Preparation and Identification of a Population of Antibodies That Recognize Carbodiimide-Modified Heparin

By Sanford N. Gitel, Victor M. Medina, and Stanford Wessler

Protein-heparin complexes, prepared by a water-soluble carbodiimide coupling technique, were used to produce anti-heparin antibodies in rabbits. Antiserums that recognized carbodiimide-treated heparin, but not untreated heparin, were obtained. Carbodiimide-treated heparan sulfate exhibited 10% to 20% cross-reactivity compared with a similarly treated heparin, whereas there was no cross-reactivity with five other carbodiimide-treated mucopolysaccharides. 3H-1-ethyl-3-(3-trimethylammoniumpropyl) carbodiimide iodide was used to demonstrate that carbodiimide forms a stable adduct with heparin and other mucopolysaccharides. Using an antibody fraction that eluted from 1-ethyl-3-(3-trimethylammoniumpropyl) carbodiimide iodide-treated heparin-Sepharose with 2 mol/L KI, it was demonstrated that, for the antibody population studied, the addition of one carbodiimide per heparin molecule resulted in complete epitope expression without loss of anticoagulant activity. The addition of up to eight additional carbodiimide molecules to heparin did not increase the extent of epitope formation, although anticoagulant activity was lost. Except for heparan sulfate, the addition of radiolabeled carbodiimide to other mucopolysaccharides did not result in epitope formation. These data demonstrate that antibodies to an epitope derived from heparin can be formed, that the epitope is fully expressed while anticoagulant activity is present, and that the antibody is specifically directed against an altered portion of the polysaccharide.

Heparin is a mucopolysaccharide widely distributed in animal tissues and possibly in the walls of blood vessels, although the majority is associated with mast cells. It has been suggested that heparin may also be present in circulating blood, but such data are inconclusive. Because of heparin’s anticoagulant and lipolytic actions, it is important to determine where in the body this mucopolysaccharide is normally found and at what concentrations. The development of an antibody to heparin would facilitate accomplishing these goals as well as provide a tool to elucidate the basis for heparin’s physiologic and pharmacologic actions.

Because heparin is a naturally occurring compound in mammals, simple immunization would not be expected to elicit an antibody. Antibodies to natural products have been produced by attaching such compounds to proteins that elicit an antigenic response, and that approach was used in this investigation. Water-soluble carbodiimides have been used to attach amine- or carboxyl-containing haptenes to proteins by way of amide bonds, and these complexes have been used successfully to raise antibodies to the haptenes.

Because heparin contains both amino and carboxyl residues, this technique was used to obtain anti-heparin antibodies. The present report describes the preparation of protein-heparin complexes, their use for the immunization of rabbits, and the demonstration that antibodies to a carbodiimide-modified heparin were obtained.

MATERIALS AND METHODS

Bovine serum albumin (BSA), ovalbumin (OA), hog mucosal heparin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMC), Staphylococcus aureus lyophilized cell powder (insoluble protein A), polyethylene glycol, and bovine fibrinogen were obtained from Sigma Chemical Co, St Louis. Methyl iodide, potassium iodide, and azure A were obtained from Fisher Chemical Co, Springfield, NJ. 1H-methyl iodide (1 mCi/mg) was a product of New England Nuclear, Boston. DEAE-Sephadex, Sephadex G-100, and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Bio-gel P4 and Bio-gel P6 were products of Bio-Rad Laboratories, Rockville Center, NY. Safety-count scintillation solvent, neutralizer cocktail, and TS2 tissue solubilizer were obtained from Research Products International, Mount Prospect, Ill. Complete and incomplete Freund’s adjuvants were products of Pel-Freeze Biologicals, Rogers, Ark.

Heparin, heparan sulfate, dermatan sulfate, hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate standards were obtained from Dr Martin Mathews, University of Chicago.

1-Ethyl-3-(3-trimethylammoniumpropyl) carbodiimide iodide (ETAC) was prepared by a published procedure, modified in that the commercial hydrochloride (EDAC) was neutralized with a 10% solution of sodium carbonate. Tritium-labeled ETAC (1H-ETAC) was similarly prepared, except that a 1:1 ratio of 1H-methyl iodide to EDAC was used. The resulting 1H-ETAC had a specific radioactivity of 41 μCi/mg.

Bovine IgG was isolated from bovine plasma using ammonium sulfate precipitation followed by chromatography on DEAE-Sephadex. Bovine thrombin was isolated from Parke Davis thrombin. Heparin was coupled to Sepharose 4B by cyanogen bromide. Human antithrombin III was isolated by a standard procedure.

OA-, BSA-, and bovine IgG-heparin complexes were prepared by

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reactions involving 40 mg of protein, 80 mg of heparin, and 100 mg of EDAC in 5 mL of water at room temperature for 90 minutes while maintaining the pH between 6.8 and 7.2. The reaction was allowed to proceed overnight without pH adjustment. The reaction mixture was chromatographed on Sephadex G-100 followed by chromatography on DEAE-Sephadex A-50. The heparin-protein fractions that co-eluted at high salt concentration were used in subsequent experiments and have been designated as OA-heparin, BSA-heparin, and bovine IgG-heparin.

The quantity of heparin was determined using an azure A binding assay. Heparin anticoagulant activity was determined in an anti-thrombin Af assay. Heparin antithrombin catalytic activity was determined by a two-stage clotting assay as follows: To a test tube containing 0.2 mL of antithrombin III (20 μg/mL in 0.14 mol/L NaCl, 0.02 mol/L TRIS /HCl, pH 7.5 [TBS]) containing BSA, 10 mg/mL) and 0.1 mL of a heparin sample (0 to 0.5 U/mL in TBS containing BSA, 10 mg/mL) at 37°C was added 0.1 mL of thrombin (ca 100 U/mL in TBS containing BSA, 10 mg/mL). The reaction mixture was incubated at 37°C for 30 seconds at which time 0.1 mL of the mixture was added to 0.2 mL of fibrinogen (10 mg/mL) in a 4% polyethylene glycol solution at 37°C and the clotting time determined.

Preparation of EDAC- and ETAC-Treated Heparin-Sepharose

Ten milliliters packed volume of heparin-Sepharose in water, 20 mL total volume, was reacted with 100 mg of either EDAC or ETAC for 45 minutes at room temperature, washed extensively with 2 mol/L NaCl, and equilibrated with TBS.

Immunization of Rabbits

Female New Zealand white rabbits (4 to 5 kg), from which pretreatment plasma samples were obtained, were immunized as follows: Rabbits were injected five times over a two-week period subcutaneously in 15 to 20 spots along the back, with 1 mL of a 1:1 mixture of complete Freund's adjuvant and an aqueous solution containing OA-heparin at a concentration of 200 to 300 μg/mL. Blood samples were obtained seven and 14 days after immunization. Two months later, a second series of immunizations were initiated with bovine IgG-heparin according to the initial schedule. Blood samples were then drawn 1, 9, and 12 days afterward. Samples were collected into 3.8% trisodium citrate (nine parts blood to one part citrate) and plasma isolated by centrifugation for ten minutes at 2,000 g and 4°C.

Booster immunizations were administered two to six months after the initial, two-stage immunization with either OA-heparin or bovine IgG-heparin as described above, except that incomplete Freund's adjuvant was used. Plasma samples were collected before reimmunization and 7, 14, and 28 days after completion of immunization.

Isolation of Rabbit IgG Fraction

The immune plasma samples were heated at 56°C for 15 minutes and the resulting precipitate removed by centrifugation for ten minutes at 2,000 g and 4°C. Immunoglobulins were isolated by precipitation at 40% ammonium sulfate saturation. The precipitate was washed twice with 50% ammonium sulfate, dissolved in deionized water to the initial plasma volume, dialyzed against TBS, and stored frozen at -70°C.

Measurement of Antibodies

Antibodies were identified by double immunodiffusion and tanned red blood cell hemagglutination techniques.

Estimation of Specificity

The specificity of compounds for the antibodies was determined using a tanned red blood cell hemagglutination inhibition technique.

Fractionation of Antibodies on Carbodiimide-Treated Heparin-Sepharose

To a 10-mL volume column of one of the heparin-Sepharose preparations was added a solution of the isolated immunoglobulins. The column was eluted stepwise with 0.14, 0.5, and 2.0 mol/L NaCl, and finally with 2 mol/L KI. The protein-containing fractions from the final eluate (ca 15 mL) were immediately desalted on a 60-mL total volume Bio-gel P4 column equilibrated with TBS.

Effect of EDAC Treatment of Heparin as a Function of EDAC Concentration

To 1 mL of heparin (1 mg/mL) in water was added 0.2 mL of EDAC in water at concentrations between 10 μg and 500 mg/mL. The reaction mixtures were maintained at room temperature for 30 minutes and dialyzed overnight against 6 L of TBS. The reactions were then twice dialyzed against the same buffer, brought to a 2-mL volume, and assayed for heparin anticoagulant activity by both the anti-Xa and anti-thrombin assays, azure A activity, and hemagglutination inhibition.

EDAC Treatment of Heparan Sulfate, Dermatan Sulfate, Chondroitin-4-Sulfate, Chondroitin-6-Sulfate, Keratan Sulfate, and Hyaluronic Acid

To 1 mL of a solution of mucopolysaccharide (1 mg/mL) was added 0.2 mL of EDAC at 5, 50, or 500 mg/mL. The reaction mixtures were treated as described above for heparin and assayed for hemagglutination inhibition.

Treatment of Mucopolysaccharides With 3H-ETAC

To 0.8 mL of mucopolysaccharide (1 mg/mL) in water was added 0.2 mL of 'H-ETAC in water and the reaction mixture kept at 28°C or room temperature for the time indicated. The reaction was stopped by addition of an imidazole/acetate mixture and stored at -70°C. Each reaction mixture was chromatographed on a 0.9 x 30.0-cm Bio-gel P6 column equilibrated with 0.3 mol/L NaCl. The P6 column had been standardized with mixtures of unreacted mucopolysaccharides and degraded 'H-ETAC. The extent of tritium incorporation was determined by liquid scintillation counting of the mucopolysaccharide peak. Hemagglutination inhibition of each isolated mucopolysaccharide was determined, and where appropriate, the azure A and anticoagulant activities were determined. Reaction products from the front and back of the mucopolysaccharide peak were applied to a 1 x 9-cm column of DEAE-Sephadex and eluted stepwise with 0.14, 0.5, and 1.0 mol/L NaCl. Samples were assayed for radioactivity. The elution positions of hydrolyzed 'H-ETAC and unlabeled mucopolysaccharides were determined by chromatography on DEAE-Sephadex under identical conditions.

Binding of 3H-ETAC–Treated Mucopolysaccharide to the 2 mol/L KI Antibody Fraction

One gram of insoluble protein A was washed three times with 10 mL of TBS and suspended in TBS to a total volume of 10 mL. To 200 μL of a nonimmune rabbit IgG fraction, or the antibody fraction that was eluted with 2 mol/L KI from ETAC-treated heparin-Sepharose, was added 200 μL of TBS and 10 μL of a solution containing 30
μg/mL of 'H-ETAC-treated mucopolysaccharide. The reaction-mixture was kept at room temperature for one hour when 150 μL of the protein A suspension was added and shaken at room temperature for one hour. The supernatant and protein A were separated by centrifugation at 4,000 g and 4 °C. Two hundred-microliter samples of the supernatant were added to 10 mL of scintillation solvent to determine the amount of 'H remaining. The solid was washed with 1 mL of TBS, centrifuged at 4,000 g, the supernatant removed, and the solid dissolved in 1 mL of TS2 tissue solubilizer. The resulting solution was added to 10 mL of neutralizer scintillation fluid and the radioactivity determined.

RESULTS

Preparation and Isolation of Protein-Heparin Complexes

Chromatography of the products of the reaction of OA, BSA, or bovine IgG with EDAC and heparin on Sephadex G-100 yielded heparin that co-eluted with the protein peak before the main heparin peak. In contrast, BSA eluted as a single peak under the heparin peak, and OA eluted at the back of the heparin peak in mixtures not treated with EDAC. Although the elution position of bovine IgG was unchanged, heparin co-chromatographed with the protein in the carbodiimide-treated reaction mixture but did not elute with the protein in untreated mixtures.

Three protein-containing fractions were obtained on chromatography of the protein-heparin complexes on DEAE-Sephadex. The first fraction eluted at 0.14 mol/L NaCl, the second at 0.5 mol/L NaCl, and the third between 0.5 and 1.0 mol/L NaCl. Chromatography of untreated mixtures of protein and heparin under identical conditions invariably resulted in the complete elution of protein (OA, BSA, or IgG) at 0.5 mol/L NaCl. In no instance was protein eluted between 0.5 and 1.0 mol/L NaCl in untreated mixtures.

The heparin-containing protein peak eluting between 0.5 and 1.0 mol/L NaCl was dialyzed against TBS for immunization or tanned red cell hemagglutination assays.

Antibody Formation

Seven and 14 days after the first immunization with OA-heparin, crossed immunodiffusion failed to demonstrate a plasma antibody to heparin or BSA-heparin, although anti-OA antibodies were obtained. However, tanned red cell hemagglutination, using cells labeled with BSA-heparin, demonstrated plasma titers between 1:40 and 1:320 after seven days and between 1:40 and 1:160 after 14 days. There was no hemagglutination of red cells labeled with BSA.

After the second immunization with IgG-heparin, plasma hemagglutination titers against BSA-heparin-labeled red cells ranged between 1:40 and 1:160 on day 1, between 1:20 and 1:640 on day 9, and between 1:80 and 1:1,280 on day 12 with no hemagglutination of BSA-labeled cells. The samples with the highest titers (1:1,280) formed two precipitin lines with either OA-heparin or IgG-heparin, one being a line of identity with BSA-heparin and the other a line of identity with either OA or IgG. Only the protein-specific precipitin bands were removed after adsorption of the immunoglobulins with OA and bovine IgG (Fig 1). Adsorption with BSA had no effect on the antibodies. Double immunodiffusion of the antibodies against heparin failed to produce a precipitin line, whereas EDAC-treated heparin formed a line of complete identity with BSA-heparin (Fig 1).

Neither heparin, heparan sulfate, dermatan sulfate, chondroitin-6-sulfate, chondroitin-4-sulfate, keratan sulfate, nor hyaluronic acid inhibited hemagglutination of BSA-heparin-labeled red cells at concentrations as high as 10 mg/mL. BSA-heparin inhibited hemagglutination, as did EDAC-treated heparin, at concentrations as low as 50 ng/mL.

Heparin, reacted with two other water-soluble carbodiimides (ETAC and CMC), also formed precipitin lines with the antibody. Each of these precipitin lines exhibited partial identity with EDAC-treated heparin. Both ETAC- and CMC-treated heparin inhibited hemagglutination, but with lower titers than EDAC-treated heparin.

When the immunized rabbits received a booster immunization with OA-heparin, hemagglutination titers ranging between 1:20 and 1:1,400 were obtained, and there was no change in the precipitin patterns noted earlier.

Three sets of rabbits of 3, 3, and 5 animals per group were immunized using this procedure. Antibodies hav-
ing the characteristics described earlier were obtained in some or all of the rabbits in each set.

**Effect of Carbodiimide Treatment of Heparin as a Function of EDAC Concentration**

Because EDAC treatment was necessary for the antibody to recognize heparin, the effect of EDAC concentration was examined. Table 1 presents data demonstrating that there are at least three concentration-dependent effects of the EDAC treatment of heparin. At the lowest EDAC concentrations, hemagglutination inhibition by heparin increased with increasing EDAC concentration, while the ability to measure heparin by dye binding (azure A) or anticoagulant activity (by both anti-Xa and anti-thrombin assays) was unimpaired. Once the EDAC concentration reached 85 μg/mL, maximum hemagglutination inhibition was obtained and heparin’s ability to act as an anticoagulant and bind azure A was impaired. There was no significant loss of hemagglutination inhibition even with a 1,000-fold increase in EDAC concentration, although azure A binding and anticoagulant activities were almost totally lost. Similar results were obtained using heparin fractions with high or low anticoagulant activity isolated by affinity chromatography on antithrombin III-Sepharose, indicating that epitope formation is not related to the ability of the heparin to bind to antithrombin III.

**Cross-reactivity of Mucopolysaccharides After EDAC Treatment**

Because maximum hemagglutination inhibition was obtained when a 1 mg/mL solution of heparin was treated with more than 1 mg/mL of EDAC (Table 1), the cross-reactivity of EDAC-treated mucopolysaccharides was investigated at three EDAC concentrations above 1 mg/mL. Table 2 presents the data obtained for a series of mucopolysaccharides, including two heparin samples, each treated with EDAC.

Hemagglutination inhibition titers were equal for the two heparin samples at the three concentrations studied. Heparan sulfate had 25% and dermatan sulfate had less than 1% of the inhibitory titer of heparin. The four other EDAC-treated mucopolysaccharides, at concentrations up to 500 μg/mL, did not cross-react with EDAC-treated heparin. It had been anticipated that heparan sulfate, a compound having a similar molecular structure to that of heparin, might show some cross-reactivity with heparin, since heparan sulfate also possesses 10% to 20% of the anticoagulant activity of heparin. Using a standard procedure, the cross-reactivity of dermatan sulfate was shown to be caused by contamination with heparin.

**Fractionation of Antibodies on Carbodiimide-Treated Heparin-Sepharose**

**EDAC-treated heparin-Sepharose.** When the IgG fractions from immune plasma were chromatographed on EDAC-treated heparin-Sepharose, three protein peaks eluting at 0.14 mol/L NaCl, 0.5 mol/L NaCl, and 2 mol/L KI were obtained. Only the material eluting with 2 mol/L KI had antibody activity as determined by hemagglutination of BSA-heparin-labeled red cells. In contrast to the unfractionated IgG, the latter fraction no longer contained anti-OA or anti-bovine IgG antibodies.

**EDAC-treated heparin-Sepharose.** Protein peaks were obtained at 0.14, 0.5, and 2.0 mol/L NaCl and 2.0 mol/L KI when the IgG fraction was chromato-

### Table 1. Effect of EDAC Concentration on Hemagglutination Inhibition, Azure A Activity, and Anticoagulant Activity of Heparin

<table>
<thead>
<tr>
<th>EDAC Concentration</th>
<th>Minimum Inhibitory Concentration*</th>
<th>Azur A*</th>
<th>Anticoagulant Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>—</td>
<td>500</td>
<td>80</td>
</tr>
<tr>
<td>1.7*</td>
<td>12.50</td>
<td>520</td>
<td>80</td>
</tr>
<tr>
<td>2.4*</td>
<td>6.20</td>
<td>500</td>
<td>80</td>
</tr>
<tr>
<td>17.0*</td>
<td>0.20</td>
<td>470</td>
<td>75</td>
</tr>
<tr>
<td>85.0*</td>
<td>0.05</td>
<td>380</td>
<td>65</td>
</tr>
<tr>
<td>170.0*</td>
<td>0.05</td>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td>850.0*</td>
<td>0.05</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>1.7‡</td>
<td>0.05</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>17.0‡</td>
<td>0.10</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>85.0‡</td>
<td>0.05</td>
<td>35</td>
<td>0</td>
</tr>
</tbody>
</table>

The actual heparin concentration was 500 μg/mL. No hemagglutination inhibition was observed at untreated heparin concentrations of up to 10 mg/mL.

*Concentration in μg/mL.

†Units per milliliter as determined by the factor Xa heparin assay. Similar results were obtained using a thrombin-based assay.

‡Concentration in mg/mL.

### Table 2. Inhibition of Hemagglutination by EDAC-Treated Mucopolysaccharides

<table>
<thead>
<tr>
<th>Mucopolysaccharide</th>
<th>Minimum Inhibitory Concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>0.025</td>
</tr>
<tr>
<td>Heparin‡</td>
<td>0.050</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>0.200</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>6.200</td>
</tr>
<tr>
<td>Chondroitin-6-sulfate</td>
<td></td>
</tr>
<tr>
<td>Chondroitin-4-sulfate</td>
<td></td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
</tr>
</tbody>
</table>

*Minimum concentration in micrograms of mucopolysaccharide per milliliter required to inhibit hemagglutination.

†Concentration of EDAC used to treat the mucopolysaccharides at 1 mg/mL.

‡Commercial heparin.

§Heparin from the mucopolysaccharide standards kit.

∥No inhibition of hemagglutination at concentrations of 500 μg/mL.
graphed on ETAC-treated heparin-Sepharose. Each of the fractions had antibody activity as determined by hemagglutination of red cells labeled with BSA-heparin, except where the initial titers in the IgG fraction were low.

Hemagglutination inhibition assays using carbodiimide-treated heparin were performed on each antibody fraction to determine the specificity of each fraction. For the antibodies eluting with 0.14 and 0.5 mol/L NaCl, EDAC-treated heparin inhibited hemagglutination as with the unfraccionated antibody. ETAC- and CMC-treated heparin, however, had no hemagglutination inhibitory activity against these antibodies.

Hemagglutination by the two antibody fractions that eluted with 2 mol/L NaCl and 2 mol/L KI was inhibited by either EDAC-, ETAC-, or CMC-treated heparin, if the carbodiimide treatment was terminated after no more than two hours. Reaction for longer periods resulted in the loss of carbodiimide-treated heparin’s ability to inhibit the antibody eluting with 2 mol/L NaCl while retaining complete hemagglutination inhibition against the antibody eluting with 2 mol/L KI. This effect was independent of the type of carbodiimide used.

**Concentration Dependence of \(^{3}H\)-ETAC Treatment of Heparin**

Heparin was treated with \(^{3}H\)-ETAC under standard conditions of reaction volume and heparin concentration. Heparin was separated from unbound radiolabeled material by desalting on a column packed with Bio-gel P6. Control experiments determined the position at which heparin eluted from the column. The sample volume-column size was selected so that a heparin plateau (ca 650 \(\mu\)g/mL) uncontaminated with \(^{3}H\)-ETAC hydrolysis products eluting in the internal volume of the column, was obtained. Any radiolabel that co-chromatographed with the \(^{3}H\)-ETAC–treated heparin, therefore, was bound to the heparin either ionically or covalently. The heparin concentration in the peak fractions was always assigned a value of 650 \(\mu\)g/mL.

Hemagglutination inhibition by \(^{3}H\)-ETAC–treated heparin was determined with BSA-heparin–labeled tanned red cells and the antibody fraction isolated by elution of ETAC-treated heparin-Sepharose with 2 mol/L KI. This antibody was chosen because it recognized ETAC-treated heparin and hemagglutination inhibition was not destroyed by extensive treatment with carbodiimide. Table 3 presents data for the treatment of heparin with \(^{3}H\)-ETAC at concentrations ranging from 1 \(\mu\)g/mL to 1 mg/mL. The results were similar to those obtained with EDAC treatment of heparin (Table 1). At low \(^{3}H\)-ETAC concentrations, hemagglutination inhibition was low, there was no effect on azure A binding or anticoagulant activity, and there was only minor incorporation of \(^{3}H\)-ETAC into heparin. As the mole ratio of \(^{3}H\)-ETAC to heparin in the reaction mixture increased, incorporation of tritium into heparin increased, as did hemagglutination inhibition. There was no effect on the azure A binding or anticoagulant activity of heparin until greater than 0.5 mol of tritium per mole of heparin was incorporated. Hemagglutination inhibition paralleled \(^{3}H\)-ETAC incorporation.

**Time Course for \(^{3}H\)-ETAC Treatment of Heparin**

Table 4 presents a typical time course for the effect of \(^{3}H\)-ETAC treatment of heparin. Maximum hemagglutination inhibition was obtained within two minutes at 28 °C with the incorporation of about one molecule of ETAC per molecule of heparin. The addition of up to eight more ETAC molecules per heparin molecule neither increased nor decreased this titer.

There was no effect on heparin anticoagulant or on azure A binding activity until two to three ETAC molecules had bound to each heparin molecule, after which there was a progressive decrease in both activities, even though the actual heparin concentration in the fractions was 650 \(\mu\)g/mL, as had been determined with control columns.
antibodies to modified heparin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid were reacted with 3H-ETAC chromatographed on DEAE Sephadex A-50 using stepwise elution of the mucopolysaccharides with 0.14, 0.5, and 1.0 mol/L NaCl solutions. Greater than 90% of the radiolabel co-eluted with each of the mucopolysaccharides. What could not be determined from these experiments indicated that 10% to 15% of the antibody could remain adsorbed by protein A. Therefore, even if all of the labeled mucopolysaccharide bound to the antibody, some radiolabeled material (10% to 15%) could remain in the supernatant after protein A adsorption. Table 6 presents data indicating that both 3H-ETAC–treated mucopolysaccharides included in the 1 mol/L NaCl eluate, whereas in control experiments, 3H-ETAC (as the reacted urea) eluted with 0.14 mol/L NaCl in the presence or absence of added heparin.

Quantitation of the Binding of 3H-ETAC–Treated Heparin and Heparan Sulfate to the Antibody

The amount of 3H-ETAC added to the mucopolysaccharides indicated that the adducts of carbodiimide form with all of the molecules of the treated mucopolysaccharides. What could not be determined from these data was whether the epitope elicited by carbodiimide treatment was associated with the majority of the heparin or heparan sulfate molecules, a small portion of them, or an impurity in the mucopolysaccharides.

Treatment of Mucopolysaccharides With 3H-ETAC

Heparin, heparan sulfate, dermatan sulfate, keratan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid were reacted with 3H-ETAC under identical conditions. Samples were obtained at the times indicated, desalted, and assayed for 3H-ETAC incorporation and hemagglutination inhibition. The data in Table 5 demonstrate that all of the mucopolysaccharides form adducts with 3H-ETAC. Although heparin incorporates the greatest amount of 3H-ETAC, several of the other mucopolysaccharides have nearly as much (70% to 80%) radiolabel bound to them.

As was the case when the mucopolysaccharides were treated with EDAC (Table 2), the only compound to demonstrate significant cross-reactivity in the hemagglutination inhibition assay was 3H-ETAC–treated heparan sulfate. As previously shown with EDAC–treated dermatan sulfate, the 3H-ETAC–treated material had less than 1% of the titer of 3H-ETAC–treated heparin. Keratan sulfate had 0.01% of the heparin titer after ETAC treatment, while the remaining mucopolysaccharides did not inhibit hemagglutination, although they did incorporate significant amounts of 3H-ETAC.

Radiolabeled samples of 3H-ETAC–treated heparin, heparan sulfate, and chondroitin-6-sulfate were chromatographed on DEAE Sephadex A-50 using stepwise elution of the mucopolysaccharides with 0.14, 0.5, and 1.0 mol/L NaCl solutions. Greater than 90% of the radiolabel co-eluted with each of the mucopolysaccharides.

Table 4. Time Course of 3H-ETAC Treatment of Heparin

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>3H-ETAC Incorporated</th>
<th>Anticoagulant Activity</th>
<th>Apparent Heparin Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>650</td>
</tr>
<tr>
<td>2</td>
<td>1.3:1</td>
<td>0.10</td>
<td>620</td>
</tr>
<tr>
<td>5</td>
<td>1.6:1</td>
<td>0.10</td>
<td>670</td>
</tr>
<tr>
<td>10</td>
<td>1.9:1</td>
<td>0.10</td>
<td>570</td>
</tr>
<tr>
<td>30</td>
<td>3.3:1</td>
<td>0.05</td>
<td>540</td>
</tr>
<tr>
<td>60</td>
<td>4.2:1</td>
<td>0.10</td>
<td>510</td>
</tr>
<tr>
<td>120</td>
<td>4.7:1</td>
<td>0.10</td>
<td>425</td>
</tr>
<tr>
<td>24 h</td>
<td>9.4:1</td>
<td>0.05</td>
<td>220</td>
</tr>
</tbody>
</table>

Heparin, 800 µg/mL, was reacted with 3H-ETAC, 440 µg/mL, at 28 °C for the time indicated. The time is given in minutes except where indicated. The actual heparin concentration was 650 µg/mL. No inhibition of hemagglutination was obtained at an untreated heparin concentration of 650 µg/mL.

*The mole ratio of 3H-ETAC incorporated into heparin using molecular weights of 300 for ETAC and 15,000 for heparin.
†The measured heparin concentration in µg/mL required to inhibit hemagglutination.
‡The minimum heparin concentration in µg/mL required to inhibit hemagglutination.
§Units/mL as determined by the factor Xa heparin assay. Similar results were obtained using a thrombin-based assay.

Table 5. Effect of 3H-ETAC Treatment on Mucopolysaccharides

<table>
<thead>
<tr>
<th>Mucopolysaccharide</th>
<th>Reaction Time</th>
<th>3H-ETAC Incorporated</th>
<th>Minimum Inhibitory Concentration</th>
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<tbody>
<tr>
<td>Heparin</td>
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<td>10 min</td>
<td>53.0</td>
<td>0.06</td>
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</tr>
<tr>
<td>60 min</td>
<td>90.0</td>
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<tr>
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<td>180.0</td>
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<td>Heparan sulfate</td>
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<tr>
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<td>Dermatan sulfate</td>
<td>24 h</td>
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<td>13.00</td>
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<td>Chondroitin-6-sulfate</td>
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Mucopolysaccharide, 800 µg/mL, was reacted with 3H-ETAC, 200 µg/mL, at 28 °C for the time indicated.
*Microgram of 3H-ETAC incorporated per milligram of mucopolysaccharide.
†The minimum concentration of mucopolysaccharide in µg/mL required to inhibit hemagglutination.
‡No inhibition of hemagglutination was obtained at a mucopolysaccharide concentration of 650 µg/mL.
obtained. A portion of these complexes was eluted co-chromatographed on gel chromatography were conditions,6t complexes of protein and heparin that had been exposed to EDAC were obtained. Pre-emptin lines of partial identity to EDAC-treated heparin were obtained when heparin was treated with other water-soluble carbodiimides, such as ETAC and CMC. Untreated heparin, however, did not form a precipitate with the antisera.

Tanned red blood cells labeled with BSA-heparin were used for hemagglutination inhibition assays. Heparin failed to inhibit hemagglutination at concentrations up to 10 mg/mL, whereas EDAC-treated heparin inhibited hemagglutination at concentrations as low as 50 to 100 ng/mL (Table 1). ETAC- and CMC-treated heparin also inhibited hemagglutination but at higher concentrations than EDAC-treated heparin. Antibodies were eluted from untreated heparin-Sepharose between 0.14 and 0.50 mol/L NaCl, whereas 2 mol/L KI was required for elution from EDAC-treated heparin-Sepharose.

These findings indicate that carbodiimide treatment, either while heparin is reacting with the proteins or in the absence of added proteins, is required for epitope formation. The data, however, do not indicate what carbodiimide-related reaction is involved in epitope formation.

Epitope formation was complete at an EDAC concentration of 85 μg/mL (Table 1). At this EDAC concentration, there was a minimal loss of heparin anticoagulant or dye-binding activity (Table 1). As the EDAC concentration was raised to as high as 85 mg/mL, the amount of epitope, measured by hemagglutination inhibition, remained constant, while anticoagulant and dye-binding activities were markedly reduced.

As with heparin, the other mucopolysaccharides (heparan sulfate, dermatan sulfate, chondroitin-6-sulfate, chondroitin-4-sulfate, keratan sulfate, and hyaluronic acid) were not recognized by the antisera as determined by immunoprecipitation and hemagglutination inhibition techniques. The specificity of the antibodies was further tested using EDAC-treated mucopolysaccharides. The mucopolysaccharides were treated with EDAC at concentrations well above that required for maximum epitope formation in heparin (Table 2). Only EDAC-treated heparan sulfate demonstrated some cross-reactivity with EDAC-treated heparin. Although dermatan sulfate did exhibit minor cross-reactivity, nitrous acid degradation demonstrated that this was caused by contamination with heparin. The lack of cross-reactivity with the remaining EDAC-treated mucopolysaccharides could be related either to the requirement of a specific sugar structure found only in heparin and heparan sulfate for epitope formation, or the inability of the other mucopolysaccharides to react with EDAC.

Heparin contains amine, alcohol, and carboxylic acid residues. When a protein-heparin mixture is treated with excess carbodiimide, besides synthesizing a protein-heparin complex, heparin can react intramo-
Antibodies to Modified Heparin

Conformational change responsible for the epitope for which are normal carbodiimide-assisted reaction products (24,25). Such reactions could be accompanied by a conformational change responsible for the epitope formation in heparin. In addition, carbodiimide adduct formation could occur, and this adduct could be the epitope. Alternatively, the adduct might be part of the epitope, with sugar residues providing the major antibody recognition site.

\(^3\)H-ETAC was used as a probe to determine whether a carbodiimide adduct could form on heparin and thereby be responsible for epitope formation. Because ETAC-treated heparin exhibited only partial cross-reactivity with the EDAC-treated compound, the isolated immunoglobulin fraction was chromatographed on ETAC-treated heparin-Sepharose. Four antibody fractions were obtained that recognized EDAC-treated heparin. Of these four fractions, only those eluting with 2 mol/L NaCl and 2 mol/L KI also recognized ETAC-treated heparin. Because the antibody population eluting with 2 mol/L NaCl could not recognize heparin that had been treated with excess carbodiimide for extended periods of time, while the 2 mol/L KI fraction was unaffected by such treatment, the latter antibody fraction was used in all subsequent experiments. Data from these experiments, therefore, are specifically related to this antibody population and may not be valid for any of the other antibody fractions.

\(^3\)H-ETAC generated an epitope in heparin (Table 3). Maximum epitope formation was obtained when the ratio of \(^3\)H-ETAC bound per heparin was approximately 1:1, at which ratio neither dye-binding nor anticoagulant activity was decreased. The incorporation of up to eight additional \(^3\)H-ETAC molecules per heparin molecule, although associated with substantive decreases in dye-binding and anticoagulant activities, did not increase the number of epitopes (Table 4). These findings indicate, for the population of antibodies studied, that although the addition of carbodiimide to heparin molecule may be required for epitope formation, it is not sufficient to generate a site that is recognized by the antibody. Rather, some specific sugar or polysaccharide sequence may be required for epitope formation.

A substantial amount of \(^3\)H-ETAC was incorporated into each of the mucopolysaccharides examined (Table 5). Only \(^3\)H-ETAC-treated heparan sulfate, however, demonstrated substantial cross-reactivity with \(^3\)H-ETAC-treated heparin. Thus, although \(^3\)H-ETAC formed adducts with the other mucopolysaccharides, there was no epitope formation. This observation is also consistent with the conclusion from previous data that carbodiimide addition to a sugar moiety is not, in itself, sufficient for epitope formation.

To exclude the possibility that an impurity, or only a small percentage, of the heparin molecules developed epitopes independent of the radiolabeling, the amount of radiolabeled mucopolysaccharide that could be bound to the antibody was determined. Both heparin and heparan sulfate were completely bound to the antibody compared with nonimmune IgG controls (Table 6). Dermatan sulfate, containing less than 1% heparin, was not adsorbed by these antibodies, as was also the case with chondroitin-6-sulfate. These findings indicate that all of the heparin and heparan sulfate molecules form epitopes that are recognized by the 2 mol/L KI eluate. It is, therefore, unlikely that the epitope is formed on a contaminant or only a small percentage of the heparin molecules.

At least one population of antibodies has been raised and isolated (2 mol/L KI eluate) that recognizes carbodiimide-treated heparin independent of the structure of the carbodiimide. This could occur if the epitope were (1) a carbodiimide adduct with recognition insensitive to the structure of the carbodiimide; (2) composed of one or more sugar residues and carbodiimide adduct with recognition insensitive to the structure of the carbodiimide; (3) composed of one or more sugar residues whose conformation had been altered by the addition of carbodiimide to the heparin molecule; or (4) composed of one or more sugar residues whose conformation had been altered due to a carbodiimide-assisted reaction (amide, ester, or anhydride formation) of the heparin molecule. Of these four possibilities, the first can be eliminated, since the addition of \(^3\)H-ETAC to all mucopolysaccharides or of more than one \(^3\)H-ETAC molecule to heparin did not result in epitope formation. The remaining three possibilities all have in common the requirement that the epitope contain a portion of the heparin molecule.

ETAC, although structurally similar to EDAC, has a positive charge, and CMC contains two bulky cyclic structures not found in either of the other two compounds. If the carbodiimide adduct were a major contributor to the epitope, such alterations would be expected to result in major differences in the recognition of EDAC-, ETAC-, and CMC-treated heparin. In fact, each of these carbodiimide-treated heparins was equally recognized by the 2 mol/L KI eluate. Thus, of the three remaining possibilities, the second would be highly unlikely unless the carbodiimide adduct were a minor contributor to the epitope. Neither of the latter two possibilities can be excluded, although the third may be favored, since epitope formation appears to correlate with the addition of a single carbodiimide molecule per heparin molecule.
Using standard immunologic techniques, the data presented herein demonstrate, for the first time, that antibodies to an epitope derived from heparin can be reproducibly formed. In addition, several of the experiments were successful in establishing that at least one fraction of the antibodies is directed specifically against an altered portion of the polysaccharide rather than against the carbodiimide adduct itself. One further observation is also of importance: that epitope formation is complete even when the carbodiimide-induced structural change in heparin is so minor that both anticoagulant and azure A binding activities are totally retained.

As convincing as the evidence in support of these statements may be, the question can reasonably be asked, Of what biologic usefulness is the availability of an antibody to an altered heparin species? The possibilities for obtaining such data are several-fold.

Because epitope formation is complete without major structural alterations in the heparin molecule, the carbodiimide-treated heparin could serve as a model for commercial heparin in determining the distribution of exogenous heparin to various tissues. The altered heparin, administered to animals intravenously, subcutaneously, or by intrapulmonary installation, and the antibodies described in this report can be used as sensitive probes to determine where the added heparin is distributed throughout the body. Because both high-activity and low-activity heparin are recognized by the antibodies, these experiments can also be performed with fractionated heparin to determine whether the distributions of these different species vary because of their functional differences.

In many investigations, heparin is bonded to artificial surfaces to render them nontrombogenic. Because heparin can be treated with carbodiimide to produce an antigenic compound without losing anticoagulant activity, carbodiimide-treated heparin could be bound to the artificial surfaces to be examined and the antibodies described in this report used to monitor the in vivo fate of the attached heparin. Alternatively, heparin could be bound to the artificial surface and then treated with carbodiimide—a procedure that was, for example, used successfully in this investigation with heparin-Sepharose.

A somewhat more speculative study would be to use the antibodies as probes for endogenous heparin, especially on cell surfaces. The reaction of heparin with EDAC to yield a heparin molecule recognized by the antibodies occurs at very low EDAC concentrations. The maximum antigenic effect is obtained at an EDAC concentration of about 100 μmol/L, and the reaction is readily observed at concentrations as low as 10 μmol/L (Table 1). Such marked sensitivity to EDAC suggests the feasibility of treating washed cells such as platelets, erythrocytes, intact endothelium, and a variety of cell types in tissue culture with EDAC and testing for the presence of heparin by standard immunofluorescent techniques, using the antibodies characterized in this report.

It is possible that the protein-heparin complexes can be used to obtain monoclonal antibodies, since these complexes have now been used successfully to obtain antibodies in rabbits. Such a result would permit identification of antibodies that recognize specific regions on the heparin molecule, such as the antithrombin III or thrombin binding sites. These antibodies could then be used to probe the structural requirements for heparin’s anticoagulant action.

Finally, because antibodies have been obtained that recognize heparin that itself has undergone only minor structural changes after carbodiimide treatment, it seems possible to anticipate that antibodies to unaltered heparin may also have formed. If this assumption is validated, the techniques described in this report for preparing antiserums, would make it possible to isolate these antibodies to native or commercial heparin.

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ANTIBODIES TO MODIFIED HEPARIN

Preparation and identification of a population of antibodies that recognize carbodiimide-modified heparin

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