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Homogeneous, Structurally Defined Heparin-Oligosaccharides with Low Anticoagulant Activity Inhibit the Generation of the Amplification Pathway C3 Convertase *in Vitro**

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This paper demonstrates that heparin-oligosaccharides with low anticoagulant activity have a high capacity to inhibit activation of the amplification pathway of complement *in vitro*. We prepared heparin-oligosaccharides by partial depolymerization of heparin using purified flavobacterial heparinase. The resulting oligosaccharide mixture was then fractionated using strong anion exchange-high pressure liquid chromatography to produce individual oligosaccharide components of this mixture, with degree of polymerization ranging from 2 to 16. These heparin-oligosaccharides were examined for both their anticoagulant activity and capacity to inhibit activation of the amplification pathway of complement. Although there was little difference among commercial heparins, a correlation between molecular weight and activity to inhibit convertase generation was clearly established for heparin-oligosaccharides between degree of polymerization 2 through 16. Heparin-oligosaccharides of degree of polymerization 10–16 (M_r 3888–5320) demonstrated up to 54% of heparin's activity on a molar basis (and up to 163% of heparin's activity on a weight basis) in inhibiting the amplification pathway of complement *in vitro* while showing almost no anticoagulant activity. These studies, for the first time, completely separate heparin's ability to inhibit complement activation from its anticoagulant activity.

Heparin is a highly sulfated, polydisperse, α-(1→4) linked copolymer of uronic acid and glucosamine (1). It is synthesized as a proteoglycan (M_r approximately one million) attached to a protein core which is removed in commercial processing to obtain glycosaminoglycan heparin (average M_r 10,000–14,000) (2). In addition to its major application as an anticoagulant (3), heparin has a multiplicity of other biological activities including the ability to regulate angiogenesis (4) and other cell growth and proliferation processes, activate and release plasma lipoprotein lipase (5), and inhibit complement activation (6–13). Although heparin can be isolated from a variety of mammalian and nonmammalian tissues, its principle location in man is in the granules of tissue mast cells and basophils. Because heparin's primary location is so closely linked to the immune response, its ability to inhibit complement activation has become an active area of interest.

In 1929 Ecker and Gross (9) first demonstrated the capacity of heparin to regulate complement activation. Subsequently, other investigators demonstrated multiple sites in the classical (10–13) as well as the alternative amplification pathways (6–8) of complement at which heparin may act (14). We previously demonstrated that heparin and heparin-oligosaccharides can inhibit the cell-bound alternative amplification pathway C3[1] convertases, C3b,Bb, and C3b,Bb,P (6) as well as fluid-phase consumption of B by D in the presence of C3b, suggesting a direct action on C3b (6).

Heparin's anticoagulant activity has been shown to be mediated through a specific oligosaccharide sequence on the heparin polymer capable of binding antithrombin III (15, 16). Heparin's structure-activity relationship on the complement system, however, is still poorly understood. Studies using fractionated heparins demonstrate that heparin with a high level of sulfation has a high level of activity in the inhibition of complement activation (17). Fractions of heparin prepared using antithrombin III-affinity chromatography have different anticoagulant activities but have little differences in their activity on complement activation (18). Studies using chemically modified heparins suggest the presence and degree of *O*-sulfation primarily controls whether heparin is inhibitory (17, 18). In contrast the presence of carboxylate groups and *N*-sulfation, which are required for anticoagulant activity, appear to play little or no role in inhibition of complement activation (17, 18). Studies in our laboratories using size-fractionated heparins and heparin-oligosaccharides first demonstrated a minimum size requirement of approximately M_r 3500 for heparin-oligosaccharides to have activity to inhibit complement activation equal on a weight basis to commercial heparin (8). This earlier study also reported that structurally defined, homogeneous, very low molecular weight heparin-oligosaccharides are capable of inhibiting the activation of complement. The potency of these very low molecular weight heparin-oligosaccharides is between 2–5% on a weight basis (0.1–0.3% on a molar basis) of heparin's activity (8).

In the present study we prepared 18 heparin-oligosaccharides having degrees of polymerization ranging from 2 to 16. Several of these oligosaccharides had potency equal to or

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[1] The abbreviations used are: C3, the third complement protein; C3b, the activated third complement protein; C4b, the activated fourth complement protein; HPLC, high pressure liquid chromatography; B, P and D are letter components of the alternative amplification pathway of complement; EAC4b,3b, sheep erythrocyte cellular intermediate containing surface C4b and C3b; EAC4b,$3b^5$, EAC4b,$3b^{20}$, EAC4b,$3b^{100}$, cellular intermediates produced from EAC1,4b using 5, 20, and 100 μg of C3b/1 $\times 10^9$ EAC1,4b, respectively.

greater than heparin on a weight basis in ability to regulate complement activation while having little or no anticoagulant activity. Activity and structural studies of these oligosaccharides establish clearly for the first time the complete separation of anticoagulant activity from activity on the complement system.

EXPERIMENTAL PROCEDURES

Materials

Commercial porcine mucosal heparin was obtained from Hepar, Franklin, OH (164 units/mg), Elkens-Sinn., Cherry Hill, NJ (1000 units/ml, H1), Fluka (140 units/mg, H2), Daiichi, Tokyo, Japan (166.6 units/mg, H3), Evans BP-Mucous (H4), Sigma (143 units/mg, H5), and U. S. Biochemical Corp., Cleveland, OH (154 units/mg, H6). Bovine lung heparin was obtained from Sigma (149 units/mg, H7) and Calbiochem (~200 units/mg, H8). Low molecular weight heparin was from Hepar (R & D, 98 units/mg, H9). Heparinase (heparin lyase, EC 4.2.2.7) was either prepared by fermentation of *Flavobacterium heparinium* (19) and purified as previously described (20) or it was purchased from Sigma. Spectrapore-6 dialysis tubing with M_r cut-off 1000 was from Spectrum Laboratories. Blue dextran, sodium azide, Sephadex G-10, purified human Factor IIa (thrombin), and carbazole were from Sigma. Sodium, 5,5-diethylbarbital and sodium 5,5-diethylbarbituric acid were from Mallinckrodt. Gelatin was purchased from Difco. EDTA, magnesium chloride, and dextrose were from Fisher. Activated thrombofax reagent was from Ortho Diagnostics, Raritan, NJ. Kabi Coatest kit used to measure antithrombin III-mediated anti-factor *X*a activity was from Helena Laboratories, Beaumont, TX. Antithrombin III was prepared from plasma (21). Fresh frozen plasma collected over 3.7% trisodium citrate was purchased from the University of Iowa Hospitals Blood Bank. Rat serum was obtained from Rockland, Inc., Gilbertsville, PA. Spherisorb strong anion exchange-HPLC columns, 4.6-mm × 25-cm and 5-μm particle size (analytical), and 1 × 25-cm and 5-μm particle size (semipreparative) were from Phase Separations, Norwalk, CT. The gel permeation-HPLC column, (TSK G2000 SW), 7.5 × 40-cm, was from Phenomenex, Palos Verdes, CA. All other chemicals were reagent-grade.

Methods

Preparation of Commercial Heparins—All heparin samples were prepared by dissolving approximately 20 mg into 1 ml of water and dialyzing (at 4 °C) first against 10 volumes of 1 M sodium chloride followed by 3 × 100 volumes of deionized water. After dialysis the heparins were removed from their dialysis bags and syringe-filtered (0.45 μm, Millipore HV) into preweighed vials, freeze-dried, and dessicated over anhydrous calcium chloride for two days. The heparin samples were then each carefully weighed and made up to concentrations of exactly 20 mg/ml with distilled water. These stock solutions were stored at −70 °C and dilutions were made for use in uronic acid, complement, and anticoagulant activity assays.

Preparation of Heparin-Oligosaccharides—Heparinase, 0.25 unit (1 μmol/min) of purified enzyme (50 units/mg) or commercial enzyme (0.23 units/mg), was added to 300 mg of heparin (167 USP units/mg, Hepar) in 37.5 ml of 250 mM sodium acetate, 2.5 mM calcium acetate solution at pH 7.0. The mixture was incubated at 30 °C for 40 h, aliquots were removed periodically and absorbance was measured at 232 nm after diluting the sample 100-fold in 0.03 N hydrochloric acid. The reaction was terminated at 30% completion (absorbance at 232 nm/final absorbance at 232 nm = 0.30) by heating to 100 °C for 1 min. The product was frozen at −70 °C, freeze-dried, and reconstituted with distilled water to a volume of 4 ml. Each sample was then desalted on Sephadex G-10, collected, freeze-dried, and reconstituted with 6 ml of distilled water to a concentration of 50 mg/ml.

Fractionation of Heparin-Oligosaccharides—Heparin-oligosaccharide mixture (20 mg) was fractionated on a strong anion exchange-HPLC semipreparative column pre-equilibrated with 0.2 M sodium chloride at pH 3.5 at a flow rate of 1.5 ml/min. A linear salt gradient (0.2–0.92 M) over a 120-min time period was used to elute the oligosaccharides. Fractions from each peak were combined, freeze-dried, reconstituted with 4 ml of distilled water, and desalted either by Sephadex G-10 permeation on a 3 × 45-cm column at a flow rate of 3 ml/min (samples with degree of polymerization <6), or by dialysis against 3 × 1000 volumes of deionized water (samples with degree of polymerization >6). The samples were freeze-dried and stored at −70 °C.

Structural Characterization of Heparin-Oligosaccharides—Analytical strong anion exchange-HPLC was used to refractionate oligosaccharides having a degree of polymerization of 10 and below. Purity was also assessed by analytical strong anion exchange-HPLC and gradient polyacrylamide gel electrophoresis and each of these heparin-oligosaccharides showed a single major component (>90%) at the expected elution time (or migration distance). Following desalting and concentration by freeze-drying, each heparin oligosaccharide was exhaustively treated with heparinase, resulting in its breakdown to a mixture of fundamental oligosaccharides of known structure (see Table I) designated *2*, *4A*, *4B*, *4C*, and *6C*. Analytical strong anion exchange-HPLC was then used to determine the identity and molar equivalents of each component in the resulting mixture. The degree of polymerization of each heparin-oligosaccharide was also established using gel permeation HPLC and gradient polyacrylamide gel electrophoresis (22).

Quantitation and Analysis of Heparin-Oligosaccharides—Freeze-dried samples were dissolved in distilled water to a volume of 1 ml, and aliquots of each were removed, added to 0.03 N hydrochloric acid, and the absorbance was measured at 232 nm. The concentration of each sample was then estimated based on its molecular weight calculated either directly from its structure or estimated from its degree of polymerization (22) and a molar absorptivity value of 5.2×10^3 M^{-1} cm (23). Based on these results the more concentrated samples were further diluted until all samples were at approximately the same concentration (1 mg/ml). Quantitation of these 1 mg/ml stock solutions relied on uronic acid determination by carbazole assay against a standard curve constructed using the heparin from which they were prepared (24). Residual sodium chloride was determined by direct measurement of conductance against a standard curve constructed using sodium chloride solution.

Complement Buffers, Complement Components, and Assays—Gelatin (0.1%) in veronal-buffered saline (0.375% sodium 5,5-diethylbarbital, 0.57% sodium 5,5-diethylbarbituric acid (veronal), 0.9% sodium chloride) was prepared at pH 7.5 and was combined 1:1 with an aqueous solution of 5.0% dextrose, 1.0 mM magnesium chloride, and 0.30 mM calcium chloride just prior to complement assays and used as the complement assay buffer. Gelatin (0.1%) in veronal-buffered saline at pH 7.5 was also mixed 1.16:1 with 86 mM EDTA,

TABLE I

Structure of heparin-oligosaccharides having degree of polymerization from 6 to 10

The abbreviations used are: ΔUA, 4-deoxy-α-L-threo-hex-4-enopyronosyluronic acid; p, pyranose; GlcA, glucuronic acid; IdoA, iduronic acid; S, sulfate.

Heparin-oligo-saccharide	Oligosaccharide[a,b] composition	M_r	No. of sulfate groups
6A	*2,4A*	1893	8
6B	*2,4B*	1893	8
6D[c]	*2,4C*	1995	9
8A	*2,2,4A*	2456	11
8B	*2,2,4B*	2456	11
8C	*2,6C*	2498	10
8D[c]	*2,2,4C*	2660	12
10A	*2,2,2,4A*	3223	14
10B	*2,2,2,4B*	3223	14
10C	*2,2,6C*	3163	13
10D[c]	*2,2,2,4C*	3325	15

[a] No sequence is implied by the order of the fundamental oligosaccharide components.

[b] The structures of the fundamental oligosaccharides making up each transient oligosaccharide are: *2*, trisulfated disaccharide, ΔUAp2S(1→4)-α-D-GlcNp2S6S; *4A*, pentasulfated tetrasaccharide, ΔUAp2S(1→4)-α-D-GlcNp2S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S; *4B*, pentasulfated tetrasaccharide, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S6S; *4C*, hexasulfated tetrasaccharide, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S; *6C*, septasulfated hexasaccharide, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→4)-α-D-Glc-NAcp6S-(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S3S6S.

[c] The oligosaccharide composition of this heparin-oligosaccharide can only be arranged in one way since 4C = *2,2*, thus this oligosaccharide's sequence is established.

pH 7.4, just prior to assays to produce 40 mM solution of EDTA; rat serum was diluted 1:20 in this buffer as a source of terminal components in the complement assays.

C3 (25), B (26), D (27), and P (28) were purified to homogeneity and quantitated as described. C3b was generated from purified C3 as described previously (6).

EAC4b,3b cellular intermediates were prepared as described previously (29, 30). Heparin and heparin-oligosaccharides were examined for ability to inhibit generation of the alternative-amplification pathway C3 convertase as previously described (6-8). Briefly, inhibition was examined in experiments that used EAC4b,3b which were prepared to have a high, intermediate, and low amount of C3 on the cell surface; EAC4b,$3b^{100}$, EAC4b,$3b^{20}$, and EAC4b,$3b^{5}$ were produced using a C3 input of 100, 20, and 5 $\mu g/10^9$ EAC4b,2a cellular intermediates, respectively. Complement assay buffer (100 μl) alone was added to tubes used for the reagent blank, no inhibition, and 100% lysis; or complement assay buffer containing a heparin or heparin-oligosaccharide dilution was added to the remaining tubes (Falcon 2052). At time 0, 100 μl of complement assay buffer containing a suspension of 1×10^7 EAC4b,3b, an excess amount of P and D (100 ng of P), and an amount of B needed to lyse the noninhibited tubes at an average of one hemolytic event/cell (0.03 ng of B for EAC4b,$3b^{100}$, 0.15 ng of B for EAC4b,$3b^{20}$, and 0.50 ng of B for EAC4b,$3b^{5}$) was added to each tube. The tubes used for the reagent blank contained no B. The mixtures were incubated for 30 min at 30 °C with shaking. Then 300 μl of a 1:20 dilution of rat serum in 40 mM EDTA was added to each tube, and incubation was continued with shaking for 60 min at 37 °C. Saline (1.5 ml) was then added to each tube except the 100% tube which was lysed with 1.5 ml of water in place of the saline. Finally, the contents of the tubes were mixed well, centrifuged, and the absorbance of the supernatant was measured at 414 nm. The percent lysis, the average number of hemolytic sites/cell (Z), and the percent inhibition were then calculated (6-8, 30). Data are expressed as either precent inhibition or as 100 times the ED_{50} (the amount of heparin resulting in 50% inhibition) for heparin divided by the ED_{50} for heparin-oligosaccharide. Concentration of heparin and heparin-oligosaccharides are expressed as $\mu g/1 \times 10^7$ cellular intermediates. The volume of the reaction tubes during convertase generation varied between 0.2 and 0.3 ml; the number of cellular intermediates was held constant at 1×10^7/tube. The amount of lysis (Z) seen in the noninhibited tube was relatively independent of the volume of buffer present in the tube (0.1-0.5 ml) during convertase generation. Similarly, the amount of inhibition of convertase generation was independent of the volume of buffer present in the tube (0.1-0.5 ml) during convertase generation.

Anticoagulant Activities—The anticoagulant activity of each heparin and heparin-oligosaccharide was determined by activated thromboplastin time (31). Antithrombin III-mediated anti-factor IIa and anti-factor Xa activities were measured using purified plasma proteins as described previously (31).

RESULTS

The partial enzymatic depolymerization of heparin produced a controlled and reproducible distribution of heparin-oligosaccharides (Figs. 1 and 2). Both commercial and purified heparinase preparations gave identical results and were used on a wide variety of commercial porcine intestinal mucosal, bovine lung, and low molecular weight heparins to prepare all of the heparin-oligosaccharides described in this paper. At 30% reaction completion, the mixture contained the maximum number and concentration of heparin-oligosaccharides with degree of polymerization between 10 and 16. This oligosaccharide mixture was then fractionated on the basis of charge using strong anion exchange-HPLC (Fig. 2) to obtain

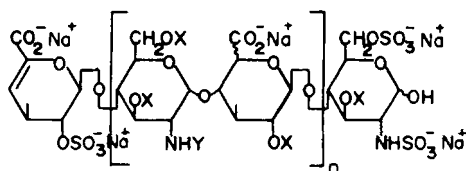


FIG. 1. Structure of heparin-oligosaccharides, where X = H, $SO_3^-Na^+$; Y = H, $COCH_3$ or $SO_3^-Na^+$, and n = 0-7.

purified heparin-oligosaccharide samples of a defined degree of polymerization and containing a single major component (32). Elution order from this column was dependent on degree of polymerization (*i.e.* disaccharide *2* followed by tetrasaccharides *4*, followed by hexasaccharides *6*, etc.), and within each size group, elution order was dependent on degree of sulfation (*i.e.* tetrasaccharide *4A* and *4B* having five sulfates followed by tetrasaccharide *4C* having six sulfates). Refractionation of these oligosaccharide components produced heparin-oligosaccharides of sufficient purity to obtain a compositional analysis (Table I). The sequence of heparin-oligosaccharide *2*, *4A*, *4B*, *4C*, and *6C* had been previously established (33, 34). Only one sequence was possible for hexasaccharide *6D*, octasaccharide *8D*, and decasaccharide *10D*, since their compositional analysis indicated they were simply oligomers of heparin-disaccharide *2*.

Next we compared the ability of heparin to inhibit EAC4b,3b containing a high surface concentration of C3b with EAC4b,3b containing a low surface concentration of C3b (Fig. *3A*). This experiment demonstrates that heparin's inhibitory activity was reduced as C3b loading was increased (6). In this experiment the amount of B input was increased as the concentration of C3b on the cellular intermediate was decreased in order to maintain an average of 1 hemolytic event/cell (Z). To determine whether the B input alone might play a role in determining the degree of inhibition, we examined the ability of heparin to interfere with the assembly of effective convertase on EAC4b,$3b^{20}$ in the presence of low, moderate, and high concentrations of B (Fig. *3B*). The amount of B present in the cellular intermediates did not have any effect on the ability of heparin or heparin-oligosaccharide to inhibit lysis.

The regulation of complement activation by heparin-oligosaccharides was first examined using a disaccharide (*2*), tetrasaccharides (*4A*-*4C*), and a hexasaccharide (*6C*) of defined sequence (22, 33, 34). These samples exhibited low activity, giving linear dose-response curves from 1 to 8 $\mu g/10^7$ cellular intermediates (Fig. 4), consistent with our earlier observations (8). Heparin-oligosaccharides of higher degree of polymerization, from 6 to 16 having molecular weights ranging from 1893 to 5320, were then tested for activity. These results were compared with heparin's activity on a molar basis. As the size of the heparin-oligosaccharides increased there was a linear increase in the inhibition of convertase activity (Fig. 5). A high molecular weight heparin-oligosaccharide (*16B*), (M_r 5320), was 54% as potent as heparin on a molar basis and 136% on a weight basis.

To further understand the relationship of oligosaccharide structure to complement regulatory activity, we also examined 11 pure heparin-oligosaccharides of defined composition ranging in size from hexasaccharide through decasaccharide (Fig. 6). These homogeneous heparin-oligosaccharides displayed lower anticoagulant activity (Table II) and virtually identical complement inhibitory activity as compared to their less purified counterparts. We also examined the ability of these homogeneous oligosaccharides to inhibit cellular intermediates prepared to have different concentrations of surface C3b. Lysis of EAC4b,$3b^{5}$ was inhibited by 32, 57, and 70% by 4 $\mu g/10^7$ cellular intermediates of dp6, dp8, and dp10 oligosaccharides, respectively. In contrast, lysis of EAC4b,$3b^{100}$ was inhibited by 15, 45, and 57% by the same concentration of dp6, dp8, and dp10 oligosaccharides, respectively.

Heparins from a variety of commercial manufacturers were also investigated for anticoagulant activity and for ability to inhibit complement activation (Fig. 7). Five porcine heparins had complement-inhibiting activities ranging from 90 to 107%

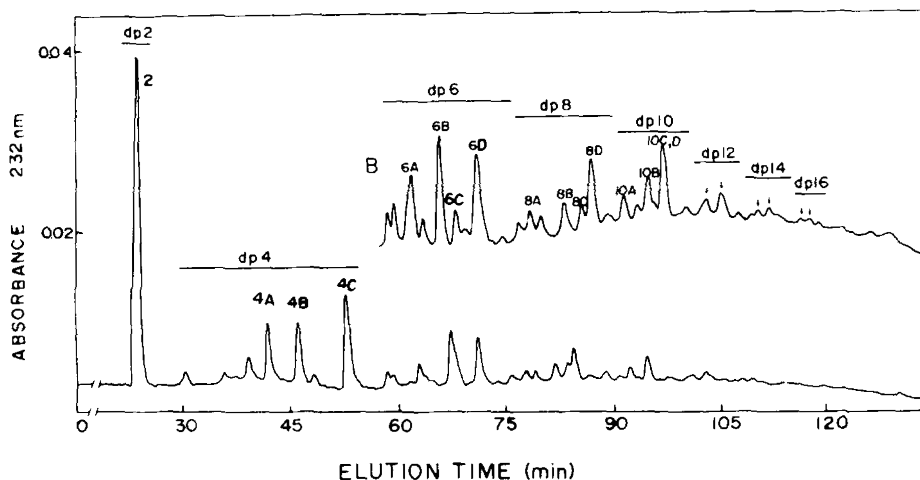
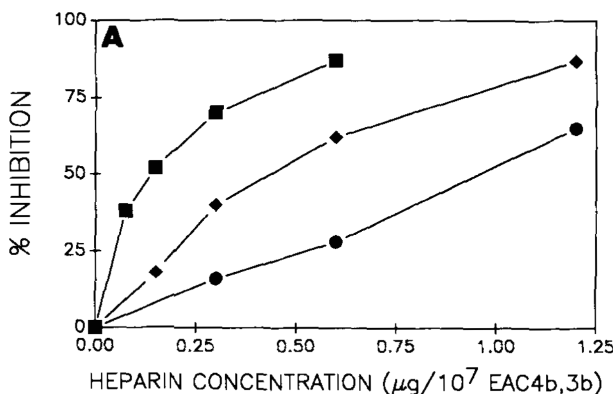


FIG. 2. **Strong anion exchange-HPLC of a heparin-oligosaccharide mixture prepared by heparinase-catalyzed depolymerization of heparin to 30% reaction completion.** Absorbance at 232 nm of 1 mg (*A*) and 4 mg (*B*) of heparin-oligosaccharide sample eluting from the column are plotted against elution time in minutes. The peaks collected from the column are labeled to identify the sample (sample number corresponds to degree of polymerization).



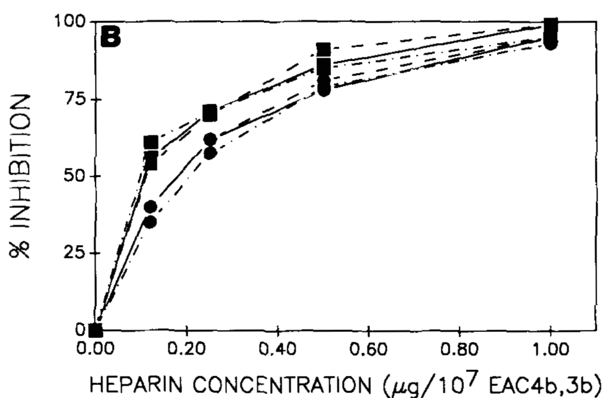


FIG. 3. **The effect of surface C3b and B concentration on the ability of commercial heparin to inhibit lysis of EAC4b,3b.** *A*, effect of surface C3b on ability of heparin to inhibit lysis of $EAC4b,3b^5$ (■), $EAC4b,3b^{20}$ (◆), and $EAC4b,3b^{100}$ (●). *B*, the effect of Factor B concentration on the ability of commercial heparin (■) and heparin-oligosaccharide (●) to inhibit lysis of $EAC4b,3b^{20}$. Three concentrations of Factor B were studied: 0.075 ng/10^7 EAC4b,3b (- - -), 0.225 ng/10^7 EAC4b,3b (—), and 0.449 ng/10^7 EAC4b,3b (-.-). Noninhibited tubes which did not receive heparin or heparin-oligosaccharide had 0.43, 0.93, and 1.52 Z of lysis (average number of lytic events/cell), respectively.

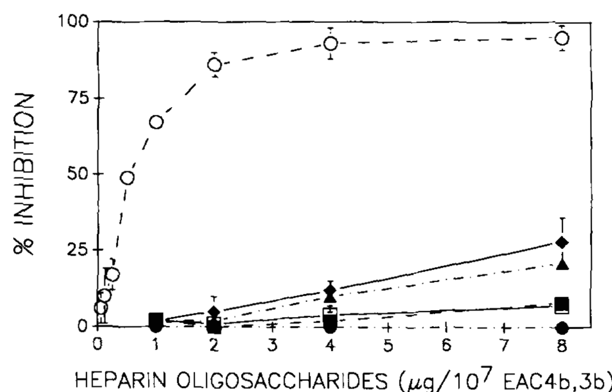


FIG. 4. **Effect of heparin and structurally characterized heparin-oligosaccharides on the alternate amplification pathway of complement ($EAC4b,3b^{100}$).** A dose-response curve is shown for heparin (○); septasulfated hexasaccharide-*6A* (◆), hexasulfated tetrasaccharide-*4C* (▲), pentasulfated tetrasaccharide-*4B* (□), pentasulfated tetrasaccharide-*4A* (■), and trisulfated disaccharide-*2* (●).

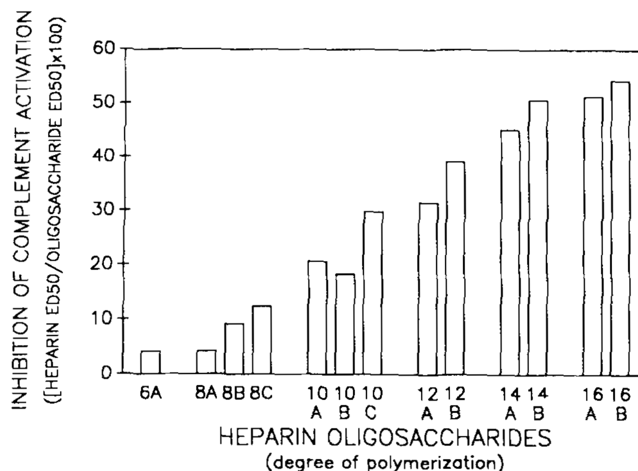


FIG. 5. **Inhibition of lysis of $EAC4b,3b^{100}$ by heparin-oligosaccharides.** Activity is expressed as [(ED_{50} heparin/ED_{50} heparin-oligosaccharide) × 100]. ED_{50} is measured on a molar basis using an average molecular weight for heparin of 14,000.

of standard porcine heparin (H1) on a weight basis. Two bovine heparins had 106 and 124% of standard porcine heparin's complement-inhibiting activity, and the low molecular weight heparin had 100% of standard porcine heparin's activity on complement activation. We also measured the anticoagulant activity of the heparin-oligosaccharides (dp 2–16) and commercial heparins using activated partial thromboplastin time and antithrombin III-mediated anti-factor IIa assays. Both assays gave similar results with the anti-factor IIa assay demonstrating slightly lower values. The anticoagulant activity of heparin-oligosaccharides and commercial heparins

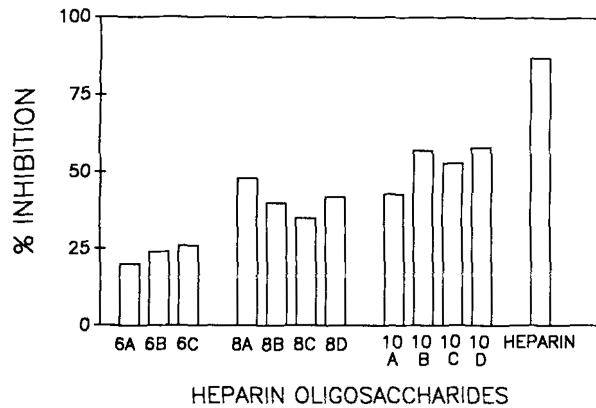


FIG. 6. **Inhibition of generation of amplification pathway C3 convertase on EAC4b,3b[20] by 4 μg of heparin and homogeneous structurally defined heparin-oligosaccharides.**

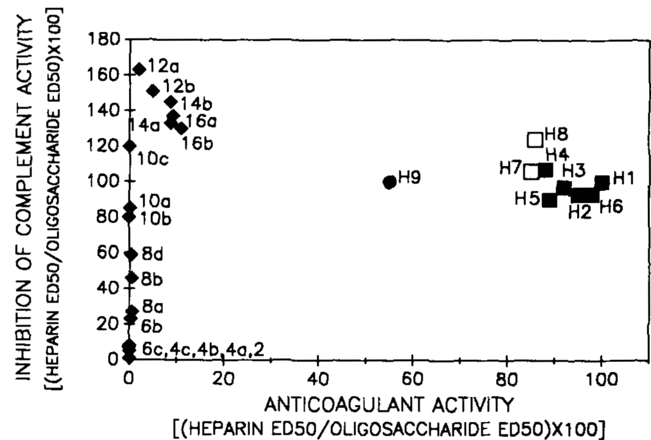


FIG. 7. **Inhibition of lysis of EAC4b,3b[100] by various commercial heparins and heparin-oligosaccharides plotted against anticoagulant activity.** Anticoagulant activity measured by antifactor IIa amidolytic assay is relative to a standard porcine heparin (*H1*) on a weight basis. The concentration of heparin or heparin-oligosaccharide required to cause 50% inhibition of coagulation or complement activity (ED_{50}) was calculated. Inhibition of complement activity is expressed as [(ED_{50} heparin/ED_{50} heparin-oligosaccharide) × 100] on a weight basis. Porcine heparins (■, H1→H6), bovine heparins (□, H7, 8), low molecular weight heparin (●, H9), and heparin-oligosaccharides (◆, 2→16b) are identified by the adjacent number.

TABLE II
Anticoagulant activity of heparin-oligosaccharides

Heparin-oligosaccharide	Activity[a]		
	aPTT	Antithrombin III mediated	
		Antifactor IIa	Antifactor Xa
		units/μmol	
2	<1	<1	<0.1
4A	2	<1	<0.1
4B	2	<2	0.1
4C	2	<2	3
6A	ND[b]	<2	<2
6B	ND	<2	4
6C	26	11	26
6D	11	<2	4
8A	16	<3	118
8B	21	<3	25
8C	ND	<3	29
8D	22	<3	32
10A	37	<3	ND
10B	45	<3	116
10C	ND	<3	61
10D	54	<3	136
Heparin	2338	2338	2338

[a] Activity is determined based on a standard curve using heparin (M_r average 14,000) with an activity of 167 units/mg. The activity of *6C* is lower than we previously reported (34).
[b] ND, not determined.

measured by anti-factor IIa assays are plotted against their ability to inhibit complement activation in Fig. 7. The porcine heparins, bovine heparins, and low molecular weight heparin cluster, showing only minor differences in activities. By contrast the heparin-oligosaccharides showed a slight increase in anticoagulant activity with a marked increase in complement-regulating activity as the degree of polymerization increased.

DISCUSSION

The effect of heparin's structure on its ability to inhibit complement activation has previously been studied only with intact heparin polymer. Chemical modification, such as the removal of *O*-sulfation and *N*-substitution (18), can markedly reduce activity while chemical transformation of other groups, such as the reduction of carboxylate groups (17), has no effect on activity. Affinity fractionation of intact heparin polymer by antithrombin III-Sepharose demonstrated that it is possible to prepare heparins with both high and low anticoagulant activity. Although low antithrombin III affinity heparin has activity toward complement comparable to both high affinity heparin and unfractionated heparin (18), it still displays substantial anticoagulant activity (approximately 30% of the anticoagulant activity of unfractionated heparin). This may be associated with incomplete fractionation or with heparin's ability to mediate coagulation through a second serine protease inhibitor, heparin cofactor II (21), resulting in anticoagulant activity that is independent of the presence of an antithrombin III-binding site. Finally, heparin with high affinity for antithrombin III may have increased sulfation and molecular weight (35), properties also associated with high complement inhibitory activity, making the separation of these activities by fractionation of intact heparin chains problematic.

In early studies (8) we completely depolymerized heparin to a mixture of its constituent oligosaccharides (*2, 4A, 4B, 4C,* and *6C*) which have only low levels of complement inhibitory activity. We examined size-fractionated heparin-oligosaccharide mixtures and demonstrated that there is a clear relationship between molecular weight and complement regulatory activity below M_r 3500. In addition we first used defined, homogeneous heparin-derived disaccharide and tetrasaccharides and demonstrated low levels of complement-inhibiting activity. These earlier studies suggested that it might be possible to investigate heparin's structure-activity relationship using a group of purified heparin-oligosaccharides having increased molecular weight.

Therefore, we partially depolymerized heparin using heparinase to prepare an array of heparin-oligosaccharides (Fig. 1) for biological testing. Heparin-oligosaccharides ranging from degree of polymerization 2-16 were purified by strong anion exchange-HPLC (Fig. 2). The molecular size of these oligosaccharides was determined using gradient polyacrylamide gel electrophoresis and gel permeation-HPLC (22). The purity of the disaccharide to decasaccharide samples were >90% while higher oligosaccharides with a degree of polymerization 12-16, were >75% pure as assessed by strong anion exchange-HPLC and gradient polyacrylamide gel electrophoresis. We have sequenced the disaccharide (*2*) which has little or no activity on complement activation (8, 33); we also sequenced tetrasaccharides (*4A-4C*) and a hexasaccharide

(6C) (22, 33, 34). Their structures are described in Table I, and their activities are given in a dose-response curve (Fig. 4). The low activity of these tetrasaccharides and hexasaccharide and our earlier observation that heparin-oligosaccharide mixtures of higher molecular weight have activity comparable to heparin on a weight basis clearly pointed out the necessity to examine the activity of pure, structurally characterized heparin-oligosaccharides of octasaccharide size and larger.

As previously reported (6–8), heparin and heparin-oligosaccharides appear to exert inhibition through their action on C3b since higher concentrations of heparin are needed to inhibit the lysis of cells containing a higher surface concentration of the activated third complement protein C3b (Fig. 3*A*) (6). This was also observed when structurally defined, homogeneous heparin-oligosaccharides were studied. However, cellular intermediates with differing amounts of C3b on their surface require varying amounts of factor B to maintain lysis with an average of one hemolytic event/cell (Z). Therefore, we determined whether the concentration of B could also effect the ability of heparin to inhibit complement activation. Fig. 3*B* indicates that when the concentration of C3 on the cells was constant, heparin and heparin-oligosaccharide inhibition was totally independent of B concentration, again suggesting that these oligosaccharides exerted inhibition through an effect on C3b.

The heparin-oligosaccharides demonstrated an uninterrupted increase in activity as degree of polymerization increased from 6 to 16 (Fig. 5). Heparin-oligosaccharide *16B* with a molecular weight of 5320 was 54% as potent as heparin on a molar basis and 136% as potent on a weight basis. Further studies on heparin-oligosaccharides having a degree of polymerization >16 are needed to determine the requirement for molar potency equivalence to commercial heparin.

The commercial heparins examined all had similar complement inhibitory activity again underscoring why it is so difficult to establish heparin's structure-activity relationship toward complement by only examining commercial heparins or fractionated, unfragmented heparins (Fig. 7). Bovine heparins are known to have a higher degree of sulfation (36); this may explain their slightly higher activity. A low molecular weight commercial heparin (H9) having slightly reduced anticoagulant activity had activity on complement activation comparable to the commercial heparins.

Homogeneous hexasaccharide through decasaccharide samples had activities (Fig. 6) nearly identical to the less purified fractions from which they were obtained. The compositional analysis for each of these heparin-oligosaccharides, and the sequences of *6D*, *8D*, and *10D* are given in Table I. Fig. 7 shows the relationship between the anticoagulant and complement inhibitory activities of the commercial heparins and heparin-oligosaccharides, clearly demonstrating that these activities can be separated. The ratio of the high complement inhibiting to low anticoagulant activities seen with some of these heparin-oligosaccharides (*i.e. 10C*, *12A*, *12B*, *14A*, *14B*, *16A*, and *16B*) suggest they may represent potential new drugs with high therapeutic indices. Particularly interesting is decasaccharide *10C*, which has little anticoagulant activity but contains the 3-sulfated glucosamine residue associated with the antithrombin III-binding site. The compositional analysis of *10C* shows that it is comprised of 2 equivalents of disaccharide *2* and 1 equivalent of hexasaccharide *6C*. The absence of significant anticoagulant activity (including a low anti-factor *X*a activity) for this decasaccharide (Table II) suggests that the hexasaccharide component *6C* is located on the reducing terminus of *10C* and thus constitutes an incomplete antithrombin III-binding site (15, 16). The absence of the complete antithrombin III-binding site in *10C* has little effect on its activity to inhibit complement activation as demonstrated by an activity greater than any of the porcine mucosal heparins examined when measured on a weight basis.

These studies have examined the ability of heparin-oligosaccharides to regulate the generation of alternative amplification pathway C3 convertase *in vitro* and are consistent with previous studies in our laboratory which show that highly charged polyionic substances can regulate multiple events in this pathway (6–8). We have not examined the complement regulatory capacity of heparin-oligosaccharide in whole serum because these studies would have been much cruder and would not have provided evidence for mechanism of action. Furthermore, the isolated system, using purified components, is a better model for the effects of these natural occurring highly charged polyions *in vivo* at the tissue level where they are released naturally.

The results presented here demonstrate a new approach to understanding heparin's activity in regulating complement using structurally defined, homogeneous heparin-oligosaccharides. A detailed knowledge of primary structure and sequence when coupled with structural modification studies on these heparin-oligosaccharides should lead to a clearer understanding of heparin's structure-activity relationship to regulate complement activation.

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