Carbohydrate Research, 255 (1994) 183–191
Elsevier Science B.V., Amsterdam

Evidence of a selective free radical degradation of heparin, mediated by cupric ion

Zhengchun Liu and Arthur S. Perlin *

Department of Chemistry, McGill University, Montreal, PQ, H3A 2A7 (Canada)

(Received February 20th, 1993; accepted July 28th, 1993)

ABSTRACT

A free radical reaction generated by a mixture of Cu^{2+}, hydrogen peroxide, and ascorbate causes an abrupt reduction in the anti Xa activity of heparin by about one-half, and in molecular weight by about one-third. The product, which has the characteristics of a "low molecular weight" heparin, differs little in constitution from the intact heparin, on the basis of NMR evidence that includes data for fractions of the polymers. The free radical attack appears to occur adjacent to, rather than directly upon, some residues of α-L-iduronic acid 2-sulfate. Substitution of the Cu^{2+} with Fe^{2+}, results in a less selective alteration of the heparin. Dermatan sulfate undergoes more extensive degradation than heparin with the Cu-reagent, although its anti Xa a potency is less drastically reduced. Overall, the results are more consistent with a high degree of regioselectivity in the interaction between heparin and Cu^{2+} ion, than with a delocalized counter-ion interaction.

INTRODUCTION

An interaction between heparin and cupric ion (Cu^{2+}) or ferrous ion (Fe^{2+}) is detectable[1–3] by NMR spectroscopy under conditions that distinguish this polymer from a variety of chemically related polysaccharides and lower molecular weight substances. That is, a pronounced paramagnetic effect induced by these ions at a level of only 1 mmol of cation per mol of disaccharide unit, caused a marked broadening of the ^{1}H-1 and ^{1}H-5 signals (and of the $^{13}COO^{-}$signal) of the α-L-iduronic acid 2-sulfate residues of heparin, whereas the other carbohydrates tested exhibited no comparable type of response.

It is not clear that these cations engage a specific binding site in heparin, in contrast to a delocalized association such as observed[4,5] with another divalent cation, Ca^{2+}. However, if a specific binding is involved, it raises the possibility of carrying out Cu^{2+} or Fe^{2+} mediated free radical reactions with heparin in a regioselective manner. As precedence, molecules of DNA appropriately modified

* Corresponding author.

SSDI 0008-6215(93)E0399-L

to chelate Cu^{2+}, have been selectively degraded[6] by peroxyl radicals generated in the presence of hydrogen peroxide and ascorbic acid. That approach is adopted here with the presumed complex consisting of a mixture of heparin and cupric sulfate or ferrous ammonium sulfate.

Several degradation procedures involving peroxyl radicals have been applied[7–12], including reactions generated by such species as Cu^{2+} and Fe^{2+}. The present study is characterized by the use of a comparatively lower proportion of the metal ion and other reactants relative to the heparin, for the specific reason mentioned.

RESULTS AND DISCUSSION

Under the experimental conditions selected (see Table I) heparin was found to sustain a rapid change, a notable indication of which was an abrupt drop in its anti Xa activity. Initially, a reaction temperature of 40°C was used, in keeping with the conditions described in a preliminary report.[13] Also, the pH of the heparin solution was first adjusted to 5.0–5.4, which appears[1] to enhance the interaction with Cu^{2+}. As seen from Table I, however, the same level of reduction in anti Xa activity was effected at room temperature and without prior acidification of the heparin solution. Furthermore, there was no significant further loss in activity when the proportions of Cu^{2+}, peroxide and ascorbate were increased several fold, or the reaction times were prolonged. It was also determined from control experiments that all three components of the reagent were needed, although it is well known[7,8,11]

TABLE I

Effect of Cu^{2+}/H_2O_2/ascorbate [a] on the anti Xa activity of heparin

Reaction time (h)	Temperature (°C)	pD [b]	Anti Xa (units/mg)	Molecular size [c]
0			152	
0.5	40	5.0	107	
2	25	5.4	81	1.14
4	25	5.4	84	1.12
4 [d]	25	5.4	88	
2	40	5.0	84	1.11
4	40	5.0	86	
15	40	5.0	85	
2	25	6.5	88	1.10
4	25	6.5	90	1.09
4 [d]	25	6.5	89	
15 [e]	40	5.0	72	1.13

[a] Ratios of 5.4 μM Cu, 1.3 mM H_2O_2, and 1.3 mM ascorbate/mM heparin (disaccharide sequence weight ~640). Controls, containing Cu *without* H_2O_2 and ascorbate, or containing the latter two *without* Cu, showed no significant reduction in potency.

[b] Of the solution of heparin in D_2O, prior to the introduction of the reagents.

[c] Molecular size relative to that of the intact heparin, expressed as relative HPLC retention times.

[d] Ratios of Cu, H_2O_2, and ascorbate to heparin were *twice* those in the other experiments.

[e] Ratios of Cu, H_2O_2, and ascorbate to heparin were *twice* those in the preceding experiment.

that heparin undergoes degradation in the presence of relatively high concentrations of hydrogen peroxide alone, or together with ascorbate.

Consequently, about one-half of the anti Xa activity of the heparin was rapidly lost — as well as its activated clotting time value (ACT)[13] — and the potency of the product then remained relatively immune towards additional exposure to the reagent. This suggests that the degradative reaction could involve a high degree of selectivity for some of the antithrombin binding sites[14] within the diverse group of individual heparin molecules. However, as the product recovered from the reaction had an average molecular weight estimated to be about two-thirds that of the intact heparin, the observed reduction in anti Xa activity may well be another example of the relatively low potencies associated with "low molecular weight heparins". A number of the latter, prepared in various ways, have about one-third of the anti Xa activity of heparin and about one-half its molecular weight (e.g., see refs 15–17).

Fractionation of the reaction product.—The product from the reaction between the Cu-reagent and hog mucosal heparin, was fractionated in the form of its barium salt by graded sedimentation from water–ethanol mixtures, as had been used[18,19] for the intact heparin. Six fractions were isolated, although 70% of the material was contained in the first, least soluble, fraction (the initial fraction obtained from heparin accounted[18] for one-half of the barium salt). The anti Xa potency of this main lot was only slightly higher than that of the unfractionated product (Table II), whereas two of the minor fractions examined (nos. 3 and 5, Table II) were much less active. A similar distribution of properties was found by gel-permeation chromatography of a second preparation, whereby material eluted early as a narrow band constituted a major fraction of higher potency than the material recovered subsequently (Table II). Hence, the unfractionated product consisted largely of molecules differing relatively little in size and accounting for most of the total anti Xa activity, admixed with fewer, readily-separable molecules

TABLE II

Fractions of heparin partially degraded by the Cu-mediated reaction

Fraction [a]	Anti Xa (units/mg) [b]	Molecular size [c]
1A	91 [172]	0.96 [c]
3A	61 [112]	1.00 [c]
5A	32 [100]	1.10 [c]
1B	115	larger
2B	69	smaller

[a] The A fractions were obtained via sequential sedimentation of the barium salt; the B fractions were obtained by gel permeation chromatography.

[b] Values in square brackets are those for the corresponding fractions prepared via the barium salt of the *untreated* heparin.

[c] Molecular size relative to that of the intact heparin. Numerical values are relative HPLC retention times.

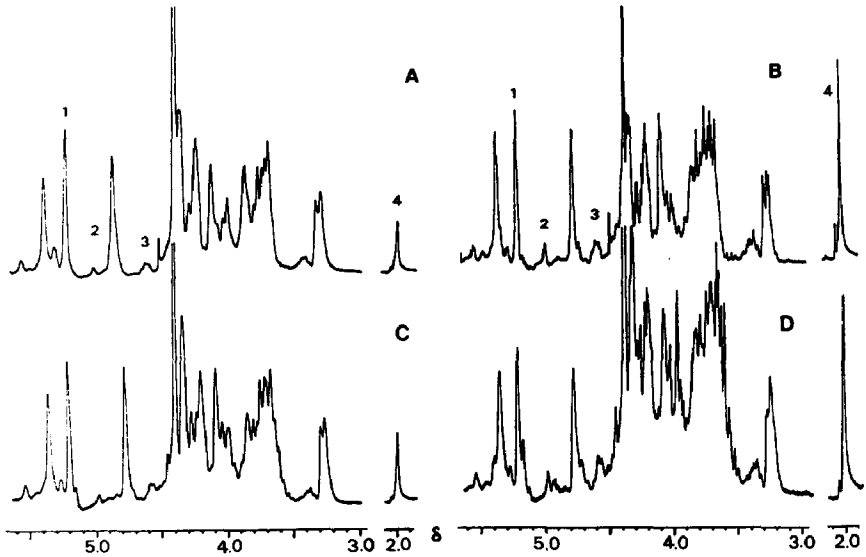
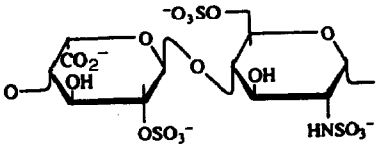


Fig. 1. ^{1}H NMR spectra (300 MHz) for solutions in D_2O at 65°C, of fractions prepared by graded sedimentation of the barium salt of porcine mucosal heparin, (A) fraction 1 and (B) fraction 5; and of the barium salt of the product recovered after treatment of the heparin with Cu^{2+}/H_2O_2/ascorbate, (C) fraction 1 and (D) fraction 5. Signal designations are for the residues of (1) α-L-iduronic acid 2-sulfate [H-1], (2) α-L-iduronic acid [H-1], (3) β-D-glucuronic acid [H-1], and (4) 2-acetamido-2-deoxy-α-D-glucose [CH_3CO].

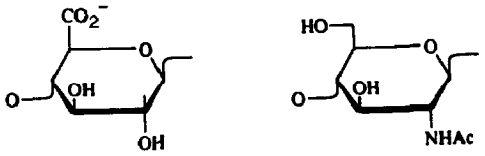
of much lower potency. Nevertheless, these lower potencies correspond qualitatively to those of the minor fractions of the intact heparin. That is, as the molecular size decreases in both series there is a decrease in anti Xa activity (Table II); (although methods other than the chromogenic assay used here may respond differently, e.g., see ref. 19).

Also noteworthy is the fact that the fractions of the Cu-treated material were characterized by differences in constitution analogous to those noted earlier[18,19] for the fractions of hog mucosal heparin itself. This was readily apparent from their ^{1}H NMR spectra, representative examples of which are presented in Figs. 1A–D. On the basis of variations in signal intensities, they showed that relative to the major constituent residues of heparin (**1** and **2**), the proportions of the residues of D-glucuronic acid (**3**) (H-1, δ 4.6), 2-acetamido-2-deoxy-D-glucose (**4**) (CH_3, δ 2.0), and L-iduronic acid (**5**) (nonsulfated) (H-1, 5.0) increased progressively with an increase in solubility (of the barium salt), and the accompanying decrease in molecular weight; i.e., the intensities of these three designated signals are relatively stronger in Fig. 1B than in Fig. 1A, and in Fig. 1D than in Fig. 1C. Hence, the major and minor fractions prepared from heparin and its Cu-treated product differ in a comparable way in constitution, as well as in potency and molecular weight.

Only minor changes in the NMR spectra were induced by the Cu treatment, most noticeable being weak signals at δ 5.17, 4.93, and 3.5–3.6 in Figs. 1C and 1D,



1 2



3 4

but not in Figs. 1A and 1B. The first of these, which is 0.03 ppm upfield of the anomeric proton signal of "normal" residues of L-iduronic acid 2-sulfate (**1**), was identified as the H-1 resonance of some additional residues of this acid. That is, a 500-MHz COSY version of spectrum (Fig. 1D) showed that the related H-2 resonance was located at δ 4.45, which is almost coincident with the major H-2 resonance of L-iduronic acid 2-sulfate. Consequently, the magnetic environment of a small proportion of these residues in heparin were affected by the Cu treatment (relatively more for the fraction represented by Fig. 1D than by Fig. 1C). If reducing end-units of L-iduronic acid 2-sulfate had been liberated by hydrolysis, their two α, β-anomeric signals would have been present near δ 5.4–5.5 (rather than at δ 5.17) in the COSY spectrum; none were detected there. Accordingly, to account for the drop in molecular size some other type of residue, positioned close to these minor iduronide residues, must have been altered chemically. The minor peaks at δ 3.5–3.6, which overlap the H-3 and H-4 signals of the various aminodeoxy-D-glucose residues (e.g., **2** and **5**), allow for the possibility that some species in that category had been subjected to radical attack, although no direct evidence of it was found.

Clearly, the small NMR spectral changes observed indicate that the proportionately large decrease in the anti Xa potency of heparin caused by the Cu-reagent was not accompanied by major changes in the constitution of the polymer. By contrast, a more extensive degradation, effected[9] by a combination of the same reactants in which the concentrations were many fold that used here, was reported to cause substantial changes in the composition of the constituent sugars of heparin. Nevertheless, the more highly degraded preparations had[9] about the same anti Xa potency as ours.

Effect of replacing Cu^{2+} by Fe^{2+}.—The abrupt drop in anti Xa activity to a level that remained relatively constant with respect to time and concentration of

TABLE III

Effect of M^{2+}/H_2O_2/ascorbate[a] on the anti Xa activity of heparin and dermatan sulfate

Polymer and metal	Reaction time (h)	Anti Xa (units/mg)
Heparin ($M = Fe^{2+}$)	0	152
	0.5	71
	8	61
	0.5 [b]	52
	2 [b]	49
	8 [b]	44
		Relative anti Xa [c]
Dermatan sulfate ($M = Cu^{2+}$)	0	1
	6	0.87 (0.85)
	15	0.81 (0.83)

[a] Ratios of 5.4 μM M^{2+}, 1.3 mM H_2O_2, and 1.3 mM ascorbate/mM polymer disaccharide unit.
[b] Ratios of M^{2+}, H_2O_2 and ascorbate to heparin were *twice* those in the preceding group of experiments.
[c] Measured for dermatan sulfate at concentrations of 12.5 and (in parenthesis) 50μg/mL, relative to a standard curve in units/mg of heparin.

reactants (Table I) suggests a highly regioselective process. Presumably, the site of attack of the peroxyl[6] (and/or other[20] species of) radicals generated in the aqueous medium during the reaction is localized by the presence of Cu^{2+}. Support for such a possibility has been obtained by the use of Fe^{2+}, which also exhibits[2] binding to heparin at a μM level, instead of Cu^{2+}. Whereas a marked decrease in anti Xa activity was observed (Table III), which paralleled observations[12] on the degradative impact of Fenton's reagent (Fe^{2+}/H_2O_2) on heparin, the downward trend continued with an increase in the concentration of reagent, and overall losses in activity were greater than with Cu^{2+}. Hence Fe^{2+} had introduced a lower degree of selectivity. This implies that the binding interactions of the two metal ions with heparin are not strictly equivalent, despite their similarities in affecting proton relaxation rates, or/and that the detailed chemistry[20] of the free radical reactions they promote are significantly different.

Reaction of dermatan sulfate with the Cu-reagent.—Also examined was the effect of the Cu-reagent on the anti Xa activity of dermatan sulfate, which is an order of magnitude lower than that of heparin. Although this polymer shows no paramagnetic response towards Cu^{2+} at the μM level[1], it was degraded to about one-half its molecular size (HPLC, uncalibrated estimate) in periods of 6–18 h. In contrast to the observation of only minor changes in ^1H NMR spectra of heparin upon degradation, here the change was striking because, as can be seen in Figs. 2A and 2B, there was a marked decrease in some of the line widths. However, this was accompanied by only a 15–18% loss in anti Xa activity (Table II). Presumably, the potency of dermatan sulfate is less sensitive to molecular size than is that of

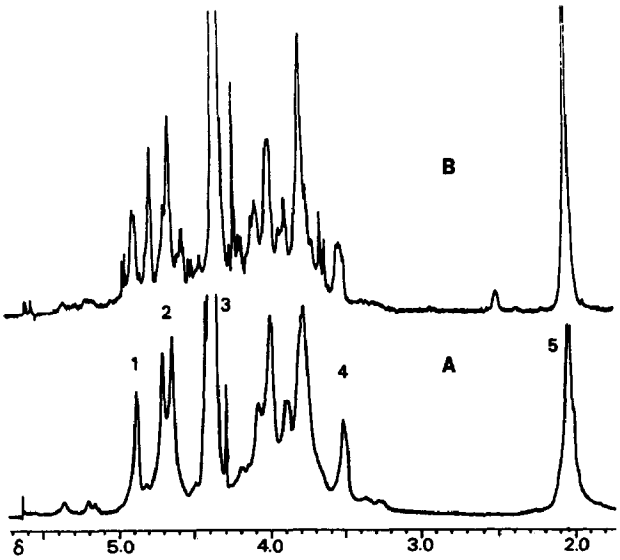


Fig. 2. ^1H NMR spectra (300 MHz) for solutions in D_2O at 65°C, of (A) dermatan sulfate, and (B) the product recovered after treatment of the dermatan sulfate with Cu^{2+}/H_2O_2/ascorbate. Signal designations for the major component residues of α-L-iduronic acid (U) and 2-acetamido-2-deoxy-β-D-galactose 4-sulfate (A): (1) U-1, (2) overlapping group for U-5, A-1, and A-4, (3) DOH, and (4) U-2; the upper portion of the acetamido methyl signal (5) is not shown.

heparin. Another possibility is that the radical attack on dermatan sulfate is less selectively focussed with respect to whatever molecular structure gives rise to the anti Xa activity, because there is no localized interaction with Cu^{2+}.

EXPERIMENTAL

General.—The NMR spectra were recorded with a Varian XL-300 spectrometer or a Varian Unity 500 spectrometer, operating at 300 or 500 MHz, respectively, for ^1H, and are referenced with respect to the signal of internal sodium, 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.0). Deuterium exchange of samples (as sodium salts) was effected by repeatedly dissolving the materials in D_2O and then evaporating the solutions prior to the NMR analysis, which was conducted with D_2O solutions containing 1–4% (w/w) of polysaccharide. The 2D (^1H, ^1H) COSY experiment was performed at 500 MHz with the Varian pulse-sequence program. HPLC analyses of molecular size were carried out with a Beckman chromatograph using an SEC 2000 column and 0.5 M NaCl as the eluate (1.0 mL/min). Measurements of anti Xa activity were performed by a chromogenic assay using an Actichrome Heparin Kit (Ortho Diagnostics, Inc., Don Mills, ON). Solutions were evaporated under diminished pressure at 40°C.

Reaction of heparin with Cu^{2+}, peroxide, and ascorbate.—In a representative experiment, a solution of sodium porcine mucosal heparin (1.0 g, 1.5 mmol) in water (12 mL) was prepared (in some experiments the pH of the solution was

adjusted to 5.4). Solutions (3 mL) containing $CuSO_4 \cdot 5H_2O$ (2.1 mg, 8.4×10^{-3} mmol), H_2O_2 (68 mg, 2 mmol), and sodium ascorbate (396 mg, 2 mmol) were then introduced in succession, and after 2 h at room temperature the mixture was subjected to dialysis (mol wt cut off, 3500) against distilled water for 24 h. The contents of the dialysis bag were passed through a short column of Amberlite IR-120 (H^+) ion-exchange resin, the effluent was neutralized with sodium hydroxide, and the product was recovered by lyophilization. Yield, 0.81 g (80%) of a pale yellow powder. Examination of the dialysate by NMR spectroscopy indicated that no heparin-like material had dialyzed out.

Fractionation of the products.—The procedure was closely similar to that described previously for the fractionation of hog mucosal heparin. The heparin (2.0 g) was converted into the barium salt by acidification in water (30 mL) with Amberlite IR-120 (H^+) ion-exchange resin, followed by neutralization (pH 7.2) with saturated barium hydroxide. Lyophilization of the solution afforded a powder, which was suspended in water, and the insoluble material was recovered by centrifugation. Ethanol was added incrementally to the supernatant, followed by centrifugation each time a precipitate appeared, a total of four times. Each of the solids recovered was converted into the sodium salt by ion-exchange, affording fractions 1 (1.46 g), 2 (0.17 g), 3 (0.16 g), 4 (0.11 g), and 5 (0.05 g).

Gel-permeation chromatography.—One g of porcine mucosal heparin, or of the product prepared from it with the Cu-reagent, was subjected to fractionation on a column (12×600 mm) of Ultrogel AcA54, using 0.3 M NaCl as the eluant, and spectrophotometric detection of the eluate absorbing at 405 nm for selecting the fractions.

Reaction of dermatan sulfate with Cu^{2+}, peroxide, and ascorbate.—A solution of dermatan sulfate (hog mucosal; Hepar Industries Ltd.) in water (1 mL), containing $CuSO_4 \cdot 5H_2O$ (84 mg, 3.4×10^{-7} mol), H_2O_2 (2.7 mg, 8×10^{-5} mol) and sodium ascorbate (15.8 mg, 8×10^{-5} mol) was stirred at room temperature for 6 h, and then subjected to dialysis (mol wt cut off, 3500) against distilled water. The contents of the dialysis bag were converted into sodium salts by ion-exchange, and recovered by lyophilization; yield, 3.1 mg (78%). The ^1H NMR spectrum of the product is shown in Fig. 2B.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council of Canada for generous support. Dr. F. Sauriol kindly carried out the 500-MHz COSY experiment.

REFERENCES

1 R.N. Rej, K.R. Holme, and A.S. Perlin, *Carbohydr. Res.*, 207 (1990) 143–152.
2 R.N. Rej, K.R. Holme, and A.S. Perlin, *Can. J. Chem.*, 68 (1990) 1740–1745.

3 Z. Liu and A.S. Perlin, *Carbohydr. Res.*, 236 (1992) 121–133.
4 A.S. Perlin and B. Casu, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 36 (1977) 108–109.
5 P. Dais, Q.-J. Peng, and A.S. Perlin, *Carbohydr. Res.*, 145 (1986) 163–179.
6 D.P. Mack, B.L. Iverson, and P.B. Dervan, *J. Am. Chem. Soc.*, 110 (1988) 7572–7574.
7 A.A. Horner, *J. Biol. Chem.*, 246 (1971) 231–239.
8 S.E. Lasker, *Adv. Exp. Med. Biol.*, 52 (1975) 119–130.
9 P. Bianchini, European Patent, 0,121,067 (1984) 1–18.
10 P. Bianchini, B. Osima, B. Parma, C.P. Dietrich, H.K. Takahashi, and H.B. Nader, *Thromb. Res.*, 40 (1985) 49–58.
11 G. Mascellani and P. Bianchini, Canadian Patent, 1,283,098 (1986) 1–29.
12 D.D. Metcalfe, H.L. Thompson, S.J. Klebanoff, and W.R. Henderson, *Biochem. J.*, 272 (1990) 51–57.
13 Z. Liu and A.S. Perlin, *Thromb. Haemostasis*, 66 (1991) 742.
14 R.D. Rosenberg, G. Armand, and L. Lam, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 3065–3069.
15 M. Verstraete, *Drugs*, 40 (1990) 498–530.
16 G.A. Neville, F. Mori, T.J. Racey, P. Rochon, K.R. Holme, and A.S. Perlin, *J. Pharm. Sci.*, 79 (1990) 339–343.
17 J. Hirsh and M.N. Levine, *J. Amer. Soc. Hematology*, 79 (1992) 1–17.
18 L. Ayotte, E. Mushayakarara, and A.S. Perlin, *Carbohydr. Res.*, 87 (1980) 297–301.
19 L. Ayotte, J.C. Lormeau, and A.S. Perlin, *Thromb. Res.*, 22 (1981) 97–102.
20 C. von Sonntag and H.-P. Schuchmann, *Angew. Chem. Int. Ed. Engl.*, 30 (1991) 1229–1253.