *or* any unidentified peak. One of the products of hydrolysis of carboxyl-reduced heparin, L-idose (VII, Figure 1), has a strong tendency to equilibrate in acid solution with its 1,6-anhydro derivative, L-idosan (VIII, Figure 1), in an equilibrium which favors the L-idosan (Sorkin and Reichstein, 1945; Perlin and Sanderson, 1970). Since L-idosan has a blocked reducing group, it is not detected directly by radiochromatography but it can be observed and measured quantitatively if the carboxyl reduction is carried out with boro[^{3}H]hydride (Taylor et al., 1973). Radiochromatograms of the products of hydrolysis and deamination of the heparin that is carboxyl reduced with boro[^{3}H]hydride again show no unidentified peaks and it is concluded that CR-heparin is completely depolymerized by reactions 3–5 of Figure 2 to yield only anhydro-D-mannose and the hexoses anticipated from uronic acid reduction.

Formation of the Unidentified Acid from L-Iduronic Acid 2-Sulfate Residues during Acid Hydrolysis. In the radiochromatographic analysis of the monosaccharide content of heparin it is necessary to include the unidentified acid with the uronic acid fraction in order to obtain ratios of uronic acid to D-glucosamine which are equivalent to those obtained in the more accurate analyses obtained on carboxyl-reduced heparin (Taylor et al., 1973). Since in the latter analyses D-glucose and L-idose (and L-idosan) were the only hexoses detected in hydrolysates of carboxyl-reduced heparin, it seemed likely that the conditions used for the acid hydrolysis of heparin (unreduced) partially convert either D-glucuronic acid or L-iduronic acid residues (or both) to the unidentified acid. To determine whether the unknown acid was derived exclusively from one of the two known uronic acids in heparin advantage was taken of the observation by Lindahl and Axelsson (1971) that the L-iduronic acid, but not the D-glucuronic acid residues in heparin are esterified with sulfate at C2; thus, while the D-glucuronic acid moieties are destroyed by periodate oxidation of heparin, the L-iduronic acid moieties are periodate resistant. Consequently, if acid hydrolysis of periodate-oxidized heparin still releases the unknown acid it cannot be derived from D-glucuronic acid which has been destroyed by periodate and must therefore arise from the L-iduronic acid 2-sulfate resi-

TABLE I: Effects of Carboxyl Reduction and Periodate Oxidation on the Appearance of the Unidentified Acid among the Products of Heparin Depolymerization.[a]

Component	Polymer			
	Heparin		CR-Heparin	
	Unoxidized	IO_4 Oxidized[b]	Unoxidized	IO_4 Oxidized[b]
Glucosamine[c]	1.29	1.21	1.10	1.13
Uronic acid[d]	1.45	1.07	0.06	0.11
Unidentified acid	0.20	0.23	0.00	0.00
Glucose			0.44	0.14
Idose + idosan			0.84	0.76

[a] Values, obtained from radiochromatographic analyses as in Figure 2, are given in μmol per mg of sample. [b] Samples (ca. 1 mg) were incubated at 5 °C in the dark for 48 h in 200 μl of 0.025 M sodium periodate in 0.05 M sodium acetate buffer, pH 3.9. Oxidized samples were dialyzed exhaustively against distilled water prior to hydrolysis and analysis. [c] Total deamination products (Shively and Conrad, 1970). [d] D-Glucuronic acid and L-iduronic acid are not separated from each other in the radiochromatographic analysis.

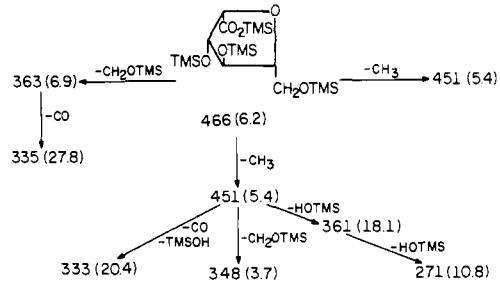

FIGURE 3: Electron impact fragmentation pattern of the 2,5-anhydrouronic acid formed in the acid hydrolysis of heparin. The structure of the molecular ion is given and the identifying *m/e* fragments are indicated. The relative abundance of each fragment is given in parentheses.

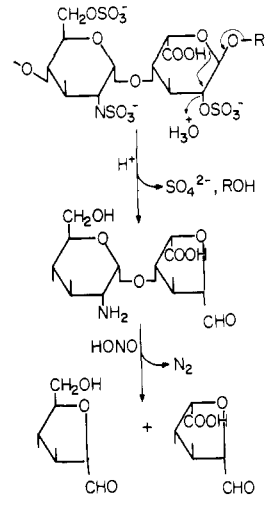

FIGURE 4: Mechanism proposed for the formation of the 2,5-anhydrouronic acid in the acid hydrolysis of heparin and its release as the free uronic acid in the deamination reaction.

dues. The data presented in Table I compare the monosaccharide contents of heparin and CR-heparin before and after periodate oxidation. The data on the CR-heparin confirm the findings of Lindahl and Axelsson that it is the D-glucuronic acid (D-glucose in this experiment) and not the L-iduronic acid that is destroyed by periodate. The heparin data also show the destruction of uronic acid residues by periodate, but it is seen that the unidentified acid is recovered quantitatively in hydrolysates of periodate-oxidized heparin. These data lead to the conclusion that the unidentified acid is formed from the L-iduronic acid 2-sulfate residues. An analogous unidentified peak derived from idose 2-sulfate residues is not observed in hydrolysates of carboxyl-reduced heparin for reasons discussed below.

A question that remains is whether the acid is formed from heparin during the acid hydrolysis (reaction 1, Figure 1) or during the deamination reaction (reaction 2, Figure 1). To answer this question, heparin was acid hydrolyzed as before and the hydrolysate was reduced with sodium boro[^{3}H]hydride *prior* to nitrous acid treatment (after reaction 1, but before reaction 2 of Figure 1) to label all reducing groups formed during acid hydrolysis. The ^{3}H-labeled oligosaccharide mixture was then treated with nitrous acid to cleave all remaining 2-amino-2-deoxy-D-glycosidic bonds. Radiochromatographic analysis of the resulting mixture without further reduction with boro[^{3}H]hydride gave maximal yields of the unknown acid. Thus, the unknown appears to be formed during acid hydrolysis and released as a free acid in the nitrous acid cleavage reaction.

Characterization of the Unidentified Acid. To prepare sufficient amounts of the unidentified acid for characterization, a heparin depolymerization mixture (product of reaction 2, Figure 1) was reduced with unlabeled borohydride and the glycitol of the unidentified acid was isolated by preparative paper chromatography as described in Methods. To determine its structure it was converted to its trimethylsilyl derivative and analyzed by gas chromatography–mass spectrometry. On the basis of its mass spectrum, the unknown acid is identified as a 2,5-anhydrouronic acid according to the fragmentation pattern in Figure 3. Figure 4 shows a mechanism proposed for the formation of the anhydrouronic acid from the L-iduronic acid 2-sulfate residues in the polymer. According to this proposal a ring contraction occurs in concert with the removal of the sulfate ester. Accompanying the ring contraction there is a cleavage of the glycosidic bond in a manner exactly analogous to the bond cleavage that occurs accompanying nitrous acid deaminative cleavage of 2-amino-2-deoxy-D-glycosidic bonds. This reaction would result in an inversion of the asymmetry at C2 to yield 2,5-anhydro-L-guluronic acid.

Products of Nitrous Acid Deamination of Standards. To study the effects of reaction conditions on the products of nitrous acid treatment of amino sugars, three standards were used: D-glucosamine, ethyl 2-amino-2-deoxy-α-D-glucoside (EAG), and CR-heparin substituted with $^{35}SO_4$ on the amino groups of the D-glucosamine residues. The former two substrates served as basic model compounds while the latter was used to determine how heparin itself reacts with nitrous acid. The use of the carboxyl-reduced form of $^{35}SO_4$-labeled heparin was dictated by the three measurements chosen to follow the heparin reaction, namely, release of *N*-sulfate as inorganic $^{35}SO_4$, the formation of anhydro-D-mannose reducing terminals, and the formation of the disulfated disaccharide, L-idose 2-sulfate → anhydro-D-mannose 6-sulfate. The percentage of the *N*-sulfate groups cleaved was determined by spotting an aliquot of the deaminated reaction mixture on a paper chromatogram and developing the chromatogram to

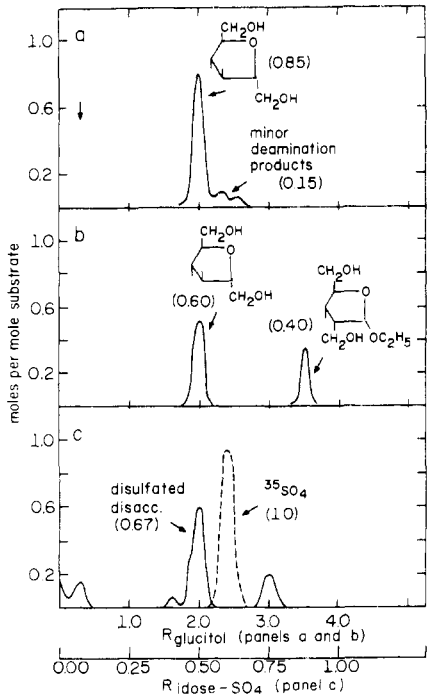

FIGURE 5: Radiochromatographic profiles of the deamination products formed in the reactions of standard compounds with nitrous acid. D-Glucosamine (a) and EAG (b) were deaminated at pH 4 by the procedure of Shively and Conrad (1970). CR-heparin (c) was deaminated by the low pH HONO procedure. Chromatograms were run on Whatman No. 1 paper in ethyl acetate–acetic acid–formic acid–water (18:3:1:4), panels a and b, or on Whatman No. 3 paper in 1-butanol–acetic acid–1 N ammonium hydroxide (4:6:3), panel c. The major peaks were identified as described in the text. Numbers in parentheses refer to the mole fraction of the ^3H or $^{35}SO_4$ recovered in the peak indicated.

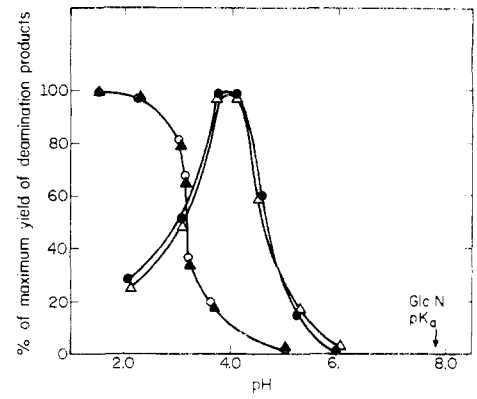

FIGURE 6: The effect of pH on the deamination of standard compounds. Reactions were run at room temperature for 60 min with 1 mM substrate in 0.20 M citrate buffers to which $NaNO_2$ was added to a final concentration of 0.05 M. The symbols refer to the release of free $^{35}SO_4$ from CR-heparin (▲), the formation of anhydromannose from CR-heparin (○), the formation of anhydromannose from EAG (●), and the formation of anhydromannose from D-glucosamine (△).

separate the free sulfate from the polymer-bound sulfate. Quantitative values for the amount of *N*-sulfate converted to free sulfate are expressed as the percentage of the total $^{35}SO_4$ cpm on the chromatogram recovered in the inorganic sulfate peak. The validity of this measurement depends upon the demonstration that all of the $^{35}SO_4$ in the substrate is linked only to the amino groups of the CR-heparin. That this requirement was met was demonstrated by the fact that the $^{35}SO_4$ could be released quantitatively as inorganic sulfate either by the N-desulfation reaction in Me_2SO (Nagasawa and Inouye, 1974) or by treatment with nitrous acid under appropriate conditions (see below), reactions in which sulfate esters are stable.

The amounts of anhydro-D-mannose and disulfated disaccharide formed were measured in separate aliquots taken from a sodium boro[^3H]hydride reduced nitrous acid reaction mixture. From this preparation, in which all of the reducing terminal anhydro-D-mannose residues of the oligosaccharides were labeled with ^3H, one aliquot was analyzed directly by radiochromatography to determine the amount of disulfated disaccharide formed and a second aliquot was acid-hydrolyzed to release free anhydro-D-[^3H]mannitol for assay by radiochromatography to determine the total anhydro-D-mannose formed. The validity of this latter assay depends upon the quantitative release of anhydro-D-[^3H]mannitol in the acid hydrolysis step, a requirement which could only be met by carrying out the study with CR-heparin in which the hexosyl bonds are much more acid labile than the hexosyluronic acid bonds of heparin itself. The aliquots analyzed in each of the three assays could be made directly comparable at all reaction time points by normalizing the ^{35}S and the ^3H cpm on each chromatogram to the same total $^{35}SO_4$ cpm on the chromatogram after correcting, where necessary, for the radioisotopic decay of ^{35}S.

Figure 5 shows the radiochromatographic profiles obtained for the deamination products formed when D-glucosamine, EAG, and [$^{35}SO_4$]CR-heparin react with nitrous acid under optimal conditions for each substrate (see below). As shown in Figure 5a, deamination of D-glucosamine yields primarily 2,5-anhydro-D-mannose, identified in this work by isolation of the major peak in Figure 5a and analyzing its Me_3Si derivative by gas chromatography–mass spectrometry. In cases where the D-glucosamine conversion is incomplete, the unreacted D-glucosamine remaining in the reaction mixture is reduced in the assay to D-[^3H]glucosaminitol which migrates with an $R_{glucitol}$ indicated by the arrow in Figure 5a. EAG (Figure 5b) yields both 2,5-anhydro-D-mannose and a second major peak, a mixture of the ribo and arabino isomers of ethyl 2-aldehydo-α-D-pentofuranoside which is reduced to ethyl hydroxymethyl-α-D-pentofuranoside in the radiochromatographic analysis. This product was identified previously (Erbing et al., 1973) as a product of nitrous acid deamination of 2-amino-2-deoxy-D-glucosides and its identity was confirmed here by gas chromatography–mass spectrometry of its Me_3Si derivative. It is important to note that, when this product is formed in the deamination reaction, the glycosidic linkage of the substrate remains intact. Previous studies (Shively and Conrad, 1970; Erbing et al., 1973) have shown that this product is formed in relatively high yields in the deamination of simple 2-amino-2-deoxy-α-D-glucosides, but in much lower yields from 2-amino-2-deoxy-β-D-glucosides. If the deamination of the α-linked glycoside residues in heparin yields a significant fraction of the analogous product, such that deamination occurs but the glycosidic bonds are not broken, the molar yield of anhydro-D-mannose reducing terminals will be much less than the amount of D-glucosamine in the polymer and the yield of disaccharide formed will be decreased proportionately. The extent to which this alternate deamination pathway occurs in the treatment of heparin with nitrous acid is discussed in the accompanying paper (Shively and Conrad, 1976). Figure 5c shows the radiochromatographic profile of the boro[^3H]hydride-reduced products formed when CR-heparin is treated with nitrous acid. The characterization of the disulfated disaccharide, the major peak on the chromatogram, is described in the accompanying paper (Shively and

Conrad, 1976). In the present work, the yield of this disaccharide is one of the criteria used to evaluate the effectiveness of the deamination conditions.

Effect of pH on the Deamination of D-Glucosamine Residues. Figure 6 shows the effects of pH on the extent of deamination of the model compounds. The deamination reagents having different pHs were prepared by addition of sodium nitrite to citric acid–sodium citrate buffers ranging in pH from 2.2 to 5.5. The reactions were run for a time interval chosen so that under the optimal pH conditions nearly quantitative deamination would just be achieved in the reaction period. At suboptimal conditions, where the rate of deamination was slower, the conversions were incomplete in this same reaction time. Results in each case are expressed as percent of the maximum yield of each product obtained when the reaction was run to completion. The data show that there are distinctly different curves for CR-heparin on the one hand and for D-glucosamine and EAG on the other. The rate of CR-heparin deamination increases as the pH is lowered and is maximal at pHs below 2.5. The curves for N-desulfation, anhydro-D-mannose formation, and disulfated disaccharide formation are superimposed upon each other, indicating that these measurements are all manifestations of the same pH-dependent reaction. The deaminations of D-glucosamine and EAG both occur optimally at pH 4.0, a pH at which CR-heparin is only slowly deaminated. Rates of deamination of these latter compounds fall sharply at both higher and lower pHs. An identical study was carried out using 0.05 M potassium phthalate buffers in place of the sodium citrate buffers. In this study virtually identical pH effects were obtained with all three standards.

Stability of the Deamination Reagent. The two most obvious explanations for the pH effects on the deamination reaction are (1) its effect on the rate of formation and decomposition of the actual deaminating species derived from the nitrous acid, and (2) its effect on the amount of the reactive ionic species of the amino sugar available for reaction with the nitrosating species. Although the nitrosating species believed to be involved in the direct reaction with the amine is N_2O_3, the effects of pH on the kinetics of its formation from HONO and its decomposition to NO, NO_2, and other inactive species are extremely complex and have not been described for conditions similar to those developed here. Consequently, a simple assay was devised to determine the loss of activity of the deamination reagent at several pHs after the reagent stood at room temperature for periods up to 1 h before addition of the amine. In each case the deamination reaction was then allowed to proceed for 30 min and the extent of deamination was determined. The data are presented in Table II. At pHs 3.5 and 4.0, the relative rates of conversion of D-glucosamine to anhydro-D-mannose were measured when D-glucosamine was added at periods up to 1 h after preparation of the nitrous acid reagent. The stability of the deamination reagent at pH 3.0 was determined by measuring the rate of N-desulfation of the [$^{35}SO_4$]CR-heparin. Two points are noteworthy. It is seen that in all cases the extent of substrate conversion in a 30-min reaction period is slightly greater when the deamination substrate is added 10 min after preparation of the nitrous acid than when the substrate is added at zero time. This is consistent with the previous finding that the rate-limiting step in the deamination reaction is the conversion of NO_2^- to the active deaminating species, N_2O_3 (Hughes et al., 1957). Thus during the 10-min preincubation period, a reservoir of the nitrosating reagent is generated faster than it is decomposed. In longer time intervals, however, there is a significant loss of reagent which proceeds more rapidly as the pH is lowered. However, the rate of decomposition of the deamination reagent at room temperature is slow enough at all pHs so that it is not a critical factor in the quantitative conversions of D-glucosamine residues or the N-sulfated D-glucosaminyl residues of heparin. Under the optimal conditions for these substrates (see below), the reactions are complete in 10 min.

TABLE II: Stability of the Nitrous Acid Deamination Reagent at Room Temperature.[a]

		Substrate Added Initially		Substrate Added Later	
pH	Deamination Substrate	Reaction Interval (min)	Deamination (%)	Reaction Interval (min)	Deamination (%)
3.0	CR-heparin	0–10	7	10–40	21
		0–30	21	30–60	17
		0–60	32	60–90	14
3.5	D-Glucosamine	0–10	10	10–40	30
		0–30	29	30–60	26
		0–60	39	60–90	23
4.0	D-Glucosamine	0–10	54	10–40	78
		0–30	73	30–60	71
		0–60	87	60–90	63

[a] The deamination reagents were prepared at room temperature by mixing 5 μl of 1 M aqueous $NaNO_2$ with 100 μl of 0.10 M potassium phthalate buffers at the appropriate pH. Reaction was initiated by addition of a 5-μl aliquot of a solution of the deamination substrate immediately (see under Substrate Added Initially) after the reagent had stood at room temperature for 10, 30, or 60 min (see under Substrate Added Later). The [$^{35}SO_4$]CR-heparin sample used for the pH 3 experiment contained 1 mg of the substrate per 10 μl of water; the D-glucosamine used for the experiments at pHs 3.5 and 4.0 was an 0.02 M solution in water. The percent deamination was determined by measuring the percent conversion of D-glucosamine to anhydro-D-mannose or, in the case of the CR-heparin substrate, the percent of the *N*-$^{35}SO_4$ released as free $^{35}SO_4^{2-}$, as described in Methods.

Comparison of the Present Deamination Conditions with Those Described Previously in the Literature. In contrast to the colorimetric indole reaction used in earlier studies of the deamination of amino sugars, the assays used in this study measure precisely defined deamination products. It is of interest therefore to use these assays to evaluate the effects of the most widely used literature variants of the reaction of nitrous acid with D-glucosamine and heparin. These include the procedure of Lagunoff and Warren (1962) and the −20 °C treatment described by Cifonelli (1968) as well as the earlier method described by us (Shively and Conrad, 1970) and its variation (Lindahl et al., 1973). These are compared with a final procedure which we have developed for heparin cleavage using as a basis the findings of the above studies on pH effects and nitrous acid reagent stability. The latter method is a modification of the Cifonelli procedure in which heparin is deaminated in aqueous glyme at −20 °C. The new method, referred to here as the "low pH HONO" procedure, uses an aqueous solution of freshly prepared nitrous acid at room temperature and has as its main advantages the high rate of reaction with heparin, the convenience of reagent preparation, and the absence of inorganic salts in the final reaction mixture. The data showing the comparisons of these methods are presented in Table III.

TABLE III: Comparison of Substrate Reactivities under Various Deamination Conditions.

Substrate	Reaction Conditions: Substrate Concn (mg/ml)[b]	NO_2^- (M)	pH	Temp (°C)	Time (min)	Deamination Products (% Yield)[a]: Anhydro-mannose[c]	Free $^{35}SO_4$[d]	Disulfated Disacc.[e]	Ref
GlcN	1.33	0.25	4.0	25	80	100			Lagunoff and Warren (1962)
EAG	1.33					100			
[$^{35}SO_4$]CR-Heparin	1.33					44	100	19	
N-Desulfated CR-heparin	0.53					40		15	
GlcN	1.11	3.90	3.9–4.0	25	10	100			Shively and Conrad (1970)
EAG	1.11					100			
[$^{35}SO_4$]CR-Heparin	1.11					13	34	8	
N-Desulfated CR-heparin	0.44					90		65	
GlcN	1.33	0.10	~1.5	−20	720	2			Cifonelli (1968)
EAG	1.33					2			
[$^{35}SO_4$]CR-Heparin	1.33					100	98	67	
N-Desulfated CR-heparin	0.56					0		0	
GlcN	1.33	0.33	1.5	25	10	2			This paper
EAG	1.33					2			
[$^{35}SO_4$]CR-Heparin	1.33					100	100	68	
N-Desulfated CR-heparin	0.56					2		0	

[a] Yields are given as percent of maximum obtained when deamination conditions that are optimal for the substrate in question are used. [b] Each substrate was also tested at one-tenth of these concentrations and in all cases the extent of substrate conversion was essentially the same as reported here. [c] Includes minor deamination products. [d] From [*N*-$^{35}SO_4$]CR-heparin. [e] Given as percent of the yield of anhydro-D-mannose obtained under deamination conditions that are optimal for the substrate in question.

The results indicate that D-glucosamine and EAG are completely deaminated by the Lagunoff–Warren and the Shively–Conrad conditions but do not react under the Cifonelli conditions or the low pH HONO conditions. On the other hand, CR-heparin is completely deaminated under the latter two conditions but not under the former two. Consistent with the failure of the Cifonelli or the low pH HONO procedures to deaminate D-glucosamine or EAG, these reagents also do not react with N-desulfated CR-heparin. This demonstration that the N-desulfated CR-heparin does not react under these conditions confirms the original conclusion that the deamination reaction at these low pHs does not occur in a stepwise fashion with hydrolytic removal of the *N*-sulfate groups followed by deamination of an N-desulfated intermediate.

The differences in the reactions of CR-heparin and N-desulfated CR-heparin with the two different pH 4 deamination reagents are difficult to explain. The Lagunoff–Warren procedure releases 100% of the *N*-sulfate groups from CR-heparin but gives only a 44% yield of anhydro-D-mannose. There is a correspondingly low yield of the disulfated disaccharide. Similar yields of anhydro-D-mannose and disulfated disaccharide are obtained from the N-desulfated CR-heparin. The data suggest that the Lagunoff–Warren method converts carboxyl-reduced heparin in large part to deamination products other than anhydro-D-mannitol. On the other hand the Shively–Conrad conditions convert N-desulfated CR-heparin nearly quantitatively to anhydro-D-mannose and disulfated disaccharide but with CR-heparin give less N-desulfation and anhydro-D-mannose than found for the Lagunoff–Warren procedure.

Discussion

Several structural features of glycosides of uronic acids substituted with sulfate at C2 influence their rates of hydrolytic glycoside bond cleavage. The inductive and steric effects of the C6 carboxyl group (BeMiller, 1967) and the substitution of one of the equatorial ring hydroxyls with the bulky sulfate group (Turvey, 1965) exert marked stabilization effects on the glycosyluronic acid bond. On the other hand it has been observed that a sulfate group at C2 of hexopyranoside residues renders the glycosidic bond much more susceptible to acid hydrolysis, presumably because of the electrostatic effects of the negatively charged sulfate hemiester which enhances the protonation of the glycosidic oxygen (Lloyd and Forrester, 1971). The end result of these effects is that the sulfate ester and the glycosidic bond of the iodosyluronic acid 2-sulfate residues in heparin exhibit similar acid labilities; consequently, the hydrolysis of the glycosidic bond of these residues may follow two pathways. The glycosidic bond and the sulfate ester of the L-iduronic acid residue are initially hydrolyzed at comparable rates, but after the sulfate group is lost the rate of glycoside hydrolysis decreases to a slower rate typical of uronic acid glycosides. The situation is analogous to that described for glycosides of 2-acetamino-2-deoxyhexoses in which the rate of glycoside hydrolysis is altered following loss of the acetyl group (Moggridge and Neuberger, 1938).

The present demonstration of the formation of a 2,5-anhydro-D-hexuronic acid during heparin hydrolysis describes still another pathway for cleavage of the glycosidic bond of L-idosyluronic acid 2-sulfate. In the normal acid-catalyzed cleavage of sulfate hemiesters, the sulfate group is removed by hydrolytic S–O bond scission. In the mechanism proposed here for the formation of the anhydrouronic acid, however, the acidic conditions render the sulfate group a good leaving group so that it is lost via scission of the C–O bond. Loss of the sulfate in this manner is accompanied by ring contraction and the eliminative cleavage of the glycosidic bond in a manner analogous to that observed in the conversion of 2-amino-2-deoxy-D-glucoside to 2,5-anhydro-D-mannose. The amount of anhydrouronic acid formed is determined by the relative rates of S–O and C–O bond scission prior to the hydrolysis of the glycosidic bond of the sulfated uronic acid. When the sulfate is hydrolytically removed before cleavage of the glycosidic bond, or when the glycosidic bond is hydrolyzed before loss of the sulfate, the elimination reaction no longer occurs.

A question that remains is why the hydrolysis of CR-heparin does not yield detectable amounts of 2,5-anhydro-L-hexose from the L-idose 2-sulfate residues. In the accompanying paper (Shively and Conrad, 1976) it is demonstrated that, when the stabilizing influence of the C-6 carboxyl is removed by reduction of the carboxyl to an hydroxymethyl group, the glycosidic bond of the resulting L-idose 2-sulfate moiety is extremely labile to acid such that it is cleaved virtually quantitatively before free sulfate is released. For reasons that are not entirely clear the elimination pathway is quantitatively important only in the hydrolysis of heparin; the anhydrouronic acid is not formed in the hydrolysis of 4-*O*-(2-*O*-sulfate α-L-idosyluronic acid)-2,5-anhydro-D-mannose, nor does hydrolysis of L-idose 2-sulfate yield the corresponding anhydrosugar.

The demonstration of the formation of a 2,5-anhydrouronic acid during heparin hydrolysis offers possible explanations for the anomalous behaviors observed with several of the color reactions used in the analysis of heparin samples containing sulfated L-idosyluronic acid residues. The anhydrouronic acid, unanticipated in the explanations sought for the anomalous color reactions, is at the same time a 2,5-anhydrosugar and a uronic acid, and, as such, might be expected to give unique color yields in the indole assay as well as the carbazole assay, both of which involve the treatment of samples with acid.

The second question addressed here is that of establishing a rationale for the differences in the reactions of heparin and amino sugars with the wide variety of nitrous acid preparations that have been employed in the study of structures containing amino sugars. Following the early demonstration that glycosides of amino sugars and heparin may be cleaved with nitrous acid whether the amino group is free or N-sulfated (Lagunoff and Warren, 1962), it has been shown that certain modifications of the deaminating reagent specifically cleave glycosides of N-sulfated amino sugars while others are specific for glycosides having a free amino group (Cifonelli, 1968; Lindahl et al., 1973). These differences are correlated here with the pH at which the deamination reaction is carried out. It has been established that the primary step in the deamination reaction is the nitrosation of the amine, a reaction which requires that the amino group be unprotonated (Hughes et al., 1957).

$$RNH_2 + HONO \xrightarrow{slow} RNH_2NO^+ + OH^- \qquad (1)$$

Subsequent conversions of this primary reaction product proceed at rapid rates and are not rate limiting in the overall deamination reaction. Since the deamination of N-sulfated and N-unsubstituted amino sugars gives the same products formed in identical yields (compare carboxyl-reduced heparin and N-desulfated carboxyl-reduced heparin in Table III), it is reasonable to conclude that both reactions share a common nitrosated D-glucosamine intermediate in spite of the fact that they take place at quite different pHs. Thus, an analysis of the reasons for the effects of pH in the deamination of these different substrates must focus on the reaction sequences leading to the nitrosated intermediate.

The actual reactant that is directly involved in the nitrosation of the amine is not nitrous acid itself but a product, NO·X, formed from nitrous acid in a sequence of inorganic reactions which are rate limiting in the nitrosation reaction. Several species of NO·X are effective nitrosating reagents, with the actual species which is involved in the nitrosation varying with the conditions under which the reaction is run (Hughes et al., 1957). For the reaction conditions that have been used in the deaminations of amino sugars, two nitrosating reagents are of primary concern. These are the nitrous acidium ion, formed rapidly at acid pHs by protonation of HONO

$$H^+ + HONO \rightleftarrows H_2ONO^+ \qquad (2)$$

and N_2O_3, formed by reaction of nitrite with the nitrous acidium ion.

$$HONO \rightleftarrows H^+ + NO_2^- \ (pK_a = 3.35) \qquad (3)$$

$$H_2ONO^+ + NO_2^- \rightleftarrows N_2O_3 + H_2O \qquad (4)$$

The formation of N_2O_3, a reaction catalyzed by acetate or other carboxylate ions that are often present in deamination reagents, occurs in the pH range of 2.5 to 4.0 where both H_2ONO^+ and NO_2^- are present in relatively high concentrations. At higher pHs the low concentration of H_2ONO^+ limits the rate of N_2O_3 formation; at lower pHs the low concentration of NO_2^- becomes rate limiting. Thus, in considering the effect of pH on the rate of deamination of D-glucosamine and EAG, it appears that the increase in the rate as the pH is lowered from 6 to 4 (Figure 6) is due to an increase in the H_2ONO^+ concentration, which gives a corresponding increase in the rate of N_2O_3 formation. The fall in these deamination rates as the pH is lowered still further is most readily explained by the fall in the level of the reactive free base form of the substrate at the lower pHs.

The reasons for the pH effects on the deamination of the N-sulfated D-glucosamine residues of heparin are not as obvious. It is clear that at pH 4 there are relatively high levels of N_2O_3 present since amino sugars with unsubstituted amino groups are rapidly deaminated, yet the N-sulfated amino sugars react very slowly. To explain the slow rate of the latter reaction two possibilities may be considered: (1) N_2O_3 is not an effective nitrosating reagent for N-substituted amino sugars; or (2) the substrate is not present in a reactive ionic form at pH 4. The substituted sulfamic acid is a strong acid with a weakly basic nitrogen, such that protonation of either the sulfate or the nitrogen would be minimal in the pH range over which the rate of deamination of N-sulfated D-glucosamine residues changes. Furthermore, the protonation of either group would serve to reduce the reactivity of substrate in the nitrosation reaction rather than to enhance it. Thus, the second possibility is ruled out and we are left with the conclusion that N_2O_3 does not react rapidly with the N-sulfated amino sugar. It appears therefore that the nitrous acidium ion formed by protonation of nitrous acid (reaction 2, above) is the most likely candidate for the nitrosating reagent in this reaction. As the pH of a solution of nitrous acid is lowered, the concentration of the nitrous acidium ion will increase (reaction 2) while the

rate of N_2O_3 formation (reaction 4) would be expected to decrease as the concentration of NO_2^- decreases. It has been shown previously that at low pHs weakly basic amines are deaminated by the nitrous acidium ion (Hughes et al., 1957). The conclusion here that the primary nitrosating agent for deamination of N-sulfated amino sugars is H_2ONO^+ is consistent with these earlier results.

Acknowledgment

The data in Table I were taken from a preliminary study of CR-heparin carried out by Dr. Roger L. Taylor in this laboratory. The authors acknowledge this contribution as well as Dr. Taylor's continued interest in this work.

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