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Accelerated Stability Studies of Heparin

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Abstract ☐ The objective of this study was to extend our understanding of the stability of heparin. Sodium heparin, derived from porcine intestinal mucosa, was first incubated in 0.1 N hydrochloric acid and 0.1 N sodium hydroxide at 30 and 60 °C and sampled at times ranging from 0 to 1000 h. The absorbance spectra of the products formed under basic conditions showed an ultraviolet maxima at 232 nm associated with chemically catalyzed β-elimination at the uronic acid residues. The products formed under acidic conditions showed a decreased staining intensity consistent with desulfation and a decrease in molecular weight corresponding to hydrolysis of glycosidic linkages when analyzed by gradient polyacrylamide gel electrophoresis. Heparin samples were next prepared in 10 mM sodium phosphate buffer at pH 7.0 in sealed ampules that had been flushed with nitrogen and incubated at 100 °C. Samples taken at times ranging from 0 to 4000 h were then analyzed. Heparin was relatively stable over the first 500 h, after which it rapidly degraded. Heparin, assayed using both anti-factor Xa and anti-factor IIa amidolytic methods retained 80–90% of its activity over the first 500 h, but these activities dropped precipitously, to ~6% and ~0.5% of the initial activity at 1000 h and 2000 h, respectively. This rapid decomposition began only after the buffering capacity of the solution was overwhelmed by acidic degradants, which caused the pH to decrease. Decomposition processes observed under these conditions included the endolytic hydrolysis of glycosidic linkages and loss of sulfation, particularly *N*-sulfate groups, and were similar to the degradation processes observed in 0.1 N hydrochloric acid. This study provides initial observations on heparin degradation pathways. More complete, quantitative studies and studies leading to the isolation and characterization of specific degradants are still required.

Introduction

Heparin is a highly sulfated, linear polysaccharide comprised of alternating hexuronic acid and glucosamine residues. It is biosynthesized as a proteoglycan inside mast cells frequently found in certain tissues, including the intestine and the lung.[1,2] Pharmaceutical heparin is isolated from this tissue following proteolysis, it contains little or no peptide component, has an average molecular weight of 12 000, and is called glycosaminoglycan heparin.[1,2] Heparin has a wide range of important biological activities[1–4] resulting from the ability of heparin to interact with various proteins causing their activation, deactivation, or stabilization.[1,2,4,5] One of these interactions, the binding of heparin to antithrombin III, is known to take place at a specific pentasaccharide sequence present within the heparin polymer.[6–8] The interaction of heparin with antithrombin III makes it a potent anticoagulant and has resulted in its widespread clinical use.[3] Despite its widespread use, heparin has a low bioavailability when administered by routes other than intravenously and causes a number of side effects, including hemorrhagic complications.[9] These problems are related, in part, to heparin's relatively high molecular weight and has fostered the development of low molecular weight derivatives. The molecular weight of heparin is reduced through its controlled chemical and enzymatic depolymerization to afford a new class of therapeutic agents called low molecular weight heparins that exhibit improved bioavailability and a higher therapeutic index.[10]

Heparin was introduced as a clinical anticoagulant in 1937 prior to the establishment of the Food and Drug Administration. Despite the routine clinical use of heparin for nearly 60 years, very little published information about heparin's stability is available. Most of the available literature on heparin stability arises from the preparation of new heparin derivatives[10–14] and little emphasis has been placed on the basic physical chemical properties of heparin.[15–17] An increased interest in the new low molecular weight heparins has heightened the need to understand their structure. This first requires an improved understanding of the parent drug, heparin. This study examines heparin's solution stability under stressed conditions as part of a continuing effort in our laboratory to further understand heparin's chemistry.

Experimental Section

Materials—Porcine mucosal heparin, sodium salt (150 USP units/mg #231704A), was from Wyeth-Ayerst Laboratories, Princeton, NJ. Hydrochloric acid (0.1 N solution) was from Mallinckrodt, Paris, KY. Sodium hydroxide (0.1 N solution), anhydrous sodium phosphate dibasic, anhydrous sodium carbonate, and copper sulfate were from Fisher Scientific, Fair Lawn, NJ. Disodium 2,2′-bicinchoninate (4,4′-dicarboxy-2,2′-biquinoline, disodium salt) and *N*-acetyl-D-glucosamine were from Sigma Chemical Company, St. Louis, MO. Fluoraldehyde protein/peptide assay reagent was from Pierce, Rockford, IL. L-Aspartic acid was purchased from Life Technologies Inc., Grand Island, NY. Absolute ethanol was from Quantum Chemical Co., Tuscola, IL. Trimmed stem, clear, score-break, lab pack, 5 mL, USP type I borosilicate ampules were from Kimble Glass Inc., Vineland, NJ. The reagents used in electrophoresis were acrylamide, *N,N,N′,N′*-tetramethylenebisacrylamide (TEMED), and Alcian Blue from Boehringer Mannheim, Indianapolis, IN, bis-*N′,N′*-methylenebisacrylamide from International Biotechnologies Inc., New Haven, CT, and ammonium persulfate from MCB Manufacturing Chemists, Cincinnati, OH. Anti-factor Xa activity kit #832 and Spectrozyme Th were from America Diagnostica, Greenwich, CT. Fibrinidex (human thrombin) was from Ortho Diagnostic Systems, Raritan, NJ, and heparin standard K3 was from the United States Pharmacopoeia, Bethesda, MD. All other organics and inorganics were reagent grade or better.

All spectrophotometric measurements were made with a Shimadzu model UV-2101PC UV-VIS spectrophotometer or a Shimadzu model UV160 spectrophotometer and all fluorescence measurements were made with a Shimadzu model RF-540 spectrofluorophotometer from Shimadzu Corporation, Kyoto, Japan. Heparin activity was measured on an ACL 3000 plus automated analyzer from Coulter Instrument Laboratory, Miami, FL. The heating mantle was from Glas-Col Apparatus Company, Terre Haute, IN, and the voltage regulator was from Standard Elect Product Company, Dayton, OH. Samples were centrifuged on a Microfuge E centrifuge, and pH measurements were made with a ϕ40 pH meter both from Beckman Instruments, Inc., Fullerton, CA. Electrophoresis analysis was performed on a 20 cm vertical slab gel with Protean II from Bio-Rad, Richmond, CA.

Methods—*Degradation of Heparin under Acidic and Basic Conditions*—Heparin (10 mg/mL) was degraded under both acidic and

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basic conditions. The acidic and basic degradations were performed in 0.1 N hydrochloric acid and 0.1 N sodium hydroxide solutions, respectively. These solutions were incubated in water baths at both 30 and 60 °C. Aliquots were removed at various time points and neutralized with an equal volume of either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid, followed by an equal volume of 50 mM sodium phosphate buffer (pH 7.0). The absorbance of each neutralized sample was determined at 232 nm and the samples were then stored frozen at −70 °C.

Stressed Storage of Heparin Solution in Sealed Ampules at Neutral pH—A 10.0 mg/mL heparin solution was prepared in 10 mM sodium phosphate, pH 7.0. Aliquots (1 mL) were put in 5 mL clear glass ampules. Ampules underwent nitrogen evacuation of oxygen and were immediately flame sealed in a nitrogen atmosphere. Several ampules were immediately frozen at −20 °C. The remaining ampules were placed in a sand bath at 100 °C, and at various time intervals two ampules were removed, cooled to room temperature, opened, combined, and stored frozen at −20 °C for further analysis.

Measurement of Sample Color—Sample (10 μL) was diluted with distilled water (990 μl) in a 1.4 mL quartz cuvette. The absorbance (from 200 to 800 nm) of the diluted sample was determined against a water blank.

Measurement of Solid Residue—A 500 μL portion of each sample was centrifuged for 1 min and the liquid was carefully decanted away from the pellet. The pellet was freeze-dried and its mass was determined.

Analysis of Reducing Capacity—Samples were analyzed[18] to determine the moles of reducing sugar present at each time-point. The assay was performed as follows.

Reagent A was prepared by dissolving 1.5 g of disodium 2,2′-bicinchoninate in 1 L of water. This was then combined with 71.6 g of anhydrous sodium carbonate and brought up to 1.15 L with water. Reagent B was prepared by dissolving 3.5 g of aspartic acid and 5.0 g of anhydrous sodium carbonate in 100 mL water. Copper sulfate (1.09 g) was dissolved in 40 mL of water. The two solutions were mixed and diluted to 150 mL with water. Reagent C was prepared by mixing 23 mL of reagent A and 1 mL of reagent B with 6 mL of absolute ethanol. Reagent C was allowed to stand for 2 h before use.

Sample (15 μL) was mixed with 285 μL of water and added to Reagent C (700 μl). The solution was vortexed and heated at 80 °C for 30 min. The solutions were then brought to room temperature and transferred to cuvettes, and the absorbance at 560 nm was measured against a water blank. A standard curve was prepared using *N*-acetyl-D-glucosamine to calculate the nanomoles of reducing sugar present in the heparin samples.

Analysis of Free Amino Groups—Fluoraldehyde proteins/peptide assay was used to determine the moles of free amino groups formed under stressed conditions. Sample (20 μL) was mixed with water (180 μL) and then added to 2 mL of fluoraldehyde protein/peptide assay reagent and mixed well. The fluorescence of each sample was measured with excitation at 360 nm and emission at 455 nm. The fluorescence value of a blank was subtracted from the fluorescence emission values of the sample to determine the net fluorescence. A standard curve was prepared using *N*-acetyl-D-glucosamine, and the net relative fluorescence was determined as a function of glucosamine concentration. The percent of free amino groups was determined using this standard curve.

Analysis of Molecular Weight—Heat-treated heparin samples were analyzed using gradient polyacrylamide gel electrophoresis (PAGE). An equal weight amount of each sample (40−50 μg) was combined with one volume of 50% (w/v) sucrose, and the mixture was loaded into a stacking gel of 5% (total acrylamide) and fractionated with a 12−22% linear resolving gel. Molecular weight was analyzed by comparing their migration to a banding ladder of heparin oligosaccharide standards obtained from the enzymatic depolymerization of bovine lung heparin.[19,20] Electrophoresis was performed at 400 V for 4−5 h. Bromophenol Blue was used as an indicator, and throughout the run, the heat was dissipated by circulating refrigerated water (4 °C). The gel was stained and fixed with Alcian Blue in 2% (v/v) acetic acid.

Analysis of Heparin Activity—The activity of heparin was determined at each time point using anti-factor Xa and anti-factor IIa amidolytic assays. Samples together with USP heparin sodium reference standard (K3) were prepared at concentrations ranging from 0.05 to 0.21 USP units/mL in pH 8.4 buffer. The pH 8.4 buffer was composed of 6.06 g of tris(hydroxymethyl)aminomethane, 2.79 g of ethylenediaminetetraacetic acid, 10.23 g of sodium chloride, and 1 g of polyethylene glycol (PEG 8000) and adjusted to pH 8.4 with either sodium hydroxide or hydrochloric acid.

The anti-factor Xa assay used a kit containing human antithrombin III, bovine factor Xa, and Spectrozyme factor Xa chromogenic substrate reconstituted according to the manufacturer's specifications, except that the substrate was reconstituted with 7 mL instead of 5 mL of water. The assay was performed on an ACL 3000 plus automated analyzer. Sample (50 μL) was first added to 250 μL of antithrombin III (4.0 nkat/mL), 50 μL of this mixture was then added to 50 μL of factor Xa and incubated for 60 s, and the reaction was initiated by adding 50 μL of substrate (0.57 μmol/mL). The absorbance at 405 nm was determined as a function of time (3 s after addition of substrate and for a period of 240 s at 1200 rpm) and the anti-factor Xa activity was calculated using a calibration curve prepared from the heparin standard. The standards and samples were each analyzed in duplicate.

The anti-factor IIa assay used frozen, pooled, citrated, normal human plasma (Biological Specialty Corp., Colmar, PA) as a source of antithrombin III, human thrombin (factor IIa, Fibrinidex), and Spectrozyme Th chromogenic substrate. Plasma (10 mL) was diluted with pH 8.4 buffer (8.3 mL) and each ampule of Fibrinidex (50 NIH units/ampule) was reconstituted with 18.3 mL of saline affording a final concentration of 5.56 NIH units/mL. Chromogenic substrate (50 μmol/vial) was dissolved in 36.6 mL of water to afford a substrate concentration of 1.37 μmol/mL. Samples and heparin standard (0.007−0.02 USP units/mL) were prepared in pH 8.4 buffer.

The assay was again performed in a ACL 3000 plus automated analyzer. Sample (250 μL) was first added to 250 μL of diluted plasma, 50 μL of this mixture was then added to 50 μL of human thrombin, the mixture was incubated for 60 s, and the reaction was initiated by adding 50 μL of substrate. The absorbance at 405 nm was determined as a function of time (10 s after addition of substrate and for a period of 150 s at 1200 rpm) and the anti-factor IIa activity was calculated using a calibration curve prepared from the heparin standard. The standards and samples were each analyzed in duplicate.

Results and Discussion

A search of the scientific and pharmaceutical literature shows very little has been published regarding the stability of heparin.[15−17] Nagasawa and co-workers[15] performed a detailed study on the solvolytic desulfation of the pyridinium salt of heparin in dimethyl sulfoxide−water and dimethyl sulfoxide−methanol at elevated temperatures. The loss of *N*-sulfate preceded the loss of *O*-sulfate and little or no hydrolysis of glycosidic linkage occurred. This study provided a method for preparing chemically modified heparins for biological studies and as enzyme substrates.[14] Conrad[16] examined the acid lability of glycosidic bonds to L-iduronic acid, using 0.5 M sulfuric acid at 100 °C and found it to be as labile to acid hydrolysis as some neutral sugars. The hydrolysis of heparin with strong acid at 100 °C has also been used to prepare unsulfated oligosaccharides as enzyme substrates.[21]

Initial experiments in the current study were directed at examining whether conditions could be found under which heparin degradation could be conveniently studied. A second important goal was to identify the chemical processes responsible for heparin's degradation. The stability of heparin was first examined under strongly acidic (0.1 N hydrochloric acid) and strongly basic (0.1 N sodium hydroxide) conditions at 30 or 60 °C for 0−1000 h. The absorbance of the product solution at 232 nm was determined at each time point and is presented in Figure 1A. The λ_{max} for unsaturated uronic acids is at 232 nm.[22] Mild base treatment of the benzyl or methyl ester derivatives of heparin results in its fragmentation through a β-elimination reaction forming a low molecular weight heparin containing an unsaturated uronic acid at the nonreducing end of each newly formed chain.[12,13] A full absorbance scan (200−800 nm) of base-treated sample prepared in 30 mM hydro-

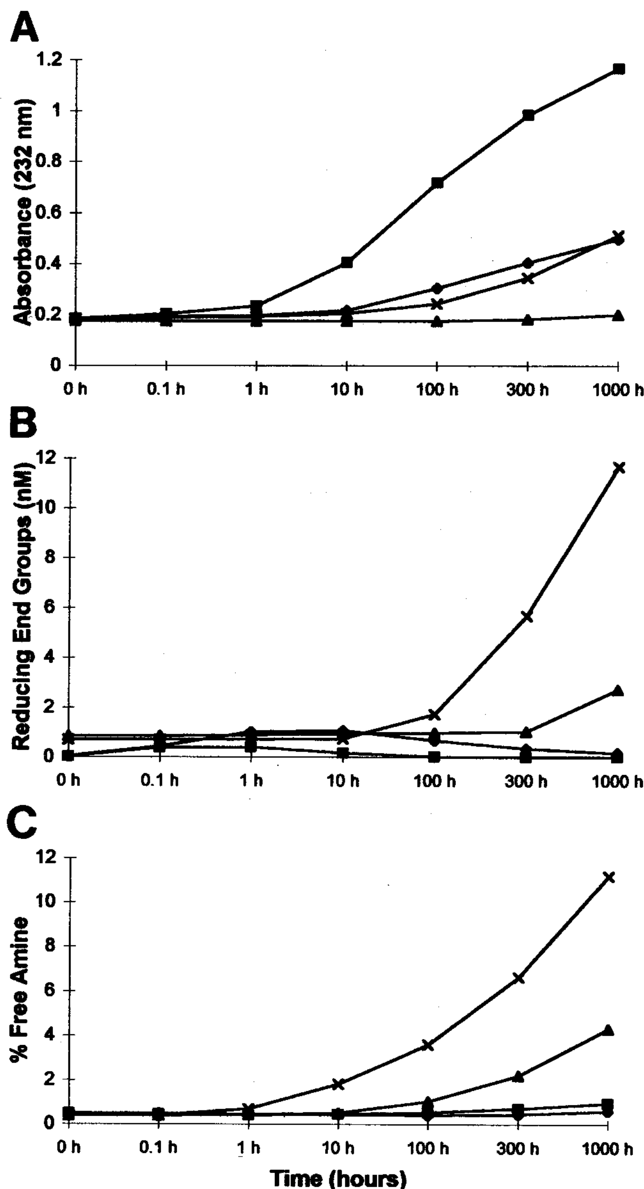

Figure 1—Absorbance at 232 nm degradation of heparin in acid and base. (A) Absorbance (232 nm) as a function of time (h). (B) Reducing ends (M) as a function of time (h). (C) percent free amino groups ($[RNH_2]/([RNH_2] + [RNHCOCH_3] + [RNHSO_3Na]) \times 100$) as a function of time (h). Panels A, B and C are plotted for heparin in 0.1 N hydrochloric acid at 30 °C (▲) and 60 °C (×) and in 0.1 N sodium hydroxide at 30 °C (◆) and 60 °C (■).

chloric acid showed a λ_{max} at 232 nm (not shown). The absorbance at 232 nm was identical before and after centrifugation, suggesting that light scattering by aggregates played little or no role in the observed increase in absorbance. These data (Figure 1A) indirectly suggest that strong base can fragment underivatized heparin through the same type of β-elimination reaction.

The degradation of heparin under strongly acidic and basic conditions was also examined using gradient polyacrylamide gel electrophoresis (PAGE). Heparin chains are separated on the basis of their molecular size and then visualized by staining with Alcian Blue dye that binds to the sulfate groups on these chains. The degradation of heparin in 0.1 N hydrochloric acid and 0.1 N sodium hydroxide as a function of time was studied at both 30 and 60 °C. Acid and base treatment both resulted in a decrease in heparin's molecular weight and the formation of low molecular weight products. Acid treatment also resulted in a marked decrease in staining intensity as a function of time, suggesting that considerable *N*- and/or *O*-desulfation had taken place.

Each heparin chain contains a reducing and nonreducing end. Each time a chain fragments, a new reducing and nonreducing end should be formed. Reducing ends can be measured using the Moppler–Gindler assay.[18] Reducing ends were measured as a function of time in both acid- and base-treated samples (Figure 1B). These data clearly demonstrate an increase in reducing ends in samples treated with acid while no such increase takes place in samples treated with base. The fragmentation of glycosidic linkages in the acid-treated samples was confirmed by gradient PAGE analysis. The results on the base-treated samples also show a decrease in molecular weight by gradient PAGE, yet surprisingly no increase in reducing ends is detected (Figure 1B). These observations can be reconciled if the aldehyde groups (detected by reducing sugar assay), at the newly formed reducing-end, are destroyed under basic conditions. The loss of reducing end aldehyde groups is widely observed when polysaccharides are treated with base in a collection of reactions known as "pealing".[23]

A reduction in heparin's sulfation, under acidic conditions, was suggested by a reduction in staining intensity on gradient PAGE analysis. The loss of *N*-sulfate groups should afford free amino groups. The rate of formation of free amino groups was determined using a fluoraldehyde-based assay. Porcine mucosal heparin has ~20 hexosamine residues per chain. It is primarily *N*-sulfated but also contains, on the average, a single *N*-acetyl group per chain.[8] Approximately 2–4% of the glucosamine residues in heparin are present as free amino groups, as determined using a sensitive fluorescence assay. This corresponds to an average of two free amino groups in every three heparin chains (assuming a molecular weight of 12 000 for heparin). The loss of *N*-sulfate groups in both acid- and base-treated products was assessed by measuring the increase in free amino groups as a function of time (Figure 1C). Examination of the change in relative fluorescence as a function of time showed a marked increase for acid-treated samples with little or no increase for the base-treated samples (data not shown). Using a standard curve, constructed with *N*-acetylglucosamine, the percentage of free amino groups ([moles of free amino groups/moles of glucosamine residues in sample] × 100) could be calculated and plotted as a function of time (Figure 1C). This shows that heparin treated with 0.1 N HCl at 60 °C for 1000 h only loses ~12% of its *N*-sulfate groups. Virtually no loss of *N*-sulfation can be detected in the base-treated samples.

These preliminary studies on the stability of heparin in 0.1 N sodium hydroxide and in 0.1 N hydrochloric acid suggested that the decomposition mechanisms observed under acidic and basic conditions were quite different. Since heparin is usually stored at neutral pH, a long term study was undertaken to examine heparin stability in a pH 7.0 solution at elevated temperature. In this study, oxygen was also excluded to eliminate oxidative degradation pathways (strong oxidative conditions have been used to prepare low molecular weight heparin[10,17]).

Samples (10 mg/mL) were prepared in sodium phosphate buffer (10 mM at pH 7.0) in ampules that were nitrogen flushed and sealed. These ampules were then heated at 100 °C for 4000 h. During this time, pairs of ampules were periodically removed, opened, combined, and frozen for subsequent analysis. All samples were analyzed as a group after the final 4000 h time point was taken.

A visual inspection of the samples all showed a yellow-brown color developed after 500 h. In addition, precipitate was clearly observed in the samples taken at the 1000, 2000, 3000, and 4000 h time points. This gave an early indication

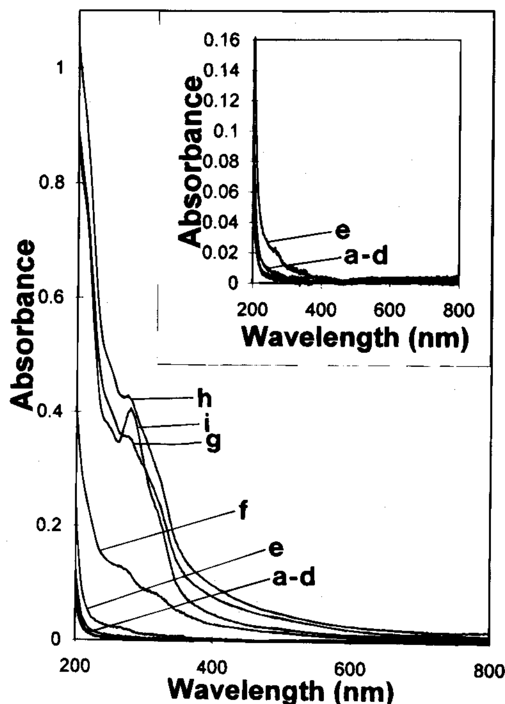

Figure 2—Absorbance is plotted as a function of wavelength (nm). Heparin treated at 100 °C. Lines a–i correspond to samples taken at 0, 10, 100, 250, 500, 1000, 2000, 3000, and 4000 h from heparin treated at 100 °C. The inset refers to enlargement of lines a-e that correspond to samples taken at 0, 10, 100, 250, and 500 h from heparin maintained at 100 °C in 10 mM sodium phosphate buffer at pH 7.0.

that substantial sample degradation had taken place after 500 h at 100 °C.

A scan of absorbance between 200 and 800 nm was performed and is shown in Figure 2. These scans confirmed that an accelerated rate of sample degradation had taken place after 500 h and suggested the formation of decomposition product(s) having λ_{max} of 210 and 280 nm. In addition to the formation of colored product(s), an insoluble residue was measured in the samples taken from 500 to 4000 h (Figure 3A). The solids formed most rapidly between 500 and 2000 h. At the end of the study the solids corresponded to 8−9 wt % of the initial sample.

The pH of the samples had been carefully adjusted to 7.0 at the beginning of the experiment. After 4000 h (167 days) the initial sample that had been sealed and stored at −20 °C had a pH of 7.1. The pH of the samples at each time point are presented in Figure 3B. The sample pH rose from 7.1 to 7.4−7.5 over the initial 500 h time period after which it dramatically decreased to a value of 4.2−3.9 from 1000 to 4000 h. While it is unclear whether the initial increase in sample pH was significant, there is little doubt that the decrease in sample pH corresponded to a degradation process initiating at 500 h and corresponding to the generation of acidic product(s), colored product(s), and insoluble particulates.

The reducing capacity of all samples was next determined using the Mopper−Gindler assay.[18] This should represent a measure of new reducing ends afforded through the breakdown of glycosidic linkages in heparin or more reactive reducing ends afforded through the chemical modification of the reducing sugar residue in a heparin chain. An increase in reducing capacity was observed throughout the first 3000 h of the reaction time course (Figure 3C). This suggests that

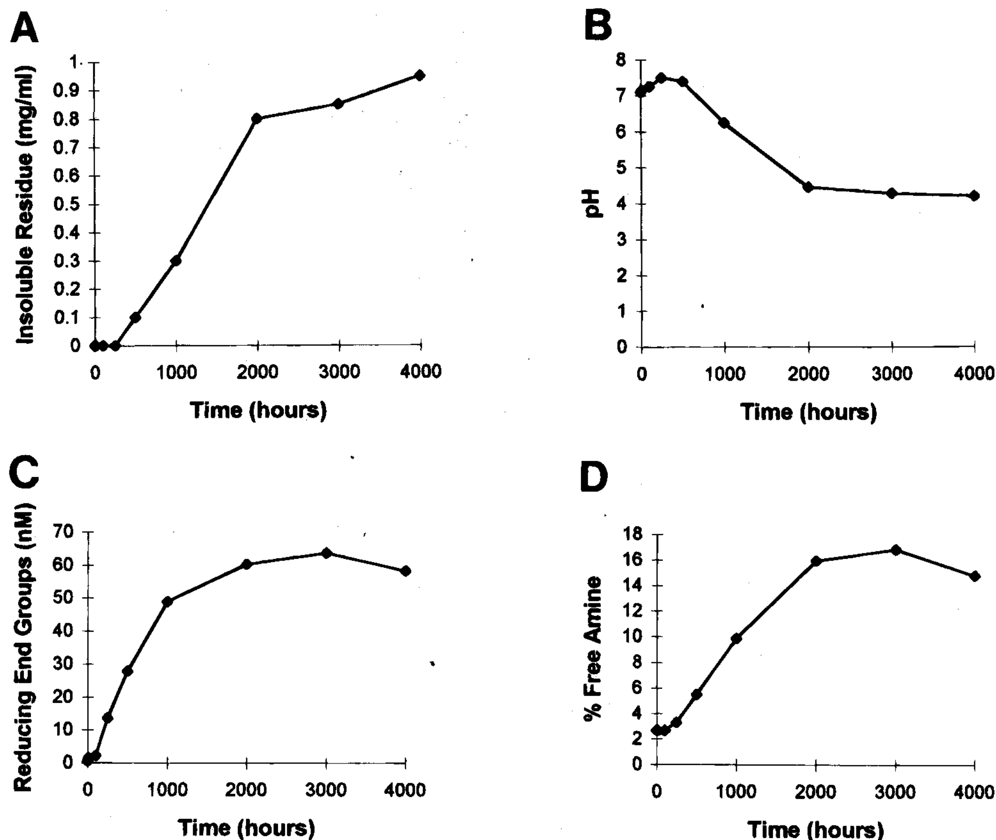

Figure 3—Degradation of heparin at 100 °C. (A) Precipitation residue (mg/mL) as a function of time. (B) pH is plotted throughout the time course of the treatment of heparin. (C) Reducing-sugar equivalents as a function of time. (D) Percent free amine as a function of time. In all cases, heparin was maintained at 100 °C in 10 mM sodium phosphate at pH 7.0.

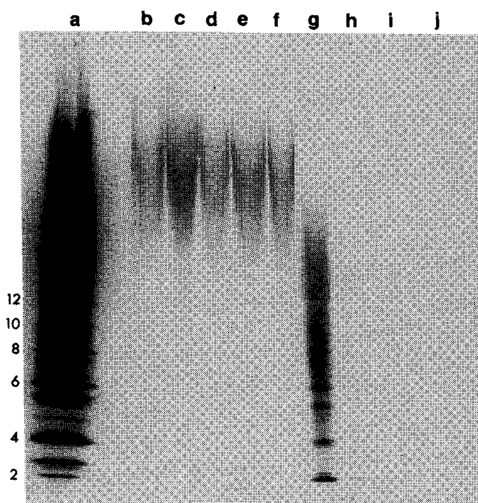

Figure 4—Gradient PAGE analysis of heparin heated at 100 °C. Lane a contains a mixture of oligosaccharide standards prepared by heparin lyase I treatment of bovine lung heparin. Numbers 2–12 correspond to dp. Lanes b–j correspond to samples taken at 0, 10, 100, 250, 500, 1000, 2000, 3000, and 4000 h. Heparin was maintained at 100 °C in 10 mM sodium phosphate at pH 7.0.

the samples are being continuously degraded over time. No substantial increase in the breakdown rate was observed after sample coloring began at 500 h. The results of this assay suggest that a continual underlying degradation process takes place at a rate independent of pH and not correlatable to the coloring of the sample or the formation of particulates.

The number of free amino groups measured by a fluoraldehyde-based assay remains unchanged during the first 250 h of the decomposition study (Figure 3D). From 250 to 2000 h there is a marked increase in free amino groups, suggesting that this decomposition process coincides with sample coloring. *N*-Sulfate groups are more labile than *N*-acetyl groups;[15,17] thus the loss of *N*-sulfation probably gives rise to the formation of free amino groups. Solvolytic loss of *N*-sulfate groups has been reported and is most pronounced at acidic pH and in the presence of amines.[15] This suggests that *N*-desulfation may be autocatalytic.

Gradient PAGE analysis of the samples showed little or no change in sample molecular weight from 0 to 500 h (Figure 4). The molecular weight, however, decreased rapidly from 500 to 1000 h, suggesting that substantial breakdown of glycosidic linkages was taking place. The staining intensity greatly diminished, particularly from 1000 to 4000 h, suggesting that not only was the sample molecular weight decreasing but substantial *N*- and *O*-desulfation was also taking place.

There are a number of assays used to measure heparin's anticoagulant activity. Heparin primarily acts through antithrombin III, a serine protease inhibitor, to catalyze the inhibition of coagulation serine proteases such as factor Xa and factor IIa (thrombin). Chromogenic assays using synthetic peptide-based substrates and purified factor Xa or factor IIa offer accurate and reliable measures of heparin activity.[24] Chromogenic assays are not subject to many of the same interferences observed in older clotting-based assays. Both anti-factor Xa and anti-factor IIa assays are dependent on the presence of a specific pentasaccharide sequence within heparin that binds to antithrombin III.[24] This sequence contains all the functional groups typically found in heparin together with an unusual glucosamine residue having a 3-*O*-sulfate group. The anti-factor IIa assay is also sensitive to the size of the heparin chain being determined, requiring a tetradecasaccharide to hexadecasaccharide length chain for full activity.[2]

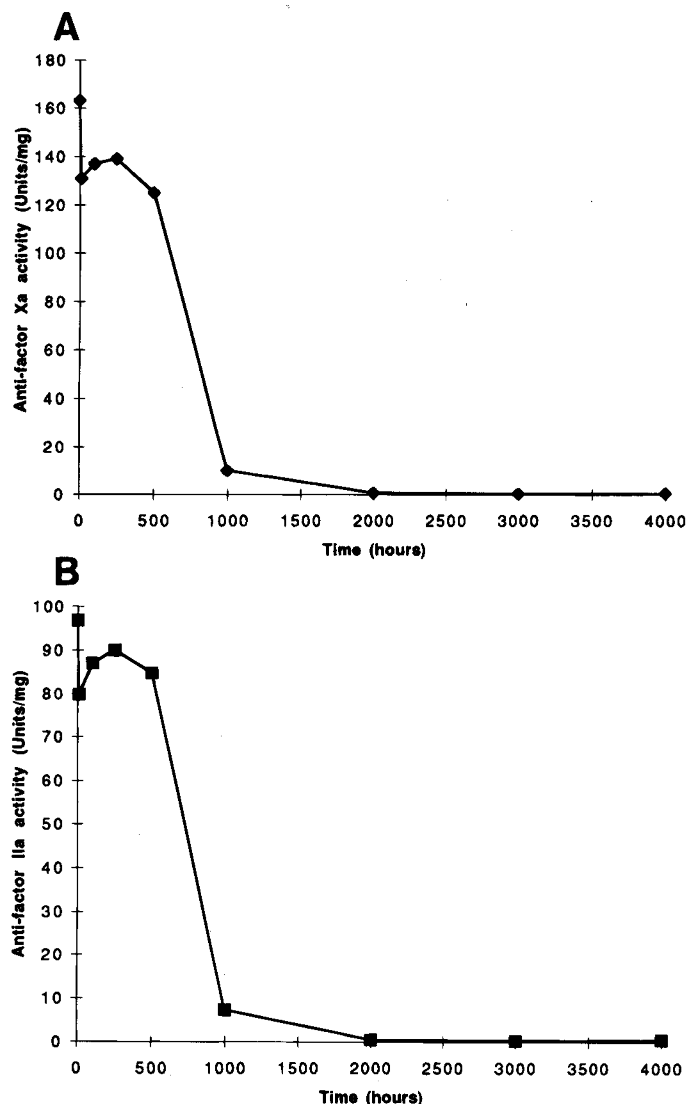

Figure 5—Heparin activity as a function of time. (A) Anti-factor Xa activity (units/mg) and (B) anti-factor IIa activity (units/mg) are plotted as a function of time in hours. The heparin samples have been maintained at 100 °C in 10 mM sodium phosphate at pH 7.0.

The anti-factor Xa activity requires only the presence of heparin's antithrombin III pentasaccharide binding site.

A plot of the anti-factor Xa and anti-factor IIa activity of heparin samples as a function of time maintained at 100 °C shows the expected drop in activity between 500 and 1000 h (Figure 5). Interestingly, the activities measured by both assays afford parallel profiles, suggesting that both anti-factor Xa and anti-factor IIa activities are lost through an identical chemical process or processes. When heparin is depolymerized through endolytic processes, to prepare low molecular weight heparin, anti-factor IIa activity is usually lost rapidly as the molecular weight of the heparin chains decrease, while anti-factor Xa activity is only lost late in the depolymerization process when the antithrombin-binding pentasaccharide sequence is destroyed.[25] Thus, while heparin has a ratio of anti-factor Xa/anti-factor IIa activity of ~1, low molecular weight heparins exhibit ratios of 2–25.[4,10] The parallel loss of anti-factor IIa and anti-factor Xa activities observed in this study suggests that (1) the increase in reducing end groups observed (Figure 3C) results from exolytic breakdown of heparin chains, (2) the loss of *N*-sulfation occurring between 100 and 500 h (Figure 3D) occurs primarily in regions of the heparin chain outside the antithrombin III binding site, and (3) catastrophic

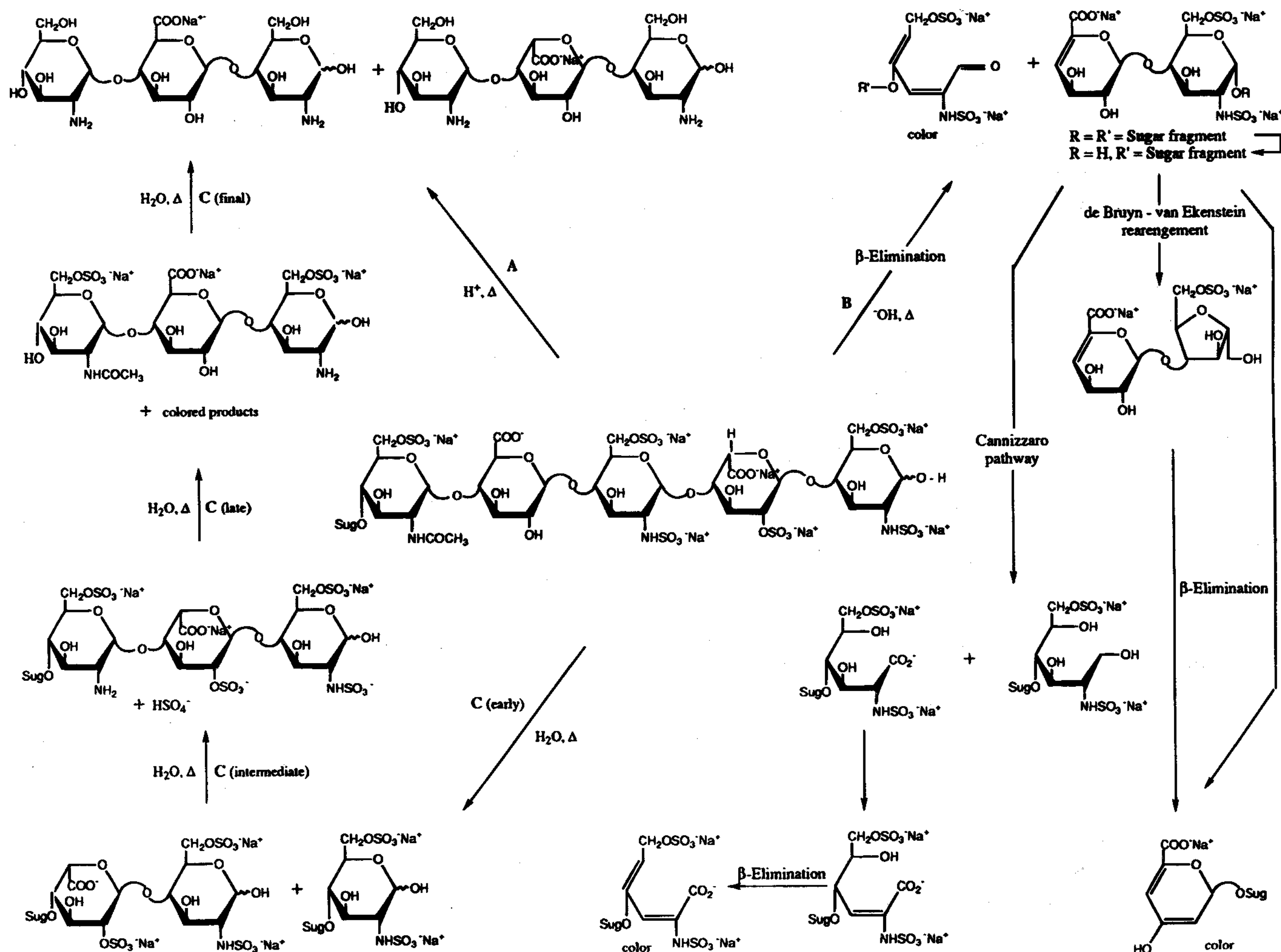

Figure 6—Proposed mechanism for heparin degradation under acidic (a), basic (b), and neutral (c) conditions. The acidic and neutral pathway terminate in a similar collection of small, desulfated oligosaccharide products that can become colored through caramelization.[26] The basic pathway is proposed to terminate in a Cannizzaro pathway[27] and a de Bruyn van Ekenstein rearrangement,[23] leading to colored products.

breakdown of the heparin chain (Figure 4) occurring at 1000 h as the sample pH drops to 6 (Figure 3B) is accompanied by destruction of the antithrombin III binding site and simultaneous loss of anti-factor Xa and anti-factor IIa activities.

The results of these detailed stability studies, summarized in Figure 6, suggest that while heparin is remarkably stable it can decompose through a number of pathways. Under basic conditions β-elimination at glycosidic linkages causes a decrease in molecular weight and the "pealing reaction" damages the reducing end aldehydic groups in the decomposition products that are formed. Little or no *N*-desulfation is observed under basic conditions. Under acidic conditions the molecular weight decreases through hydrolysis of glycosidic linkages resulting in an increased number of reducing end groups. Little or no β-elimination takes place. Loss of sulfation (probably both *N*- and *O*-sulfation) results in a decreased staining intensity on PAGE analysis. Loss of *N*-sulfates can be quantified using a fluorescence-based assay.

Sample breakdown at neutral pH begins by an increase in the reducing capacity of the sample. Since little change in molecular weight was observed by gradient PAGE or in heparin activity, it is likely that this increased reducing capacity results from either exolytic chain breakdown or from another chemical modification of the chain's reducing end, making it more susceptible to oxidation (more sensitive to the Mopper–Gindler assay[18]). Little or no change in sample pH occurs during this initial decomposition process. Between 250 and 500 h, a second process begins. Colored product(s) with λ_{max} of 210 and 280 nm and an insoluble residue begin to form. This second process is also accompanied by an increase in free amino groups probably through *N*-desulfation. Little or no change in sample pH is observed between 250 and 500 h. At 500 h a third decomposition process begins with a drop in pH resulting from acidic products, probably formed through desulfation, that overwhelm the buffering capacity of the solution to afford an acidic medium and initiate this decomposition mechanism. The molecular weight of the heparin decreases rapidly, probably through endolytic, acid-catalyzed hydrolysis of glycosidic linkages. This results in the rapid and simultaneous loss of both anti-factor Xa and anti-factor IIa activity. Following this rapid decrease of molecular weight and activity, nearly complete *N*- and *O*-desulfation takes place. These studies provide no information on the effect of heparin's decomposition on its secondary structure. Further studies are also required to identify the precise decomposition mechanisms and their kinetics.

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