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HEPARIN AND MODIFIED HEPARIN INHIBIT COMPLEMENT ACTIVATION IN VIVO[1]

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Heparin regulates C activity in vitro, but has not been examined for this activity in vivo. The present study investigated the ability of commercial heparin and derivatized (*N*-desulfated, *N*-acetylated) heparin (Hep-NAc) with greatly diminished anticoagulant activity to inhibit C activation in guinea pigs. Catheters were placed in the right atrium of guinea pigs and kept patent with frequent saline flushes. The next day, heparin, Hep-NAc, or saline was given and 2.5 min later cobra venom factor or saline was given. Blood was drawn at intervals and assayed for total hemolytic C, C3 hemolytic activity, free hemoglobin, and activated partial thromboplastin time. Total hemolytic C and C3 activity decreased less rapidly in heparin- and Hep-NAc-pretreated animals than in non-pretreated animals, indicating that both heparins inhibited C activation. Heparin and Hep-NAc also inhibited cobra venom factor-induced hemolysis. This study demonstrates that commercial heparin and modified heparin inhibit C activation in vivo. This represents an important step in the development of an oligosaccharide drug to regulate C activation.

Heparin, an acidic polysaccharide produced by mast cells and basophils, is widely used clinically as an anticoagulant (1, 2). Heparin has also been shown to activate and release lipoprotein lipase, aggregate platelets, cause thrombocytopenia, cause tumor regression when given with steroids, and regulate angiogenesis (1, 3–7). Heparin was first shown to regulate C activity by Ecker and Gross (8) more than 6 decades ago, and subsequent studies have shown that glycosaminoglycan heparin in vitro may act on the classical pathway of C (9–13), potentiating the action of C1 esterase inhibitor (14), interfering with C1q[3] binding to immune complexes (10), inhibiting C1s interaction with C4 and C2 and C2 binding to C4 (11, 12), and suppressing reactive lysis (15). In addition, both fluid phase native proteoglycan and commercial heparin in vitro have been shown in our laboratory to inhibit generation of cell bound or fluid phase alternative pathway convertase, to inhibit preformed alternative pathway convertase, and to impair factor H-mediated decay of the alternative pathway convertase (16). The ability of heparin to inhibit the generation of alternative pathway convertase is independent of antithrombin binding activity and requires the presence of *O*-sulfation (17, 18). Our studies have shown that the size of the heparin fragments correlates directly with the ability to inhibit C activity with the minimally effective structure being a tetrasaccharide and a decasaccharide having activity almost equal to the activity of commercial heparin on a weight basis (18). These studies have shown that heparin acts on multiple proteins in the C cascade with its most important activity on C3b to inhibit the alternative pathway convertase (16, 18–21).

Despite these extensive in vitro studies to define the capacity of heparin to regulate the alternative and classical pathways of C, no in vivo studies have examined the consequences of heparin administration on C activation. The present study was designed to examine the ability of heparin to inhibit CoVF activation of the alternative pathway of C in vivo.

MATERIALS AND METHODS

Preparation and characterization of heparin and derivatized heparin. Porcine mucosal heparin, sodium salt, was obtained from Hepar Industries Inc., Franklin, OH. Derivatized heparin (Hep-NAc) was prepared as previously described from the pyridinium salt of this commercial heparin (17). Briefly, 2.5 g commercial sodium heparin (Hepar Industries) were passed through a column containing 30 g Dowex AG-50W-X8 cation exchange resin (H^+ form); the eluate was neutralized with pyridine to produce the pyridinium salt. After dialysis and lyophilization, the heparin salt was *N*-desulfated by dissolving in 120 ml DMSO/5% methanol and heating 1.5 h at 50°C. The mixture was then diluted with 125 ml of distilled water, the pH was adjusted to 9.1 with NaOH, and the mixture was dialyzed and lyophilized. The resulting *N*-desulfated heparin was *N*-acetylated by adding a total of 0.8 ml acetic anhydride over 2 h (115 μl were added every 20 min) to the *N*-desulfated heparin dissolved in 100 ml 10% methanol/0.05 M Na_2CO_3 at 25°C. The Hep-NAc product was diluted in 2 volumes of distilled water, exhaustively dialyzed against distilled water, and then lyophilized, which resulted in 1.8 g of final product (yield = 70%). The anti-Xa anticoagulant activities of heparin and Hep-NAc were then examined as described previously (22).

Heparin and Hep-NAc were digested using heparinase (EC 4.2.2.7) purified from *Flavobacterium heparinum* (23, 24) and analyzed using PAGE (25). Heparin was also fractionated using an antithrombin III affinity column to select heparin molecules with high and low affinity for antithrombin III (26). Heparinase-digested heparin was fractionated by strong anion exchange HPLC to prepare a heptasulfated hexasaccharide that included a portion of heparin's antithrombin III binding site (22). ^{1}H-Nuclear magnetic resonance spectroscopy

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[3] Abbreviations used in this paper: CoVF, cobra venom factor; CH50, total hemolytic C; Hep-NAc, *N*-desulfated, *N*-acetylated heparin; $DGVB^{2+}$, half isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin, which contains 2.5% dextrose, 0.5 mM magnesium and 0.15 mM calcium; C1q, C1s, C2, C3, C3b, C4, C5, C6, C7, C8, C9, classical pathway C components; PTT, partial thromboplastin time.

at 360 MHz was used to characterize the Hep-NAc (10 mg Hep-NAc in 500 μl of ^2H_2O).

Guinea pig model for C activation. Male guinea pigs, 543 to 766 g in size (mean weight, 641 g), were observed for at least 3 days before treatment to ensure that they were healthy. One day before treatment, animals were anesthetized using ketamine hydrochloride (35 mg/kg; Aveco, Ft. Dodge, IA) and Rompun (5 mg/kg, xylazine; Haver Pharmaceuticals) i.m. Next, a 0.08-cm inside diameter × 0.17-cm outside diameter × 20-cm long silastic catheter (Dow Corning, Midland, MI) was inserted into the external jugular vein by cutdown and passed into the right atrium (27). The wound was sutured and a four-way stopcock was attached to the catheter that was kept patent by flushing with saline every 8 h. On the following day, the animals were weighed and this weight was used to determine CoVF and heparin dosages based upon the calculated blood volume, which is 7.25% of the guinea pig's total body weight (28).

Then the experimental timetable shown in Table I was followed. At each sample point, 1.5 ml of blood were drawn into syringes containing 3.8% sodium citrate (w/v) with a final citrate/blood concentration of 1/10. At −2.5 min, 3, 5, or 10 mg of heparin/ml animal blood volume or 5 mg Hep-NAc/ml blood volume or saline (CoVF alone control) were administered by catheter. Then at zero time, 0.3 U of CoVF per ml of blood volume or saline (heparin alone control) was administered by catheter; CoVF for these experiments had a concentration of 1.47 mg/ml and 1 U/4 μg. Blood was kept on ice until all of the specimens for one animal were collected, and then plasma was separated using an Eppendorf centrifuge. Plasma was stored at −70°C until assay. Each group included at least three animals and the results were averaged. The total fluid removed by 45 min was less than 20% of the calculated maximum total vascular volume, and all fluid that was removed was replaced with saline.

C buffers and components. Half-isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin and $DGVB^{2+}$, was used as buffer in the hemolytic assays. CoVF was purified from cobra venom using the technique of Ballow and Cochrane (29). C2, C5, C6, C7, C8, and C9 were purchased from Diamedix, Miami, FL. Classical pathway sheep cellular intermediates containing C1 and C4 were prepared as described previously (30).

Guinea pig plasma was assayed for C3 hemolytic activity. Briefly, the plasma was diluted in $DGVB^{2+}$ and incubated with 1×10^7 classical pathway sheep cellular intermediates containing C1 and C4, 2 U of C2, and 10 U each of C5, C6, and C7 for 30 min at 30°C. Then, 10 U each of C8 and C9 were added in 0.1 ml $DGVB^{2+}$, and incubation was continued for 60 min at 37°C. Finally, saline was added and the tubes were centrifuged. Lysis was determined by measuring A_{414}. Total hemolytic C (CH50) activity in the guinea pig plasma was also examined by standard methods (31).

Activated PTT assay. Plasma obtained from representative guinea pigs was centrifuged to obtain platelet-poor plasma. The plasma was then diluted 1/10, 1/100, and 1/1000 in pooled normal guinea pig plasma, and the activated PTT was determined by a standard technique (32) using an automated activated PTT reagent with micronized silica activator (Organon Teknika Corporation, Durham, NC). The fibrin generation was detected using a fibrometer (BBL, Division of Becton Dickinson, Cockeysville, MD).

Free hemoglobin assay. Guinea pig plasma was assayed for free hemoglobin levels using the Plasma Hemoglobin Assay Kit from Sigma Diagnostics (St. Louis, MO).

RESULTS

First, we compared commercial heparin with Hep-NAc for anticoagulant activity and for susceptibility to heparinase digestion. The commercial heparin had 167 U of anti-Xa assay per mg, whereas the Hep-NAc had only 1.4 U of activity per mg. Thus, the Hep-NAc possessed less than 1% as much in vitro anticoagulant activity as commercial heparin. When the two heparins were digested with heparinase (Fig. 1), Hep-NAc was only minimally fragmented as compared with the commercial heparin, indicating that nearly all the *N*-sulfated glucosamine residues had been converted to *N*-acetyl glucosamine (24). ^{1}H-nuclear magnetic resonance spectroscopy at 360 MHz confirmed the structure of Hep-NAc. The anomeric proton of *N*-acetylated glucosamine (5.42 ppm) had an integration of 1 to an integration of 3 for the methyl proton of *N*-acetylated glucosamine (2.04 ppm), demonstrating that the glucosamine residues of Hep-NAc were fully *N*-acetylated. The remaining peaks in the Hep-NAc spectrum were very similar to those of heparin, suggesting that the chemical modification had been specific for the glucosamine residues.

TABLE I
Experimental timetable

Procedure	Time (min)
Baseline sample	−10
Heparin, Hep-NAc, or saline	−2.5
CoVF or saline	0.0
Sample	5
Sample	10
Sample	15
Sample	20
Sample	30
Sample	45

We compared the ability of these two heparin preparations to inhibit CoVF activation of C (Figs. 2 and 3). We found that animals pretreated with heparin and Hep-NAc had less CoVF-induced C activation than control animals; Hep-NAc inhibited CoVF-induced C activation about half as well as commercial heparin on a weight basis (Figs. 2 and 3).

Animals that received heparin without CoVF demonstrated that heparin interfered with the CH50 assay (Fig. 2*a*). This reduced heparin's apparent effectiveness in inhibiting CoVF consumption of CH50 activity. In contrast, heparin did not interfere with the C3 assay in the concentrations that were used in these experiments (Fig. 3*b*).

It was obvious that plasma obtained from animals that received CoVF and heparin had less hemolysis than animals that received CoVF alone. Consequently, we examined the plasma specimens for free hemoglobin (Fig. 4). We found that free hemoglobin levels in animals receiving CoVF alone quadrupled over 45 min, whereas there was no increase in hemolysis in any of the animals pretreated with either heparin or Hep-NAc at 3 mg/ml of blood volume or greater (Fig. 4). In preliminary studies, we observed more hemolysis after administration of CoVF and 0.1 mg heparin/ml blood volume than was seen after CoVF and 3 mg heparin/ml blood volume, but less hemolysis than in animals treated with CoVF alone. Free hemoglobin increased from baseline to 173 and 203% at 20 and 30 min after 0.1 mg heparin and CoVF as compared with increases of 287 and 357% at 20 and 30 min after CoVF alone.

Finally, we compared commercial heparin with Hep-NAc for anticoagulant activity. This experiment shows that Hep-NAc had much less anticoagulant activity in vivo than commercial heparin (Table II). Guinea pig plasma required dilution to determine the activated PTT. A 1/10 dilution of the heparin and Hep-NAc plasma samples in normal guinea pig plasma had activated PTT of >250 and 81 s, respectively. A 1/100 dilution of the heparin plasma sample still had an activated PTT of >250 s. Finally, a 1/1,000 dilution of the heparin plasma sample had an activated PTT of 130 s and the Hep-NAc had an activated PTT of 27 s, identical to normal guinea pig plasma.

DISCUSSION

Heparin has been studied extensively and found to regulate C activity in vitro (8–21, 33). Heparin binding sites have been described on C proteins, including factor

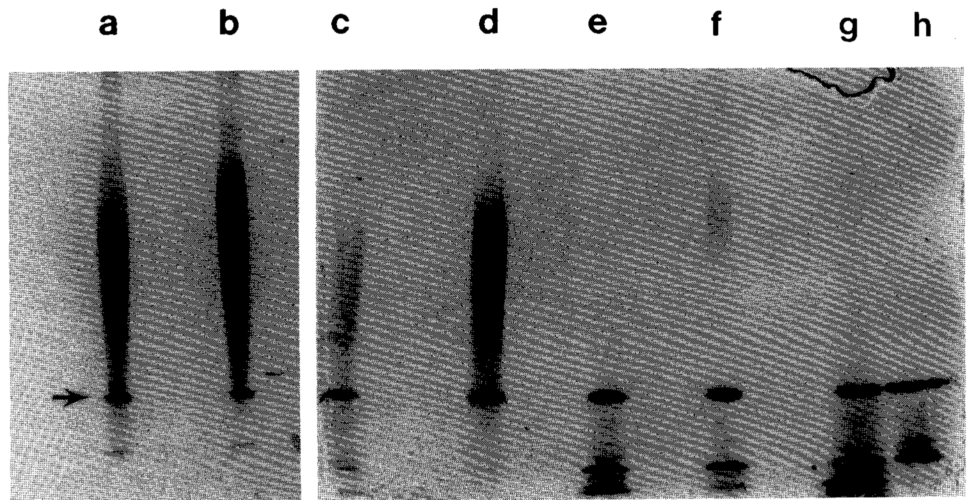
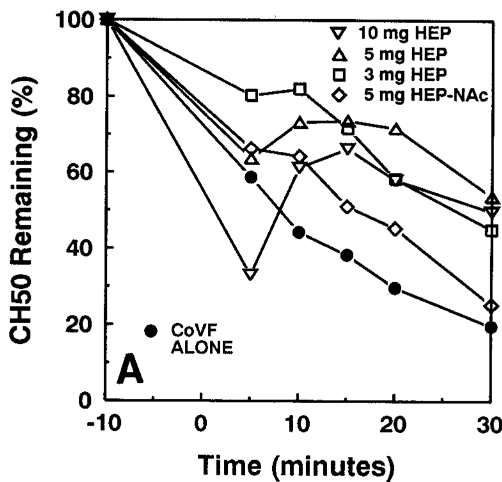


Figure 1. Gels contain: nondigested Hep-NAc (*a*), nondigested commercial porcine heparin (*b*), heparinase-digested commercial porcine heparin (*c*), heparinase-digested Hep-NAc-derivatized heparin (*d*), heparinase-digested commercial heparin with high affinity for antithrombin III (*e* and *f*), heparinase-digested commercial heparin with low affinity for antithrombin III (*g*), and a heparin-derived heptasulfated hexasaccharide standard (22) that arises from heparin's antithrombin III binding site when heparin is treated with heparinase (*h*). Note that the band in *h* is not seen in *d*. *Arrow*, bromphenol blue dye marker.



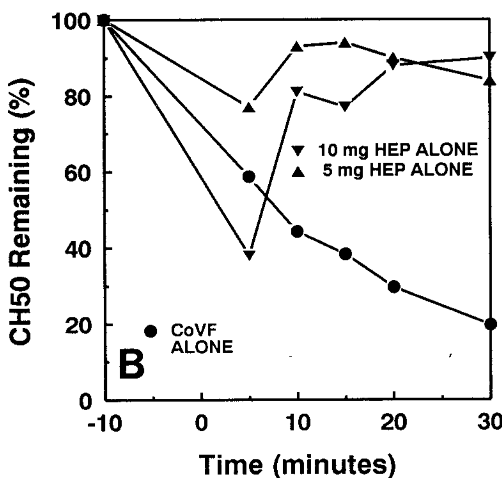
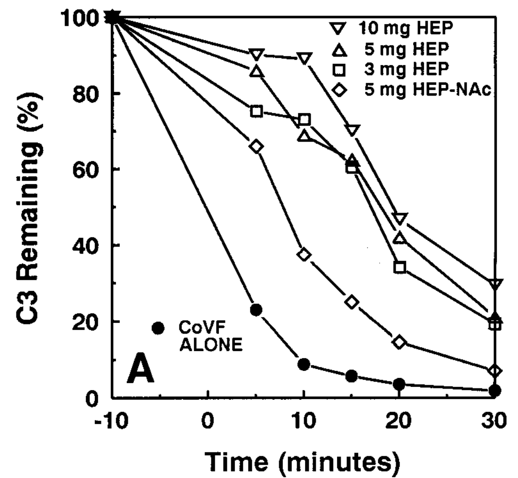


Figure 2. Heparin effect on CH50 in guinea pigs treated with CoVF. In *a*, commercial heparin and derivatized heparin (Hep-NAc) were examined for ability to retard the decrease in CH50 hemolytic activity. Both heparin and Hep-NAc inhibited CoVF-induced activation. In *b*, the activity of commercial heparin alone without CoVF was examined for its effect on the CH50 assay. Heparin interference with the CH50 assay was most pronounced in the 5-min sample. This interference decreased the apparent effectiveness of heparin to retard the decrease in residual CH50 activity. Thus, the effects seen in *a* underestimate the ability of heparin to inhibit C activation early after its administration.



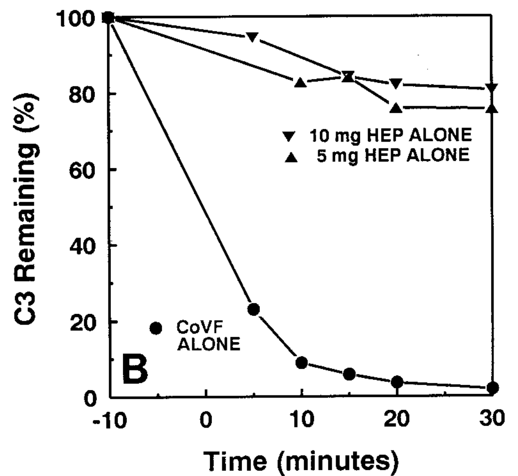


Figure 3. Heparin effect on C3 in guinea pigs treated with CoVF. In *a*, commercial heparin and derivatized heparin (Hep-NAc) were examined for ability to retard a decrease in C3 hemolytic activity. Both heparin and Hep-NAc inhibited CoVF induced activation. In *b*, the activity of commercial heparin alone without CoVF was examined for its effect on C3 activity. Heparin did not interfere with the C3 determination.

H and C4 binding protein (34, 35). Functionally, it is clear that heparin interacts with C3b to inhibit its participation in the alternative pathway (16, 18–21). Based upon these studies, we previously concluded that the activity of heparin on C is complex (19, 20); the net effect of heparin in serum or in vivo had not been examined previously. Consequently, the present studies were designed to determine heparin's capacity to regulate CoVF activation in a whole animal. In companion studies, we

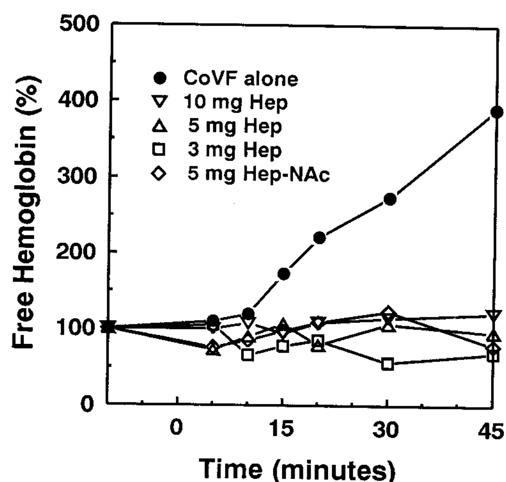


Figure 4. Heparin effect on hemolysis in animals treated with CoVF alone (●), 10 mg heparin (▽), 5 mg heparin (△), 3 mg heparin (□), or 5 mg Hep-NAc (◇).

TABLE II

Anticoagulant (activated PTT) activity of commercial heparin and derivatized heparin

Treatment Group	Dilution of Plasma		
	1:10	1:100	1:1,000
Commercial heparin, 5 mg	>250[a]	>250	130
Hep-NAc, 5 mg	81	37	27

[a] All activities are in seconds and were determined on the last specimen. Control guinea pig plasma activity was 27.

also examined the ability of heparin to regulate C activation in whole human serum and in whole guinea pig serum; those experiments also demonstrated that heparin inhibited C activation (36).

In the studies presented in this report, we compared commercial heparin with a derivatized heparin, having diminished anticoagulant activity (17). Derivatized heparin (Hep-NAc) was only minimally digestible by heparinase, whereas the commercial heparin was readily digested by heparinase, demonstrating that nearly all the *N*-sulfated glucosamine residues in heparin had been chemically converted to *N*-acetylated glucosamine residues (Figs. 1 and 5). This was confirmed using ^{1}H-nuclear magnetic resonance spectroscopy, which also suggested that little or no structural damage had been done to other functional groups in the molecules. The Hep-NAc possessed only about 1/120th the anti-Xa anticoagulant activity in vitro as compared with the commercial heparin; in vivo this meant that the Hep-NAc had greatly reduced anticoagulant activity as compared with the activity of commercial heparin (Table II). This reduced level of anticoagulant activity may be important if heparin or a heparin derivative is ultimately to be used in vivo to regulate C activity.

CoVF was used as an activator because of its profound ability to activate C (37). The CoVF,B complex has a half-life measured in hours and ultimately activates virtually all the C3 in plasma (37). Thus, we assumed that this would provide a good test for the ability of heparin to inhibit C activation in vivo. As demonstrated in Figures 2 and 3, both heparin and Hep-NAc inhibited C activation as measured by residual CH50 and C3 hemolytic activity. Figure 2 shows that heparin inhibited consumption of CH50 activity although heparin itself interfered with the CH50 assay, making this experiment somewhat difficult to interpret during the early time points after CoVF administration. Figure 3 shows that heparin also inhibited C3 consumption and did not interfere with the C3 assay. Figure 6 shows the dose effect relationship of heparin on residual CH50 and C3 activities, again demonstrating that commercial heparin at all tested concentrations had an effect that could be distinguished from the non-heparin-treated group. Although the Hep-NAc-treated animals had less residual C activity than did commercial heparin-treated animals, the animals treated with this derivatized heparin had more residual CH50 and C3 activity than animals that did not receive heparins.

These findings are consistent with those of Ekre (38), who reported in 1985 that heparin inhibits C activation as measured by crossed immunoelectrophoresis in whole human serum independent of heparin's affinity for antithrombin III. In contrast, in guinea pig serum the ability to inhibit hemolysis was related to m.w. and was reduced in heparin preparations with low affinity for antithrombin III. In the present study, we found that Hep-NAc possessed approximately half the C inhibitory activity in the guinea pig model as compared with commercial heparin (Fig. 6). This is consistent with the Ekre study. These studies suggest that a population of heparin molecules may exist that is highly active in inhibiting C similar to the ability of high affinity antithrombin III heparin to inhibit coagulation. This possibility is further supported by studies in our laboratories that have shown that heparin can be separated on a C3b affinity column (Weiler, J., R. E. Edens, R. J. Linhardt, manuscript in preparation). We are presently characterizing this heparin to determine its composition and sequence and the site on the C3b molecule to which it binds.

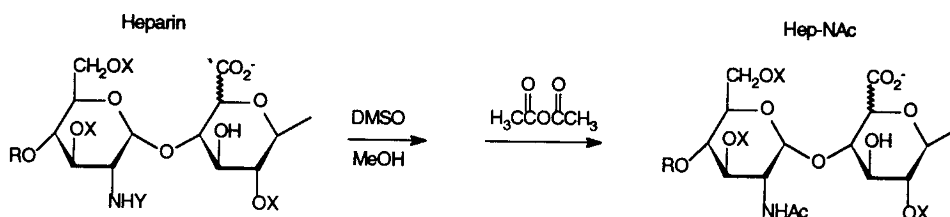


Figure 5. Conversion of commercial heparin to the *N*-desulfated, *N*-acetylated form (Hep-NAc).

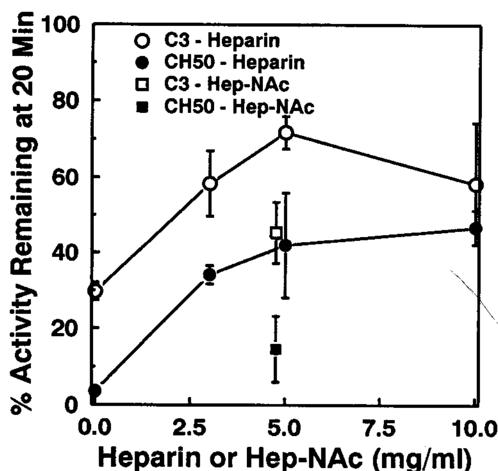


Figure 6. Dose-response effect of heparin and Hep-NAc on residual CH50 and C3 activity 20 min after CoVF administration. *Bars* demonstrate that all heparin groups had more residual CH50 and C3 activity than the CoVF-alone (placebo) group.

Ekre et al. (39), in 1986, also reported that heparin inhibits the wheal and flare response in skin induced by intradermal injection of heat-aggregated γ-globulin similar to the ability of mepyramine, an antihistamine, to block this activity. However, mepyramine, but not heparin, inhibited trypsin, compound 48/80, and histamine-induced wheal and flare in skin. The ability of heparin to inhibit the wheal and flare reaction was independent of its antithrombin III binding activity or its m.w., again suggesting that the capacity to regulate C does not parallel the ability to interfere with coagulation (13).

The present studies are also consistent with previous cardiopulmonary bypass experiments performed in animals and humans (40–44): Nilsson et al. (40) reported that perfusion of a hollow fiber membrane oxygenator coated with heparin caused less C activation as compared with a noncoated oxygenator in pigs. In that study the C3d to C3 ratio increased from 1 at time zero to 1.89 and 3.47 in the animals perfused using heparin-coated and uncoated hollow filter oxygenators, respectively. This shows that heparin coating of a hollow fiber membrane oxygenator decreases C activation in a clinically relevant setting. Nevertheless, the heparin in this setting was bound to the oxygenator, which itself is a C activator. Thus, the Nilsson et al. (40) study did not prove whether the heparin actually inhibited C activation or simply blocked C-activating sites on the oxygenator.

C levels have been studied in patients undergoing cardiopulmonary bypass; these patients receive heparin, protamine, and a variety of other drugs in addition to having their blood exposed to membrane oxygenators (41–44). In this setting, it is difficult to dissect the role that heparin plays in regulating C activation. Previous studies do show that the combination of heparin and protamine may activate C similar to an immune complex (9). Nevertheless, heparin is always given to these patients for anticoagulation, and protamine is virtually always used to "reverse" this activity of heparin near the end of the procedure. No control group has been studied in which these drugs are not given during cardiopulmonary bypass to examine the individual role of these drugs in mediating C activation.

The most profound activity of the heparin was to inhibit hemolysis in the animals (Fig. 4). Indeed, in animals that were studied as long as 1 h after CoVF administration, there was no increase in plasma free hemoglobin in any of the heparin-treated animals as compared with the increase seen in control animals that received only CoVF. Preliminary in vivo studies also showed more hemolysis after administration of CoVF and 0.1 mg heparin/ml blood volume than was seen after CoVF and 3 mg heparin/ml blood volume, but less hemolysis than in animals treated with CoVF alone. This demonstrates that heparin more profoundly inhibited hemolysis than consumption of residual CH50 or C3 activity. It is likely that CoVF caused lysis of the guinea pig E by the action of C, which was inhibited by the presence of heparin (45). The mechanism by which the heparin inhibited in vivo hemolysis is now being explored with in vitro studies to determine whether the effect is on reactive (bystander) lysis or some other event after classical pathway activation.

Taken together, these observations indicate that heparin regulates C activity in vivo. This suggests that although heparin regulates multiple steps in the C cascade, its net effect is inhibitory. Future studies with heparin in vivo and in vitro will examine monodisperse heparin preparations with high affinity for C3b to determine whether this activity can be increased. Ultimately these oligosaccharides might be used to regulate C activation in vivo in situations such as in joints, where it would be preferable for C activation not to occur.

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