Randomness in the Heparin Polymer: Computer Simulations of Alternative Action Patterns of Heparin Lyase

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SYNOPSIS

Heparin is a mixture of linear polysaccharides of undetermined sequence. Both biosynthetic data and computer simulation studies have established that each heparin polymer chain is comprised of oligosaccharides of defined sequence, representing ordered domains. One such ordered domain is a pentasaccharide corresponding to heparin's antithrombin III binding site. Previous computer simulation studies, performed under the assumption that heparin lyase (heparinase, EC 4.2.2.7), has a random endolytic action pattern, suggested that certain of these ordered oligosaccharide domains may themselves be nonrandomly arranged in the heparin polymer. The present work presents computer simulations of alternative action patterns for heparin lyase while assuming a random distribution of these oligosaccharide units within the heparin polymer. We consider action patterns that are determined solely by the primary structure of the substrate molecules. Results of the simulations are compared to (1) the experimental measurements of product chains formed throughout the reaction and (2) the change in weight average molecular weight $\overline{M}_w$ as a function of reaction completion as determined by absorbance at 232 nm.

From the simulation of 60 action patterns for heparin lyase, we infer that one of the following statements concerning heparin and heparin lyase is true: (1) Heparin is a random arrangement of a small number of structurally defined oligosaccharide units. Heparin lyase changes its action pattern during the depolymerization of heparin (perhaps influenced by the secondary structure of substrate). (2) Heparin contains clusters of oligosaccharide sequences that are present in low concentrations (overall) in the polymer. Heparin lyase has a specificity for cleaving glycosidic linkages either exolytically at the nonreducing terminus of a chain or (endolytically) at the reducing side of these rare oligosaccharide sequences.

INTRODUCTION

Heparin is a polydisperse microheterogeneous polymer whose anticoagulant activity has wide therapeutic applicability.[1] Nevertheless, the detailed chemical structure of the heparin polymer chains remains incompletely known. Mathematical modeling and computer simulation have been used to test hypotheses and refine our understanding of the mode of action of heparin lyase,[2] the distribution of bonds susceptible to cleavage in the presence of heparin lyase,[3] and the arrangement of specific oligosaccharide sequences in the heparin polymer.[4]

Computer simulation studies have now been extended, based on information concerning the molecular weight and structure of oligosaccharide products that are not cleaved in the presence of heparin lyase.[5] We use a simulation of the kinetics of depolymerization that allows us to vary the simulated action pattern of heparin lyase and the sequence of the heparin polymer. On the basis of measurements of (1) the appearance of certain free oligosaccharide products, (2) the predominance of certain types of oligosaccharides at the point of 30% completion of heparin depolymerization by heparin

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lyase, and (3) the weight average molecular weight of the heparin mixture during its depolymerization, we make predictions about the structure of heparin and the action pattern of the enzyme heparin lyase. In particular, we propose that either (1) the action pattern of heparin lyase with respect to the primary structure of heparin changes during the course of the depolymerization of heparin (perhaps influenced by secondary structural aspects of heparin molecules); or (2) certain oligosaccharide products of the depolymerization reaction occur in clusters within the heparin polymer, and that heparin lyase cleaves a linkage either exolytically, at the nonreducing termini of the polymer chains, or endolytically, between specific oligosaccharide sequences (which we describe).

THEORY

The mathematical model is based on the demonstration by Rice and Linhardt[6] that after depolymerization of heparin by heparin lyase, 80–90 wt% of the end products are members of a set of six specific oligosaccharides (**1–6**). The chemical structures of these oligosaccharides are presented in Figure 1. This set of so-called final oligosaccharides was used in simulation studies employing the assumptions that (1) heparin lyase has a random endolytic pattern of action and (2) that the heparin polymer chains consist of a random arrangement of these six final oligosacchraides.[4] After creating a simulated population of heparin chains and simulating their depolymerization catalyzed by heparin lyase, it was concluded that these two hypotheses were not consistent with experimental observations.[4]

Heparin's polydispersity and microheterogeneity were included in a computer simulation of the action of heparin lyase on a population of heparin molecules. Suppose that **M** final oligosaccharide units comprised the initial population of polymer chains. (The value of **M** used in our simulations was 16384.) A probabilistic algorithm was used to assign the type of oligosaccharide unit to each of the **M** units, which were implicitly given a linear order. The experimentally determined composition of final oligosaccharides in heparin was used to assign the probabilities.[4] The first and last oligosaccharide units of each chain in the population were determined by generating a random number for each final oligosaccharide unit and using a probability p as a cutoff value. If the random number generated for a particular final oligosaccharide unit did not exceed p then that particular unit was designated the end of a polymer chain, and the succeeding oligosaccharide unit (in the implicit ordering of units) was designated the beginning of the (next) polymer chain. The value of p selected was 0.059, in order to give a

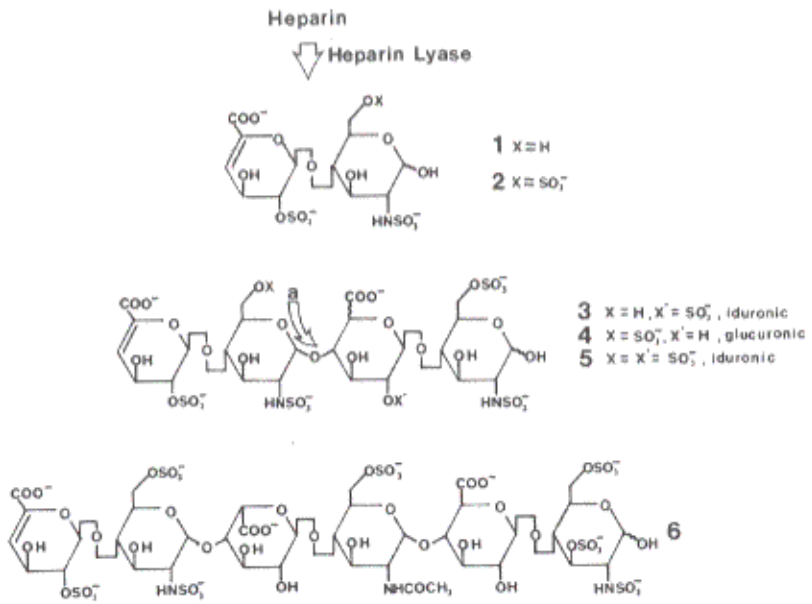

Figure 1. Chemical structures of the principal products ("final oligosaccharides") of heparin lyase-catalyzed depolymerization of porcine mucosal heparin. Glycosidic linkage (a) can be cleaved when either oligosaccharide **3** or **5** is contained within a larger oligomer as a "bound final oligosaccharide."

population of polymer chains with an expected value of 13000 for the number average molecular weight.[4] Four repetitions of the simulation of the initial population of chains (using different random number seeds) resulted in a value of 899.5 for the mean of the number of chains in the initial population of chains (the coefficient of variation equaled 3.05%). It can be shown[4] that the distribution of oligosaccharide units in the simulated population of polymer chains will ideally follow a binomial distribution, with mean $p\mathbf{M}$ and variance $p(1-p)\mathbf{M}$.

The current simulation extends the results of previous simulations[4] by varying the mechanism of cleavable bond selection by the enzyme molecules. Using one of seven choices of algorithms (Table I), the simulated enzyme selects the chain to be cleaved, either (1) probabilistically, in proportion to the number of cleavable bonds in the chain; or (2) with each chain equally likely to be selected; or (3) deterministically or probabilistically choosing one fragment from the last chain cleaved by the enzyme molecule. Next, the bond to be cleaved within the preselected chain is chosen, in one of the following ways: (1) with each bond equally likely (random endolytic mechanism); (2) favoring bonds closer to one terminus of the chain; (3) favoring bonds closer to the center of the chain; (4) deterministically or probabilistically choosing one terminus of the chain and cleaving the bond closest to that terminus (exolytic mechanism); or (5) probabilistically cleaving the bond at the reducing side of a **1**, **4**, or **6** oligosaccharide (if it occurs within the chain) or cleaving the bond closest to the nonreducing end of the chain.

In selecting chains or bonds to be cleaved probabilistically, we use the following procedure (see Figure 2). First we make a list of the **N** possible choices of chains (or bonds) in decreasing order of probability. Using a linear congruential random number generator,[7] we compute R, a pseudorandom number in the interval (0, 1). Then we divide the open interval (ϵ, 1) into a sequence of **N** subintervals, which are monotonically decreasing in length. We let the length of the jth subinterval be proportional to the probability of selecting item j from the list. (Here j is an integer, and $1 \leq j \leq \mathbf{N}$.) The constant ϵ is a nonzero floating point number whose value is less than the smallest pseudorandom number generated by the algorithm.[7] If the value of R lies in the jth interval, then the jth chain (or bond) in the list is selected. In this manner, we use a uniformly distributed random variable (the random number generator) to assign a probability distri-

Table I Algorithms for Selection of Polymer Chains and Bonds Within Those Chains (to be Cleaved)

Code for algorithms for the selection of a chain
- 1 = The probability of choosing a chain is proportional to its length.
- 2 = Longer chains are more likely to be selected.
- 3 = Shorter chains are more likely to be selected.
- 4 = Each chain containing a cleavable bond is equally likely to be chosen.
- 5 = The nonreducing-end fragment of the last cleavage is chosen.
- 6 = The reducing-end fragment of the last cleavage is chosen.
- 7 = One of the two fragments of the last cleavage is chosen.

Code for algorithms for the selection of a bond
- 1 = Each bond is equally likely to be chosen.
- 2 = Bonds near the nonreducing end of the chain are preferred.
- 3 = Bonds near the reducing end of the chain are preferred.
- 4 = Bonds near either one of the two ends of the chain are preferred.
- 5 = Bonds near the center of the chain are preferred.
- 6 = The bond closest to the nonreducing terminus is selected.
- 7 = The bond closest to the reducing terminus is selected.
- 8 = A bond closest to the one of the two termini is selected.
- 9 = Either the bond at the reducing side of oligosaccharide **1**, **4**, or **6** is cleaved, or the bond closest to the nonreducing terminus of the chain is cleaved, probabilistically, as follows:

  Probability of cleavage at oligosaccharide **1**, **4**, or **6** = $A/(A+B)$

  where A equals the number of oligosaccharides **1**, **4**, and **6** not at the reducing end of a chain and B equals the number of chains in the population.

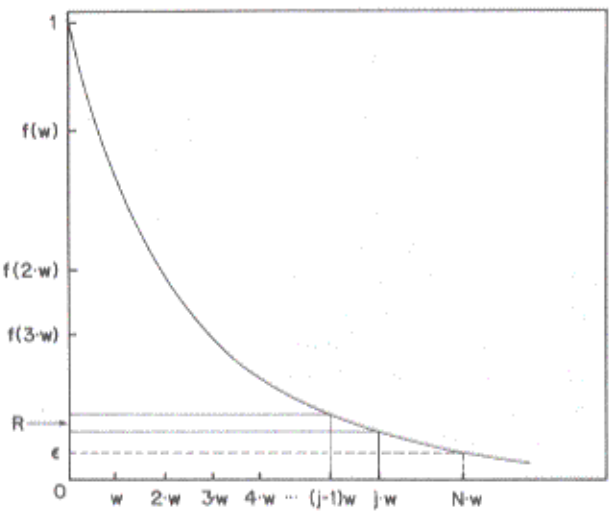

Figure 2. Illustration of the algorithm for randomly selecting an item in a list of **N** items, assuming that the items are arranged in the list in decreasing order of probability. The function $f(x) = e^{-x}$ is used to map equal intervals on the abscissa (horizontal axis) to a sequence of monotonically decreasing intervals on the ordinate (vertical axis). A random number R in the range $(\epsilon, 1)$ is generated. The interval in which it lies on the ordinate determines the ordinal number of an interval on the abscissa, which is the ordinal number of the item to be chosen from the list. In the example shown, $f(j*w) < R \leq f((j-1)*w)$, so that item number j is selected from the list. The abscissa is divided into **N** intervals of width w, where $w = -\ln(\epsilon)/\mathbf{N}$ and ϵ is discussed in the text.

bution to items in a list so that the probabilities decrease with increasing distance from the head of the list.

Two of the final oligosaccharides are dimers of other final oligosaccharides: **3** is **1** → **2** dimer and **5** is **2** → **2** dimer. Experimentally it has been demonstrated that **3** and **5** each contain a glycosidic linkage between their internal glucosamine and iduronic acid residue (linkage labeled "a" in Figure 1) that is stable under the conditions used for enzymatic treatment.[6] However, when oligosaccharide **3** or **5** is contained within a larger oligomer (as a bound final oligosaccharide), this linkage (Figure 1, linkage a) contained in **3** or **5** can be cleaved. Disaccharide **2** and tetrasaccharide **5** are the predominant oligosaccharides formed upon treating heparin with heparin lyase. A polymer composed entirely of either of these oligosaccharides can be written as $(\mathbf{2})_n$. If cleavage of a $(\mathbf{2})_n$ chain occurs randomly, then the product distribution at the end point can be proven[4] to be ideally 50 mol % **2** and **50** mol % **5**.

The mathematical relationships among free and bound final oligosaccharides can be described as follows. Let $y_i(t)$ represent the number of molecules of oligosaccharide i incorporated within a larger chain at time t. Let $x_i(t)$ equal the number of molecules of oligosaccharide i not incorporated within a longer chain at time t, and let K_i denote the total number of number of molecules of oligosaccharide i in the population of chains. To avoid double counting, we define $y_3(t) \equiv 0$ and $y_5(t) \equiv 0$ for all times t. We can use conservation of mass to write the following equations:

$$K_1 = x_3(t) + x_1(t) + y_1(t) \quad (1)$$

$$K_2 = x_2(t) + 2 \times x_5(t) + x_3(t) + y_2(t) \quad (2)$$

$$K_4 = x_4(t) + y_4(t) \quad (3)$$

$$K_6 = x_6(t) + y_6(t) \quad (4)$$

Adding Eqs. (1) and (2) and rearranging, we get

$$x_1(t) + x_2(t) = -2 \times (x_3(t) + x_5(t)) + K_1 + K_2 - (y_1(t) + y_2(t)) \quad (5)$$

This equation and the relationship between **1**, **2**, and **3** and between **2** and **5** (remember **3** = **1** → **2** and **5** = **2** → **2**) suggests plotting

$$x_1(t) + x_2(t)$$

on the vertical axis against

$$x_3(t) + x_5(t)$$

on the horizontal axis, where t is the time since the beginning of the depolymerization reaction. At time t_{end}, the end point of the depolymerization, the resulting graph for points representing different simulated action patterns of heparin lyase will be a straight line. Note that we may rescale the results of the simulation by multiplying the conservation Eqs. (1)–(4) by a constant factor. This will enable us to compare the results of experimental data with the simulation, even though the number of moles of final oligosaccharides will be different between the experimental and the simulated data.

We wish to compare the effects of varying the pattern of action of heparin lyase on the ratio of $x_1(t) + x_2(t)$ to $x_3(t) + x_5(t)$. The values of constants K_1 and K_2 are 613 and 14,535, respectively,

for all of the simulations. The laboratory values of K_1 and K_2 neglecting oligosaccharides **1** and **2** contained within chains other than the "final oligosaccharides" (a mass balance of 80–90% is obtained based on oligosaccharides **1–6**), are 5.62 nmol and 134.44 nmol, respectively. The ratios of simulated to observed K_1 and K_2 are used to determine the scale factor of 108.6 $nmol^{-1}$ used to convert experimental values for 100 μg heparin undergoing depolymerization[4] to simulated values.

MATERIALS AND METHODS

Chemicals

Heparin sodium salt, from porcine intestinal mucosa (160 units/mg), was obtained from Hepar Industries (Franklin, OH). Heparin lyase (heparinase, EC 4.2.2.7) was purified from *Flavobacterium heparinum* [5 m-units/μg (1 m-unit = 1 nmol of product formed/min)] or purchased (11 m-units/μg) from Sigma Chemical Co. (St. Louis, MO), and both were free of contaminating activities that could act either on the heparin polymer or on its depolymerization products.[8] All other chemicals were reagent grade.

Equipment

Strong anion-exchange (SAX) high-pressure liquid chromatography (HPLC) was performed using two LDC-Milton Roy (Riveria Beach, FL) Constametric III pumps with gradient control by digital to analogue interface using an Apple IIe microcomputer running Chromatochart software from Interactive Microware Inc. (State College, PA). The system was equipped with a Rheodyne (Cotati, CA) 7125 injector and an ISCO (Lincoln, NE) Model 1840 variable-wavelength uv detector. Separations were performed on a 4.6 mm × 25 cm SAX column of 5-μm particle size from Phase Separations (Norwalk, CT). Ultraviolet spectroscopy was performed on a Shimadzu (Tokyo, Japan) Model UV-160 spectrophotometer.

Methods

Heparin Depolymerization. Heparin (16 mg in 1 mL) was depolymerized at 30°C with 1 m-unit/mL heparin lyase in a solution of 0.2 *M* sodium chloride/5 m*M* sodium phosphate at pH 7.0. During the reaction time course (at 0, 60, 120, 180, 240, 300, and 600 min), 100-μL aliquots were collected and immediately frozen on dry ice. The percent of reaction completion was determined by measuring the absorbance of the product, obtained at each time point, at 232 nm after a 1 : 100 dilution in 30 m*M* hydrochloric acid. This absorbance was divided by the final product absorbance measured at 600 min, and the resulting fraction was multiplied by 100.

Kinetic Analysis by SAX-HPLC. The aliquots were collectively heated in a water bath at 100°C for 1 min to thermally inactivate the enzyme. SAX-HPLC was performed by injecting a 10-μL sample containing 160 μg of product or 10 μL of diluted sample (1 : 20 in distilled water) containing 8 μg of product into a fixed 200-μL sample loop attached to the SAX column. The column was preequilibrated with 0.2 *M* sodium chloride and eluted using a 165-mL linear gradient from 0.2 to 1.5 *M* sodium chloride, pH 3.5, at a flow rate of 1.5 mL/min.

RESULTS AND DISCUSSION

Accumulation of information on the structural regularities of the heparin polymer allows more sophisticated and realistic mathematical models to be constructed, which in turn, can guide or redirect experimental investigations. In 1982 Linhardt et al.[2] showed that a simple model of the structure of heparin demonstrates that the enzyme heparin lyase has a random endolytic pattern of action. The data used to support that conclusion were simultaneous measurements of viscosity and absorbance at 232 nm during the course of the depolymerization. Linhardt et al.[2] measured viscosity from which $\overline{M}_w$ was calculated during the course of the depolymerization of heparin.[9,10] Their mathematical model represented each heparin polymer chain as a sequence of identical disaccharide units, with bonds between successive disaccharides susceptible to cleavage by heparin lyase. The simulated value of $\overline{M}_w$ was computed at various times during the simulation of the depolymerization of heparin and compared with the experimentally measured values. Two independent characteristics of the selection of the bond to be cleaved were considered: (1) random or nonrandom and (2) endolytic (anywhere within the polymeric chain) or exolytic (only at a terminus of a polymeric chain). Of the four combinations of these characteristics, random endolytic gave the best agreement with experimental data. Each chain was given equal probability of selection, independent of its length.

In 1985 Linhardt et al.[3] used more detailed experimental data to propose that heparin lyase cleavable α-glycosidic linkages were randomly distributed within the heparin polymer. Experimentally, they classified the products of depolymerization into six groups: disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides, and oligosaccharides having degree of polymerization of 12 or more. Three types of distributions of cleavable bonds were compared, using a two-state Markov model for determining the bond at the nth site in a chain of disaccharide units. One state represented the occurrence of a cleavable bond at the $(n-1)$th site and the other state represented the occurrence of a noncleavable bond at the $(n-1)$th site. After determining the locations of the cleavable bonds in the population of simulated heparin chains, the depolymerization of the population was simulated, assuming the random endolytic action of heparin lyase. Each member of the population of simulated chains at the end point of the reaction was placed in one of the six categories (based on the number of monosaccharide moieties) and the distribution of molecules in the categories compared with experimental findings. The case of random distribution of cleavable bonds gave better agreement with experimental data on the distribution of lengths of products of depolymerization by heparin lyase than did the cases of clustering of cleavable or of noncleavable bonds.

In 1989 we utilized data on the rate of appearance of six oligosaccharide products of depolymerization and a simulation of the kinetics of depolymerization to argue that heparin lyase does not select bonds to be cleaved randomly and endolytically.[4] These six oligosaccharides accounted for 81% of the weight of the product mixture after 600 min of depolymerization, and were termed "final oligosaccharide fragments" (Figure 1). This mathematical model had two simplifying assumptions: (1) heparin polymer chains consist of sequences of final oligosaccharides, randomly arranged; and (2) heparin lyase has a random endolytic pattern of action. The conclusion of this study was that the experimental data did not support the coexistence of these two assumptions.

We have now tested the effects of varying the action patterns of heparin lyase while maintaining the assumption that heparin is a random arrangement of final oligosaccharides. In Figure 3, we present the experimentally determined concentrations of final oligosaccharides during the time course of the depolymerization.[4] Note that the oligosaccharide **2** contributes 48% of the weight of the product chains. In addition, **3**, **4**, and **6** each contain a disaccharide structure corresponding to **2**, while **5**

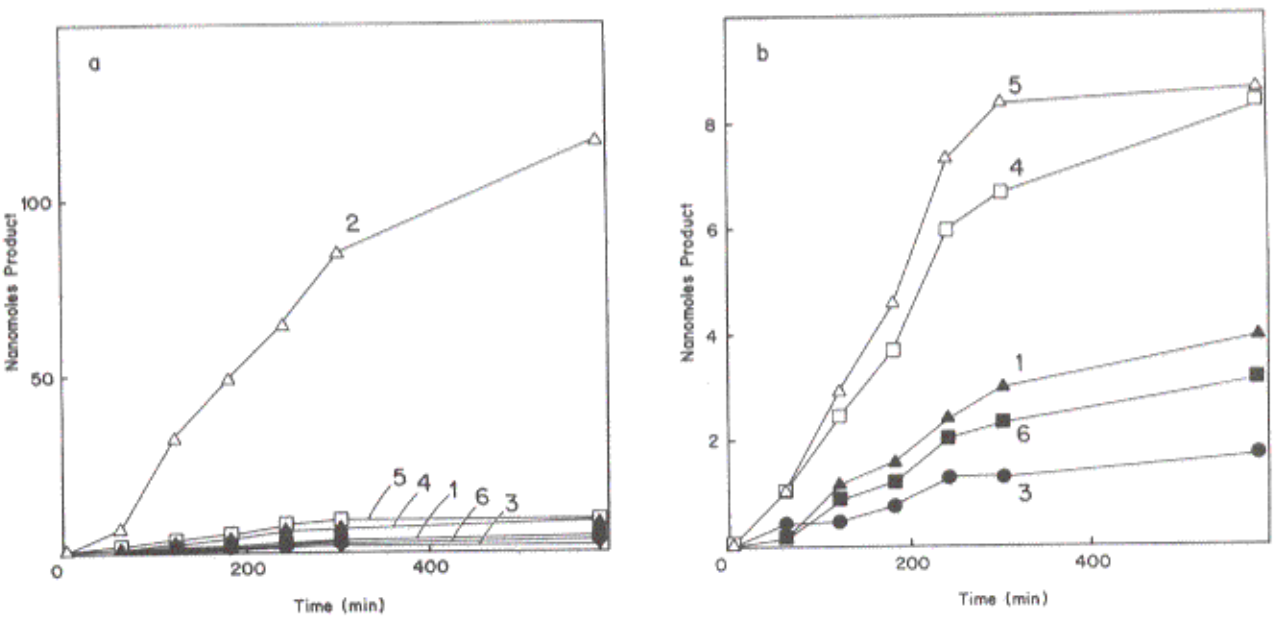

Figure 3. Plot of experimentally determined concentrations of final oligosaccharides (**1–6**) vs time during heparin lyase-catalyzed depolymerization of heparin. Panel (a) shows all six oligosaccharides plotted in the same scale while (b) removes oligosaccharide **2** from the plot. Measurement of the kinetics was performed exactly once, owing to the 90 min of time required for the analysis of each data point. The coefficient of variation of these HPLC determinations in our laboratory is $\leq 5\%$.[5,6]

contains two such structures, bringing the heparin polymer to a total of 89.5 wt % and 87.8 mole % content of disaccharide comprising **2**. The experimentally determined nanomoles of final oligosaccharide product chains per 100 μg of heparin has been reported[4] as follows: **1** (3.94), **2** (115.62), **3** (1.68), **4** (8.32), **5** (8.57), **6** (3.15), and unidentified products (14).

The results of the simulations were analyzed by plotting the sum of end point concentrations of oligosaccharides **1** and **2** vs the sum of end point concentrations of oligosaccharides **3** and **5**, using Algorithm 1 (Table I) for selecting a chain and each of the nine algorithms for selecting a bond (see Figure 4). Variation of the algorithm for selecting a chain to be cleaved did not appreciably affect the results (i.e., the rate of product formation) of any of the simulations that were performed (data not shown). Chain selection algorithm 1 was chosen for all the simulations shown since it is consistent with mass action. It selects a chain probabilistically, by assigning to each chain a probability that is proportional to the number of final oligosaccharides it contains. As proven in the theory section, the simulated data points lie on a straight line. Thus Figure 4 is useful to cluster the bond selection algorithms by their tendency to form free disaccharides **1** and **2**. The ordering of the action patterns (1–8, from Table I) with respect to increasing concentration of **1** + **2** formed at the end point of the simulated depolymerization is the following: (1) preference for bonds near the center of the chain (bond selection algorithm 5); (2) random endolytic cleavage (algorithm 1); (3) probabilistic exolytic cleavage, preference for bonds near one of the termini of the chain (algorithms 2–4); and (4) deterministic exolytic cleavage (algorithms 6–8).

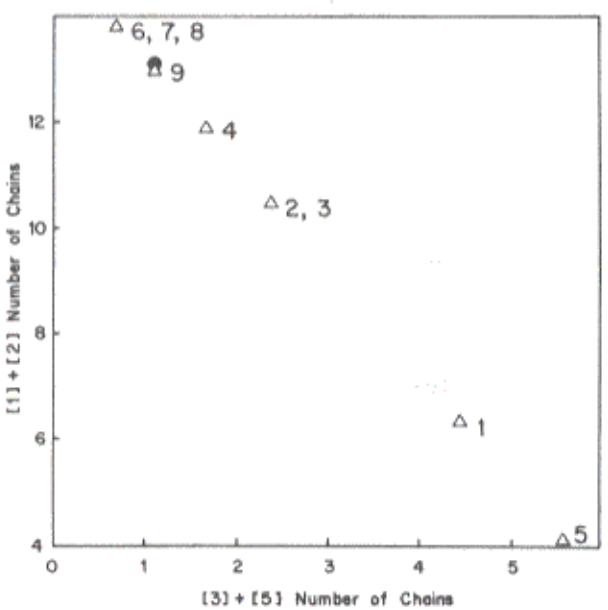

Figure 4. Plot of sum of number of chains (in thousands) of free final oligosaccharides **2** and **1** vs sum of number of chains (in thousands) of free final oligosaccharides **3** and **5** at the end point of the depolymerization of heparin by heparin lyase. The results of experimental measurements (●) are shown, as well as the results of simulating action patterns 1–9 (△) for selecting a bond within a chain. See Table I for the codes of the action patterns. Each simulated data point is the mean of four independent simulations (using different random number seeds). The coefficient of variation is ≤ 5% for every simulated data point.

At 30% of completion of the depolymerization of heparin, the products of the reaction are oligosaccharides **1**–**6** as well as higher oligosaccharides still containing heparin lyase-cleavable sites. A preponderance of these higher oligomer chains are comprised of oligosaccharide **2** with either oligosaccharide **1**, **4**, or **6** at their reducing terminus (i.e., $(\mathbf{2})_x \rightarrow \mathbf{1}$, $(\mathbf{2})_x \rightarrow \mathbf{4}$, or $(\mathbf{2})_x \rightarrow \mathbf{6}$, where $x = 1, 2, 3, 4, \ldots$).[11] In order to favor the formation of oligomers of structure $(\mathbf{2})_x \rightarrow \mathbf{1}$, **4**, or **6**, we chose to simulate heparin lyase cleavage of linkages either at the reducing side of oligosaccharide **1**, **4**, or **6**, or at the nonreducing terminus of a polymer chain (bond selection algorithm 9). The experimental datum (after suitable rescaling) lies closest to the action pattern (9) in which the enzyme probabilistically cleaves either at the nonreducing terminus of a chain, or at the reducing side of oligosaccharide **1**, **4**, or **6** within a chain. Thus on the basis of the relative concentrations of oligosaccharides **1**, **2**, **3**, and **5** at the end point of the depolymerization, and on the preferential formation of $(\mathbf{2})_x \rightarrow \mathbf{1}$, **4**, or **6** oligomers at 30% reaction completion, we find that action pattern (9) for heparin lyase most closely agrees with experimental data.

As another means of selecting from among the possible action patterns of heparin lyase, we compared change in weight average molecular weight $\overline{M}_w$ of heparin during its depolymerization by heparin lyase (taken from Ref. 2) with values of weight average molecular weight at various times during the simulated depolymerization. The graphs of $\overline{M}_w$ vs percent completion of depolymerization for the simulation of the nine bond selection algorithms (and using algorithm 1 for chain selection) are shown in Figure 5. The mechanisms for selecting a bond form a set of nonoverlapping curves, from de-

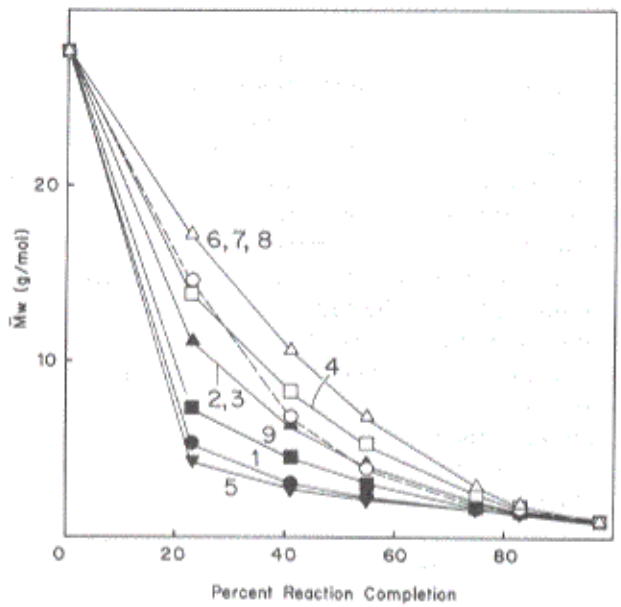

Figure 5. Graph of weight average molecular weight in thousands vs percent reaction completed for several action patterns (1–9) and for the experimental values (– – –) calculated from viscosity measurements. The experimental data are an average of 2 determinations. Each simulated data point is the mean of four independent simulations (using different random number seeds). The coefficient of variation is ≤ 5% for every simulated data point.

terministic exolytic (6–8, with the greatest value of $\overline{M_w}$ throughout the depolymerization reaction), to probabilistic exolytic (2–4), to random endolytic (1), to probabilistically choosing the central region of a chain (5, with the smallest value of $\overline{M_w}$ throughout the depolymerization reaction). We infer that the exolytic mechanisms decrease the value of $\overline{M_w}$ of the population of chains more slowly than does the random endolytic mechanism or preference for bonds near the center of the chains. (This fact will be used later.)

We can compare the simulated values of $\overline{M_w}$ with the calculated values of $\overline{M_w}$ obtained from previously reported values of viscosity changes during depolymerization of heparin, suitably rescaled (Figure 5). The molecular weight of heparin can be estimated within 2% by a linear transformation of the relative viscosity of heparin, in the molecular weight range of 4000–10,000 amu.[2,9,10] From zero to approximately 20% completion of reaction, the experimentally obtained value of $\overline{M_w}$ lies between the deterministic exolytic and probabilistic exolytic mechanisms (between bond selection algorithms 6–8 and 2–4, respectively). After approximately 20% of the reaction is completed, the observed value of $\overline{M_w}$ falls off more rapidly than either of the exolytic mechanisms would allow, suggesting that a change to a different mechanism has occurred. The mechanism (bond selection algorithm 9) that most closely agrees with experimental observations of oligosaccharides **1**, **2**, **3**, and **5** at the end point of depolymerization (see Figure 4) causes the value of $\overline{M_w}$ to decrease more rapidly between 0 and 20% completion than is observed experimentally.

Simulation of $\overline{M_w}$ vs percent of completed reaction provides a second criterion for evaluation of a proposed action pattern of heparin lyase, and should be viewed together with the data on the relative amount of oligosaccharides **1** + **2** and **3** + **5** at the end point of the simulation. Since $\overline{M_w}$ can be simulated as a continuous function of time, it provides a more detailed (although indirect) examination of the depolymerization reaction than simply looking at the concentrations of end products of the reaction. The graphs of $\overline{M_w}$ vs percent of completed reaction for the prototypical mechanisms, described by the algorithms given in Table I, may suggest answers to questions related to the pattern of action of heparin lyase. The experimental data are compatible with an action pattern (9) that cleaves linkages either exolytically or at the reducing end of oligosaccharides **1**, **4**, or **6** (Figure 4). However, Figure 5 does not agree with this mechanism, but indicates the occurrence of a biphasic mechanism, which is initially exolytic-like and progressively changes to a mechanism that prefers to cleave near the center of chains. We speculate that the helical coil of the heparin polymer (absent in oligomers) may be responsible for a change in the enzyme's action pattern as the reaction progresses.

We may use the predominance of chains of the form

$$(\mathbf{2})_x \rightarrow \mathbf{1}, \mathbf{4}, \text{ or } \mathbf{6}$$

(where x is 1, 2, 3, or 4) at 30% of completion of the depolymerization, to argue that heparin lyase possesses some specificity for linkages at the reducing side of oligosaccharide **1**, **4**, or **6**. Based on this specificity of heparin lyase, we can conclude that the locations of these types of final oligosaccharides within the polymer chains will determine the location of many of the linkages to be cleaved during the first 30% of the depolymerization reaction. In all the models discussed in this paper thus far, we assume that the final oligosaccharides are randomly distributed within the polymer chains, in amounts

proportional to their mole fractions (which are observed after depolymerization). Our present results of computer simulations suggest the apparent insufficiency of randomly distributed final oligosaccharides with respect to agreement with experimental data on (1) concentrations of final oligosaccharides at the end point of depolymerization and (2) the change of $\overline{M}_w$ (determined from measurements of viscosity) over time.

Consider the hypothetical case of clustering of oligosaccharides **1**, **4**, and **6** within the heparin polymer. Assuming the random distribution of these *clusters* (or regions of low density of oligosaccharide **2**) within the population of polymer chains and the specificity of heparin lyase for linkages **2** → **1**, **2** → **4**, or **2** → **6**, each cluster results in one cleavage of the "random endolytic" type, followed by a sequence of "pseudoexolytic" cleavages within the cluster, which release oligomers of the form

$$(\mathbf{2})_x \rightarrow \mathbf{1}$$

$$(\mathbf{2})_x \rightarrow \mathbf{4}$$

$$(\mathbf{2})_x \rightarrow \mathbf{6}$$

where x is a small positive integer. Let A_c denote the average number of final oligosaccharides (excluding oligosaccharide **2**) per cluster. Then as A_c increases, the number of random endolytic cleavages decreases, because for a fixed size population of final oligosaccharides, the number of clusters varies inversely with A_c. As A_c increases, the beginning of the depolymerization of heparin by heparin lyase will result in more chains of short length, because each cleavage is more likely to occur within a cluster. Thus the reaction becomes more like an exolytic reaction with respect to the sizes of the product chains. Furthermore, owing to the action pattern of heparin lyase, the concentrations of oligosaccharides **1**, **2**, **3**, and **5** at the end point (see Figure 4) will be independent of the value of A_c. Clustering of oligosaccharides **1**, **4**, and **6** thus changes the graph of $\overline{M}_w$ vs percent completion (Fig. 5) without changing the quantities graphed in Figure 4.

The results of the simulations lead us to conclude that either (1) heparin is a random arrangement of free final oligosaccharides and heparin lyase changes its action pattern during the depolymerization reaction (perhaps using information contained in the secondary structure of the substrate molecules); or (2) final oligosaccharides **1**, **4**, and **6** are clustered within the heparin polymer, and heparin lyase has a specificity for cleaving the linkage at the reducing side of oligosaccharides **1**, **4**, and **6** as well as for exolytically cleaving the linkage at the nonreducing terminus of a chain.

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