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The Purification and Mechanism of Action of Human Antithrombin-Heparin Cofactor*

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SUMMARY

A procedure is presented for purifying antithrombin-heparin cofactor from human plasma. The final product is homogeneous as judged by disc gel electrophoresis, sodium dodecyl sulfate gel electrophoresis, and immunoelectrophoresis. The final yield averages 12%. A specific antibody directed against pure inhibitor preparations precipitates virtually all of both antithrombin and heparin cofactor activity from defibrinated plasma. The purification, the immunoprecipitation, and other data, indicate that both activities are properties of a single molecular species.

The inhibitor and thrombin form a 1:1 stoichiometric complex which cannot be dissociated with denaturing and reducing agents. Addition of heparin, a widely used anticoagulant which specifically accelerates the action of our inhibitor, increases the rate of formation of this complex without altering its stoichiometry or its dissociability. Interaction of thrombin with antithrombin-heparin cofactor requires the presence of the active center serine of the enzyme and arginine residue(s) on the inhibitor, since chemical modification of either of these critical residues inhibits complex formation both in the presence and absence of heparin. We suggest that, in analogous fashion to trypsin-trypsin inhibitor systems, a specific interaction occurs between the active center serine of thrombin and a unique arginine-x reactive site on the antithrombin-heparin cofactor.

Furthermore, lysyl residues of the inhibitor probably serve as a binding site for heparin, since chemical modification of these residues virtually eliminates heparin cofactor activity with only minimal reduction of antithrombin activity. We postulate that heparin binds to the inhibitor and causes a conformational change which results in a more favorable exposure of the arginine reactive site, allowing a rapid interaction with thrombin.

At the beginning of this century, Contejean (1), Morawitz (2), Howell (3), and others recognized that thrombin gradually lost activity when added to defibrinated plasma or serum. On this basis, they suspected that a specific inactivator of this enzyme, antithrombin,[1] must be present in plasma under normal physiological conditions. Over the next 50 years, many investigators attempted to show that the antithrombin capacity of plasma represents a true molecular entity.

In 1916 McLean (5) isolated heparin from the liver, as well as the heart, and demonstrated its potent anticoagulant action. Confusion about its inhibitory effects on purified procoagulants was resolved by Brinkhous *et al.* (6) in 1939, who showed that heparin was effective as an anticoagulant only in the presence of a plasma component which they termed heparin cofactor. In the 1950's the work of Waugh *et al.* (7) and Monkhouse *et al.* (8) indicated that plasma antithrombin activity and plasma heparin cofactor activity are intimately related. These investigators suggested that heparin acts to accelerate, by 50- to 100-fold, the rate at which antithrombin neutralizes thrombin. In 1968, this hypothesis was substantiated by Abildgaard (9) who obtained small amounts of a purified human plasma protein which functions in both capacities. Furthermore, several groups (10–12) of investigators have shown that, in several species, this inhibitor acts in similar fashion to inhibit Factor X_a.

Our own investigations, summarized in this communication, provide the first reproducible means for obtaining large amounts of this inhibitor from human plasma and advance the most convincing evidence to date that both plasma antithrombin and plasma heparin cofactor activities reside in the same molecular species. In addition, we present the first mechanistic analysis of the thrombin-inhibitor interaction. Thus we provide a framework for understanding the heretofore mysterious action of heparin as an anticoagulant.

METHODS AND MATERIALS

Column Chromatographic Materials

Sephadex G-50 and G-200, Sepharose 4B, and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Pis-

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[1] Seegers *et al.* (4) have proposed a widely adopted classification of mechanisms responsible for thrombin inhibition: antithrombin I was defined as the ability of fibrin to adsorb thrombin; antithrombin II designated the plasma cofactor required for heparin's anticoagulant action; antithrombin III was employed to describe the entity, present in plasma and serum, which neutralizes thrombin in a slowly progressive fashion; antithrombin IV was recommended as the name for a postulated inhibitor generated during clotting. In this terminology antithrombin-heparin cofactor would be designated as antithrombin III-antithrombin II.

cataway, N. J. DEAE-Sephadex A-50 was swollen for 72 hours at 4° in 0.1 M Tris-HCl (pH 9.0) prior to use. Bio-Gel P-200, DEAE-cellulose, and Cellulose-PO_4 were purchased from Bio-Rad Laboratories, Richmond, Calif. DEAE-cellulose (DEAE-32 microgranular) was obtained from Whatman, defined, washed to pH 12 with 0.5 M NaCl in 0.5 M NaOH, to pH 2 with 0.5 M NaCl in 0.5 M HCl, and equilibrated with 0.1 M Tris-HCl (pH 8.3).

Chemicals

[^{3}H]DFP[2] with a specific activity of 4.9 mCi per mg was purchased from New England Nuclear. Unlabeled DFP, 1,2-cyclohexanedione, and *O*-methylisourea were purchased from K and K Laboratories, Plainview, N. Y. 2,3-Butanedione was obtained from Eastman-Kodak Company, Rochester, N. Y.

Plasma

Human plasma was obtained by plasmaphoresis of normal donors using ACD as an anticoagulant. Protein purifications were initiated within 2 hours of plasma collection.

Heparin and Protamine Sulfate

Heparin and ^{35}S-labeled heparin of specific activity 6 mCi per g were purchased from Organon, West Orange, N. J. and Calatomic, Los Angeles, Calif., respectively. Heparin used in preparing heparin-Sepharose was obtained in crude form (Stage 14) from the Wilson Chemical Corporation, Chicago, Ill. It was further purified by three successive precipitations from 1.2 M NaCl with cetylpyridinium chloride as described by Lindahl *et al.* (13). The resulting preparations averaged 175 U.S.P. units per mg.[3] Protamine sulfate (10 mg per ml) was obtained from Eli Lilly and Company, Indianapolis, Ind.

Proteins

Ribonuclease, chymotrypsinogen, ovalbumin, aldolase, albumin, and phosphorylase were purchased from Worthington Biochemical Corporation, Freehold, N. J. Human fibrinogen (Grade L) was obtained from AB Kabi, Stockholm, Sweden. Crude hirudin was obtained from the Sigma Chemical Company, St. Louis, Mo. and purified by an affinity column method.[4]

Human prothrombin was prepared from plasma by DEAE batch fractionation, barium citrate adsorption-elution, and $(NH_4)_2SO_4$ precipitation according to Shapiro and Waugh (14). Crude human Factor V (25 times purified) was obtained from plasma by isoelectric precipitation (15). Human brain thromboplastin was prepared by the method of Quick (16). The human prothrombin was rapidly and quantitatively converted to thrombin with optimal concentrations of thromboplastin and Factor V. The resulting serine protease was purified by DEAE-cellulose, cellulose-PO_4, and Sephadex G-100 chromatography as described by Rosenberg and Waugh (17). The final product is homogeneous as judged by disc gel or sodium dodecyl sulfate gel electrophoresis and has a specific activity of 3200 NIH units per mg.

To alkylphosphorylate the active serine of thrombin, 10 ml of the enzyme solution at a concentration of 0.165 absorbance units per ml were treated with 10 μl of 1 M DFP in 2-propanol. This inactivation was conducted at an ionic strength of 0.15 to 0.35, pH of 7.5 or 8.3, and 24°. The 350-fold molar excess of DFP produced a time-dependent inhibition of the enzyme so that 1% or less of esterolytic and proteolytic activity is present after 4 hours of incubation. Under these conditions, approximately 1 mole of DIP is incorporated per mole of thrombin.

Preparation of Thrombin-Sepharose, DIP-Thrombin-Sepharose, Ethanolamine-Sepharose, and Heparin-Sepharose

Sepharose 4B was activated with cyanogen bromide (18) and suspended in an equal volume of 0.1 M $NaHCO_3$ (pH 8.5). Twenty to 50 ml of native or DIP-thrombin were exhaustively dialyzed against 4 liters of 0.1 M $NaHCO_3$ (pH 8.5) and added to activated Sepharose at a concentration of 0.14 absorbance unit per ml of "packed gel." A bland control gel was prepared by adding 0.1 ml of ethanolamine per ml of packed gel. After stirring for 16 hours at 4°, the resulting matrix was washed successively with 2 liters of 0.01 M Tris-HCl in 0.15 M NaCl (pH 7.5), 1 M glycine (pH 8.5), and 0.01 M Tris-HCl in 0.15 M NaCl (pH 7.5).

To determine whether thrombin-Sepharose and DIP-thrombin-Sepharose contained the same quantity of protein per ml of matrix, purified hirudin was employed. This specific inhibitor of thrombin interacts equally well with the native and alkylphosphorylated enzyme (19). Several preparations of thrombin-Sepharose and DIP-thrombin-Sepharose were titrated with hirudin using clotting time assays, and in no case did the amount of hirudin absorbed differ by greater than 7%. Furthermore, these titrations suggested that approximately 70% of the thrombin or DIP-thrombin bound to Sepharose 4B is available for interaction. Both the DIP-thrombin-Sepharose and the thrombin-Sepharose appeared to be stable for long periods of time as judged by their ability to absorb a given amount of pure hirudin. However, both types of gels were usually employed within 4 days of their preparation.

Heparin-Sepharose was prepared essentially as described above with 2.67 mg of the sulfated mucopolysaccharide added per ml of packed gel (20). Since amino groups are essential in binding heparin to the matrix, purified heparin with covalently attached amino acids and polypeptides was employed. To quantitate the amount of heparin bound to Sepharose 4B, varying concentrations of free heparin were mixed with a constant amount of defibrinated plasma and heparin-Sepharose. Judging from the concentration of free heparin required to prevent 50% of the inhibitor (measured immunologically) from binding to this matrix, 50 to 70 units of heparin are bound to 1 ml of packed gel. This finding is in excellent agreement with the recently published data of Iverius (21), obtained by direct measurement of hexosamine and sulfate released on mild acid hydrolysis of heparin-Sepharose.

Ultrafiltration

The ultrafiltration units and the Diaflo membranes were purchased from the Amicon Corporation, Lexington, Mass.

Conductivity Measurements

Conductivity measurements were made with a Radiometer conductivity meter, type CDM 2d. Salt concentrations were obtained by comparison with NaCl solutions of known molarity and buffer composition.

[2] The abbreviations used are: DFP, diisopropylphosphorofluoridate; DIP, diisopropylphosphoryl; DTT, dithiothreitol; ACD, 0.44 g of citric acid, 1.32 g of trisodium citrate, 1.47 g of dextrose/100 ml of H_2O.

[3] The heparin preparations were assayed by the South Mountain Laboratories, Maplewood, N. J.

[4] Hirudin is purified by affinity chromatography using thrombin-Sepharose. The final product of this procedure is homogeneous by disc gel electrophoresis. This method will be reported in a subsequent communication.

Isotopic Measurements

Radioactivity determinations were carried out with a Nuclear Chicago liquid scintillation counter equipped with an automatic external standardization system. Samples were counted in a toluene-2,5-diphenyloxazole (PPO) (0.4%)-1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (0.005%) scintillation fluid.

Amino Acid Analysis

Appropriate quantities of protein were dialyzed extensively to remove salts and hydrolyzed in 6 N HCl in sealed tubes at 110° for 20 hours. After removal of HCl, the amino acid content of the hydrolysate was determined with a Beckman 121 amino acid analyzer equipped with an expanded scale attachment and a CRS-100 AL Infotronics integrator.

Measurement of Protein Concentration

In most instances protein concentrations were determined by absorbance measurements at 280 nm. The extinction coefficient of human thrombin was assumed to be 16.2 (22). The extinction coefficient of human antithrombin was estimated as 10.5. This was obtained by comparing the protein content of samples determined by the method of Lowry *et al.* (23), using bovine serum albumin as a standard (Protein Standard Solution, Armour Pharmaceutical Company, Chicago, Ill.), to their absorbance at 280 nm.

In a few instances, antithrombin concentrations were determined fluorometrically with a Baird-Atomic spectrofluorometer. Samples were irradiated at 295 nm and the radiation emitted at 340 nm was quantitated (24).

Gel Electrophoresis

Disc Gel Electrophoresis—The procedure of Davis and Ornstein (25), as modified by Rosenberg and Waugh (17), was used. Samples were applied with 10% glycerol added.

Sodium Dodecyl Sulfate Gel Electrophoresis—The electrophoretic method of Weber and Osborn (26) was used with the gel solution containing 11.1 g of acrylamide and 0.6 g of methylenebisacrylamide per 100 ml. The gels were stained for protein with Coomassie brilliant blue, and molecular weights were calculated according to the technique of Weber and Osborn (26) using ribonuclease, chymotrypsinogen, ovalbumin, aldolase, albumin, and phosphorylase as marker proteins. Each molecular weight represents an average of at least three separate determinations. In some cases band intensity was quantitated by densitometry at 550 nm using a Gilford spectrophotometer equipped with a model 2410 linear transport. The relative areas beneath the peaks were determined planimetrically.

Assays of Inhibitor Activity

Assay of Antithrombin Activity—Progressive antithrombin activity was measured in a two-stage assay similar to that of Gerendas (27). Both the sample and human thrombin were, unless otherwise indicated, either dialyzed against or extensively diluted with 0.1 M Tris-HCl in 0.1 M NaCl (pH 8.3). A 0.1-ml aliquot of human thrombin (0.010 absorbance unit per ml) was added to 0.4 ml of appropriately diluted sample, and the mixture was incubated in albuminized plastic tubes at 37° for 3 min. A 0.1-ml aliquot was removed, added to 0.3 ml of fibrinogen solution (5 mg per ml) dissolved with 0.1 M Tris-HCl in 0.1 M NaCl (pH 8.3) or to diluted bovine plasma, and the clotting time (θ) was recorded. A buffer time (θ_{buffer}) of approximately 18 s was obtained by substituting buffer for sample. All determinations were randomized, performed in triplicate, and compared to a dilution series of either defibrinated plasma (initial stages of purification) or pure antithrombin maintained at −70° or 4°. The progressive antithrombin activity was obtained from the relationship:

$$\text{Log}\left(\frac{\theta}{\theta_{buffer}}\right) = (\text{constant})\ (\text{antithrombin concentration}).$$

Assay of Heparin Cofactor Activity—The heparin-catalyzed immediate action of antithrombin was measured in a one-stage assay similar to that of Abildgaard (28). All samples, fibrinogen, human thrombin, and heparin were either extensively dialyzed against or diluted with 0.15 M NaCl in 0.01 M Tris-HCl (pH 7.5) unless otherwise indicated. To a 0.1-ml aliquot of diluted sample were added 0.1 ml of heparin (25 units per ml) and 0.2 ml of fibrinogen solution (5 mg per ml). The mixture was incubated at 37° for 1 min, 0.1 ml of human thrombin (~0.002 absorbance unit per ml) was added, and θ was immediately recorded. θ_{buffer} of approximately 20 s was obtained by substituting buffer for sample. If buffer was substituted for heparin, the observed θ was virtually identical with θ_{buffer}. All determinations were randomized, performed in triplicate, and compared to a dilution series of pure inhibitor. Heparin cofactor activity was obtained from a relationship functionally identical with that used for antithrombin. When modifying the inhibitor, by dialysis against *O*-methylisourea or 2,3-butanedione, in the presence and absence of heparin, trace amounts of ^{35}S-labeled heparin were added to samples which contained the acidic mucopolysaccharide. After completion of dialysis, aliquots of these samples were counted for ^{35}S. Based upon the radioactivity measured, a portion of each sample was diluted to a heparin concentration of 12.5 units per ml and assayed directly for heparin cofactor activity. Samples dialyzed in the absence of this acidic mucopolysaccharide were assayed for heparin cofactor activity in the usual fashion at an identical heparin concentration. Dialysis-dependent alterations in heparin concentration were in accord with volume-concentration changes established by the weighing of sacs before and after dialysis. Heparin was quantitatively retained by the dialysis membranes.

Assays of Thrombin Activity

Assay of Clotting Activity of Thrombin—The specific activities of thrombin preparations were determined, according to Waugh and Rosenberg (29), with National Institutes of Health (Lot B-3) thrombin standard. The usual log-log relationship of thrombin concentration and clotting time was employed.

Assay of Esterolytic Activity of Thrombin—The esterolytic activity of thrombin was measured with tosyl-L-lysine methyl ester (Cyclo Chemical Company, Los Angeles, Calif.) by continuously recording the uptake of 0.01 M NaOH in a pH-stat fitted with an automatic burette and chart recorder. The buffer used for this assay was 0.005 M Tris-HCl in 0.15 M NaCl (pH 8.3). All protein preparations used in these studies were extensively dialyzed against this assay buffer. The concentration of substrate was 0.015 M, and the reaction velocity at 37° ± 0.1° was recorded for the first 3 min. The composition of each incubation mixture is indicated in the text and consisted of 0.15 ml of which 0.1 ml was added to 0.9 ml of substrate in the assay buffer. Each assay sequence was randomized and included a series of standard thrombin dilutions. Plots of thrombin concentration *versus* reaction velocity were linear with remarkably constant slope and intercept.

Immunoassay of Inhibitor

The antiserum was initially purchased from Nyegaard and Company, Oslo, Norway, but later was obtained by pooling sera from rabbits immunized with our purified antithrombin preparations. In the latter case, 20 to 40 μg of protein in 0.2 ml were mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and were injected subcutaneously into 5- to 6-pound rabbits at biweekly intervals for 2 months. Immune sera were harvested 1 week after the last injection. In both instances, the antisera were monospecific as judged by immunoelectrophoresis (30) and Ouchterlony (31) double diffusion.

Immunological quantitation of the inhibitor was performed with a single radial immunodiffusion micromethod employing Mylar-backed cellulose acetate membranes (Helena Laboratories, Beaumont, Tex.) as described by Nerenberg (32). Three separate dilutions were employed for each immunologic determination, and these were randomly placed on different areas of the cellulose acetate membrane. Purified antithrombin was utilized as a standard.

The γ-globulin fraction of antisera was obtained by filtering 60 to 80 ml of serum through columns of DEAE-cellulose (5 $\times$ 30 cm) at 60 ml per hour according to the method of Stanworth (33). The resultant "low salt" eluate was concentrated to 6 to 8 ml, dialyzed against 0.1 M Tris-HCl in 0.1 M NaCl (pH 8.3), and heated to 60° for 1 hour. These preparations had no detectable antithrombin or heparin cofactor activity.

Immunoelectrophoresis

Immunoelectrophoresis was performed according to the method of Scheidegger (30) utilizing 1.2% agar and a buffer system composed of 0.0075 M barbituric acid, 0.0424 M sodium barbital, and 0.0018 M calcium lactate at pH 8.3. The antigen was placed in the circular well and subjected to electrophoresis at 4 ma per slide for 3 hours. The antibody was then placed in the trough and incubated overnight.

Statistical Procedures

These procedures were as described by Bennett and Franklin (34). In most instances, mean values are given with their associated standard errors.

Dialysis

During all dialysis the initial and final weights of the sacs were used to correct samples for dialysis-dependent concentration changes.

RESULTS

Purification of Antithrombin

In essence, antithrombin was purified from fresh human plasma by heat defibrination, treatment with barium carbonate, aluminum hydroxide adsorption-elution, gel filtration on Sephadex G-200, chromatography on DEAE-Sephadex A-50, chromatography on DEAE-cellulose, and preparative isoelectric focusing in sucrose density gradients. This highly reproducible purification method is presented, in detail, with average specific activity and recovery estimates for each fractionation step. Individual steps have been performed 20 to 35 times and have not deviated markedly from the average results presented below (see Table I).

Step I. Preparation of a Plasma Concentrate by Adsorption-Elution from Aluminum Hydroxide—Barium carbonate was added to 1,000 to 1,500 ml of plasma at a concentration of 50 mg per ml. The plasma was gently stirred for 10 min at 24° and centrifuged for 15 min at 4° and 2,000 $\times$ *g*. The resulting supernatant solution was defibrinated in aliquots of 15 ml by rapidly heating to 54° and holding there for 3 min. The samples were placed on ice for 5 min and centrifuged at 2,000 $\times$ *g* for 15 min. To the combined supernatant solutions, 20% (v/v) aluminum hydroxide, prepared according to Loeb (35), was added while stirring. The resulting creamy suspension was stirred continuously for 15 min at 24° and centrifuged at 2,000 $\times$ *g* for 15 min at 4°. The liquid was discarded and the aluminum hydroxide precipitate was washed with 500 ml of 0.15 M NaCl. To elute inhibitor activity, 0.36 M ammonium phosphate, pH 8.1, was added to the aluminum hydroxide precipitate in a volume equivalent to 25% of the initial heat-defibrinated supernatant solution, and the suspension was gently agitated for 15 min at 24°. The cloudy solution was centrifuged at 2,000 $\times$ *g* for 15 min at 4°. The supernatant solution was clarified by centrifugation at 27,000 $\times$ *g* for 30 min at 4°. The ammonium phosphate elution of antithrombin from the aluminum hydroxide was repeated two additional times. However, the third elution utilized only half of the usual volume of ammonium phosphate. All three aluminum hydroxide eluates were combined and concentrated from approximately 540 ml to about 35 ml. This concentration step required 12 to 24 hours and employed an ultrafiltration apparatus equipped with a PM-30 membrane. A final protein concentration of about 150 absorbance units per ml was achieved. The recovery of antithrombin as judged by either immunoassay or activity determination averaged 55% for Step I. The specific activity of these Step I products averaged 10 units per absorbance unit (average specific activity of plasma = 2.0 units per absorbance unit). The use of fresh plasma is mandatory for optimal yield and maximal specific activity. If frozen plasma is employed, a 25 to 50% reduction in recovery is observed.

For reproducible results, multiple elutions of the inhibitor from aluminum hydroxide are of critical importance. Although, the first eluate may, on occasion, contain 50 to 65% of plasma antithrombin, it customarily averages 26%. In two careful sequential studies of the Step I elution process, immunoassay and activity determinations showed that the first eluate contains 30% of plasma antithrombin (specific activity = 9.4 units per

TABLE I

Summary of purification of human antithrombin-heparin cofactor

These data represent average results obtained employing 1 liter of plasma. Except for the appropriate adjustment of total protein, identical results can be obtained utilizing 1.5 liters of plasma.

Step	Total protein	Specific activity	Yield
	absorbance units	*units/absorbance unit*	%
Plasma[a]	50,000	2.0	100
Aluminum hydroxide adsorption-elution	5,500	10.0	55
Sephadex G-200	2,700	13.0	35.8
DEAE-Sephadex A-50	190	134	26.3
DEAE-cellulose	34.0	494	17.3
Isoelectric focusing	12.1	900	11.3

[a] Both antithrombin and heparin cofactor determinations were employed with plasma arbitrarily set at 100 units per ml.

absorbance unit), while the second and third eluates yielded 15% (specific activity = 10.0 units per absorbance unit) and 5.2% (specific activity = 10.0 units per absorbance unit), respectively. Therefore, three elutions have been employed routinely in the purification procedure. Approximately 41% of plasma antithrombin is not adsorbed by aluminum hydroxide and is discarded prior to the first elution. Attempts to adsorb this remaining inhibitor with additional aluminum hydroxide significantly reduce the final specific activity of this product. If Step I preparations are frozen at either −20° or −90° for 20 hours to 7 days, there is a 60% loss of inhibitor activity. However, these preparations are stable at 4° for at least 4 weeks.

Step II. Sephadex G-200 Gel Filtration—Sephadex G-200 columns (2.5 × 100 cm or 5.0 × 100 cm) were equilibrated at 4° with 0.05 M Tris-HCl in 1.0 M NaCl (pH 8.3). The gel matrix was conditioned by an initial filtration with 4 ml of defibrinated plasma per cm² of column cross-section. After the passage of 1 column volume of buffer, the sample was applied and flow rates of about 3 ml per cm² of column cross-section per hour were maintained with a peristaltic pump. Fractions of 5 to 10 ml were collected and assayed for antithrombin. Although small amounts of antithrombin activity were present in the first protein peak, heparin cofactor activity was absent and these fractions were discarded. The great bulk of antithrombin and heparin cofactor activity emerged in the albumin peak. When these fractions were pooled, total inhibitor recovery averaged 65% and specific activity averaged 13 units per absorance unit. Prior attempts to use unconditioned Sephadex G-200, polyacrylamide P-200, or Sepharose 6B at varying ionic strength, pH, and temperature led to inhibitor recoveries below 35%. Since immunological assays are in excellent agreement with those dependent on activity, this substantial inhibitor loss is probably due to adsorption of the protein by the gel matrix. Clearly, conditioning the gel matrix reduces but does not eliminate this undesirable phenomenon.

On occasion, at the completion of a gel filtration, a peak of inhibitor activity is seen with minimal absorbance at 280 nm. This artifact is usually eliminated by the high speed centrifugation employed in Step I and may be due to residual aluminum hydroxide. Step II preparations are stable for many weeks at 4° but lose significant activity if frozen at −20° to −90°. Prior to the initiation of Step III, these preparations were dialyzed for 8 to 12 hours against 10 to 15 liters of 0.1 M Tris-HCl (pH 9.0).

Step III. DEAE-Sephadex A-50 Chromatography—Fig. 1 shows a typical DEAE-Sephadex chromatogram at pH 9.0 with the inhibitor eluted over a range of 0.02 M NaCl and with the peak of activity centered at an added ionic strength of 0.11 M NaCl. With different lots of DEAE-Sephadex A-50, the added NaCl concentration required to elute maximal amounts of the inhibitor varied from 0.11 M NaCl to 0.14 M NaCl. However, the range of ionic strength over which the inhibitor was eluted, the purity, and the yield of the Step III product were independent of the DEAE-Sephadex A-50 lot number. This step has an average yield of 73% and an average specific activity of 134 units per absorbance unit. If stored at 4°, Step III fractions were stable for 4 to 6 weeks. Prior to Step IV, Step III preparations were dialyzed for 8 to 12 hours against 4 liters of 0.1 M Tris-HCl (pH 8.3).

Step IV. DEAE-cellulose Chromatography—Fig. 2 shows a typical DEAE-cellulose chromatogram at pH 8.3 with the inhibitor emerging over a narrow ionic strength range of 0.008 M NaCl and with the peak of activity centered at an added ionic strength of 0.068 M NaCl. Both of these chromatographic parameters have been remarkably constant for several lots of DEAE-cellulose. The recovery of inhibitor activity was 66% for Step IV and the specific activity averaged 494 units per absorbance unit. Since only fractions with specific activity of 360 units per absorbance unit or greater were pooled, true inhibitor recovery is underestimated by approximately 15%. Rechromatography of Step IV preparations on DEAE-cellulose yielded only a modest 15% increase in specific activity and was therefore not utilized.

The pooled Step IV fractions were concentrated from 100 ml to about 4 ml by ultrafiltration with a PM-30 membrane.

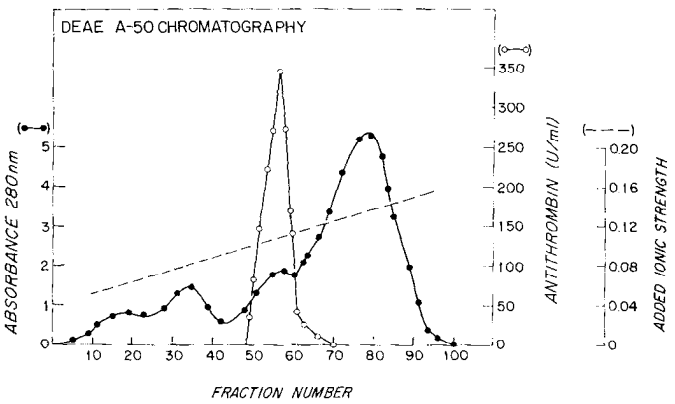

FIG. 1. Ion exchange chromatography of the inhibitor on DEAE-Sephadex A-50. DEAE-Sephadex A-50 was packed into a column (5.0 × 30 cm). Two thousand absorbance units of dialyzed Step II material were applied and a linear salt gradient was employed for fractionation with the reservoir containing 1200 ml of 0.25 M NaCl in 0.1 M Tris-HCl (pH 9.0) and the mixing chamber containing 1200 ml of 0.1 M Tris-HCl (pH 9.0). Chromatography was conducted at 4° with flow rates maintained at 67 ml per hour with a peristaltic pump. Fractions of 13 ml were collected. The original defibrinated plasma, stored at −90°, was assumed to contain 100 activity units per ml and was used as the standard. For other experimental details see "Results."

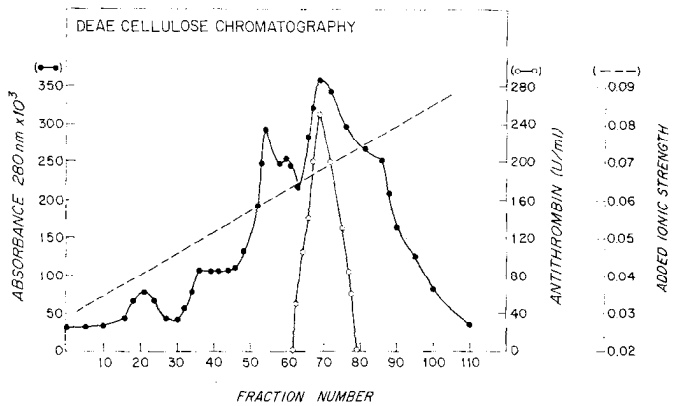

FIG. 2. Ion exchange chromatography of the inhibitor on DEAE-cellulose. DEAE-cellulose (DEAE-32) was packed into a column (2.5 × 40 cm). One hundred forty absorbance units of the dialyzed Step III fraction were applied. This purification step employed a linear salt gradient with the mixing chamber containing 950 ml of 0.1 M Tris-HCl buffer (pH 8.3), and the reservoir containing 950 ml of 0.1 M NaCl in 0.1 M Tris-HCl (pH 8.3). During the chromatography at 4°, flow rates were maintained at 40 ml per hour with a peristaltic pump and fractions of 10.5 ml were collected. The original defibrinated plasma, stored at −90°, was assumed to contain 100 activity units per ml and was used as the standard. For other experimental details, see "Results."

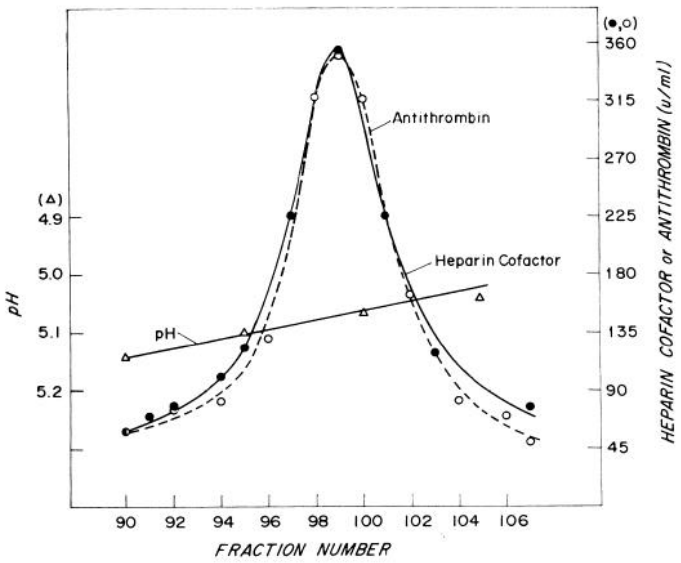

FIG. 3. Isoelectric focusing of the inhibitor in sucrose density gradients. The sucrose (Ultra Pure, Schwarz-Mann, Orangeburg, New York) density gradient was prepared manually with a 1.7% ampholyte concentration. The cathode solution of monoethanolamine was placed on the bottom of the column. The fractions were introduced sequentially with the protein sample in this case containing 17 absorbance units (dialyzed against two changes of 0.5% ampholyte solution for 2 hours) applied in mid-column after being mixed with an equal volume of dense solution. The sulfuric acid anode solution was applied at the top of the column. During isofocusing, the column was maintained at 6°. An initial potential of 250 volts was established for 10 to 12 hours. The voltage was increased gradually to a limiting value of 750 volts in 10 to 12 hours. The total time for isofocusing was 38 hours and the current approached a limiting value of 4.5 ma prior to the termination of each run. The contents of the column were removed at a flow rate of about 2.5 ml per min. To maintain a constant hydrostatic head, the space above the upper electrode compartment was filled with water at the same rate. Fractions of 2.2 ml were collected. Pure inhibitor, obtained by a previous fractionation, was adjusted to contain the identical activity as pooled human plasma (100 units per ml) and employed as the activity standard. For other experimental details, see "Results."

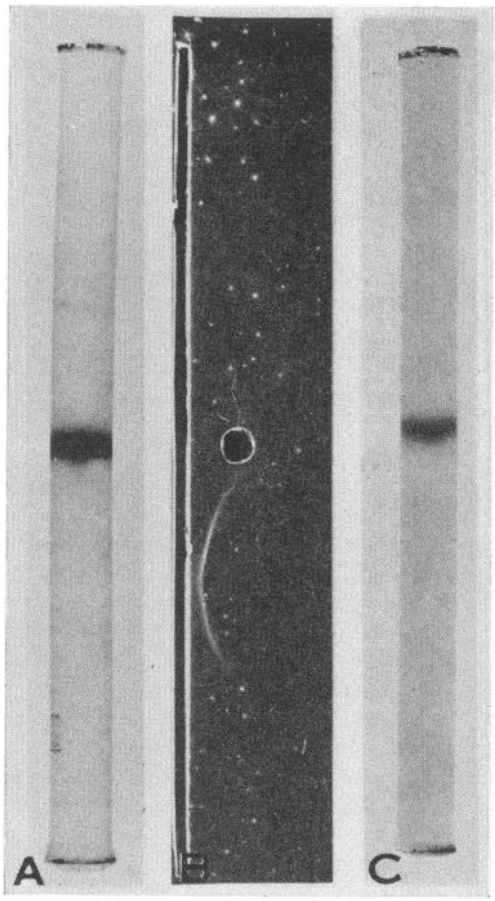

FIG. 4. Electrophoresis and immunoelectrophoresis of the purified inhibitor. The migration of protein is towards the anode (bottom of figure). *A*, a disc gel electrophoretic pattern obtained with 40 μg of protein; *B*, an immunoelectrophoretic pattern obtained with 10 μg of protein in the circular well and 50 μl of concentrated γ-globulin prepared from rabbit antihuman sera (courtesy of Dr. Chester Alper, Childrens Hospital, Boston, Massachusetts). *C*, a sodium dodecyl sulfate gel electrophoretic pattern obtained with 40 μg of protein. See text for additional experimental details.

Step V. Isoelectric Focusing—Isoelectric focusing in an LKB 8102 column was performed, as described by Vesterberg and Svensson (36), with pH 4 to 6 carrier ampholytes (LKB-Produckter, AB, Sweden). As shown in Fig. 3, the inhibitor has an isoelectric point of 5.11 and distributes predominantly over 0.15 pH unit. The recovery of inhibitor activity has averaged 65% and its specific activity has averaged 900 units per absorbance unit. Measurements of antithrombin and heparin cofactor activity from pH 5.0 to 5.15 yield superimposable gaussian profiles. When Fractions 93 to 102 were examined by polyacrylamide disc gel electrophoresis, sodium dodecyl sulfate gel electrophoresis, or immunoelectrophoresis, a single sharp band was seen (Fig. 4). Occasionally, trace amounts of a more rapidly moving impurity (I_1) were found. However, I_1 has never constituted more than 1% of the major band when gels were analyzed by densitometry. Fractions above 102 had increasing quantities of I_1 which may amount to 5% of the major band. Fractions below 93 had a second component (I_2) which had a mobility slightly lower than antithrombin on disc gel electrophoresis. In this region of the pH gradient, antithrombin and I_2 were perceived as a closely split doublet. Preliminary evidence suggests that I_2 may signify inhibitor polymorphism. This final step of the fractionation yields identical results when the protein content of the sample is increased from 17 absorbance units (Fig. 3) to 35 to 50 absorbance units.

Ampholytes have been removed from inhibitor preparations either by extensive dialysis or by filtration through conditioned Sephadex G-50 columns (1.25 × 60 cm).

Immunoprecipitation of Inhibitor from Pooled Defibrinated Human Plasma

The γ-globulin fraction directed against the inhibitor or 0.15 M NaCl in 0.01 M Tris-HCl (pH 7.5) was mixed in ratios of 1:2, 1:1, 2:1, and 3:1 with heat-defibrinated pooled human plasma. These mixtures were incubated at 37° for 1 hour and at 4° for 16 hours. They were centrifuged at 5000 × *g* for 20 min and assayed for antithrombin as well as heparin cofactor activity. As compared to their buffer controls, the activities of antithrombin and heparin cofactor remaining in the mixtures were 43% *versus* 43%, 24% *versus* 32%, 23% *versus* 24%, and 10% *versus* 8%, respectively. Furthermore, the γ-globulin fraction of normal rabbit serum was equivalent to the buffer control when employed in the place of the γ-globulin directed against the inhibitor. In addition, a maximum of 75 to 80% of the antithrombin and heparin cofactor activities could be immunoprecipitated from pooled human plasma defibrinated with small

amounts of purified thrombin (5 NIH units per ml). Therefore our inhibitor is responsible for the major portion of both of these plasma activities.

Interactions of Thrombin and DIP-thrombin with Inhibitor

Analysis of Thrombin-Inhibitor Interactions Using Esterolytic Assays—The inactivation of thrombin by antithrombin was studied at 37° in the presence and absence of heparin. All of the components of this system were either exhaustively dialyzed against or extensively diluted with buffer consisting of 0.15 M NaCl in 0.005 M Tris-HCl (pH 8.3). To examine the antithrombin-thrombin interaction, the inhibitor was initially incubated with buffer for 3 min at 37°; then thrombin was added for varying periods of time. The final concentrations of the enzyme and inhibitor were 0.162 absorbance unit per ml and 0.164 absorbance unit per ml, respectively. To analyze the interaction of thrombin with antithrombin and heparin, the inhibitor was incubated with heparin for 3 min at 37° before the addition of thrombin. The final concentration of the enzyme and inhibitor were maintained at their previous levels, while heparin was 12 units per ml. The small substrate tosyl-L-lysine methyl ester was utilized to measure the initial esterolytic activity of the thrombin and to follow its decay after 10 s, 10 min, and 20 min of incubation in the presence or absence of heparin. For each time point, three separate incubation mixtures were employed. In the presence of heparin, 39% ± 2%, 34% ± 1%, and 36% ± 1% of the initial activity of thrombin remained at 10 s, 10 min, and 20 min of incubation, respectively. In the absence of heparin, 82% ± 1%, 20% ± 0.3%, and 23% ± 1% of the initial activity of thrombin was present at 10 s, 10 min, and 20 min of incubation, respectively.

A statistical analysis of these data revealed: (*a*) in the absence of heparin, more thrombin is inactivated by antithrombin at 10 min of incubation than at 10 s of incubation (20% *versus* 82%). However, additional inactivation of thrombin is not observed at 20 min of incubation (20% *versus* 23%). (*b*) In the presence of heparin, the inactivation of thrombin by antithrombin is complete at 10 s of incubation (39% *versus* 36%). (*c*) Although the immediate (10 s) inactivation of thrombin by antithrombin is significantly greater with heparin (39% *versus* 82%), the final level of thrombin inactivation is significantly greater without heparin (23% *versus* 36%).[5]

When the concentration of antithrombin was increased to 0.240 absorbance unit per ml, residual thrombin could not be detected by esterolytic assay at 10 min in the absence of heparin or at 10 s in the presence of heparin. Thus the active site of thrombin was completely hidden from the small substrate tosyl-L-lysine methyl ester. Therefore, we chose to examine the relationship of the active center serine of thrombin to the interaction with antithrombin.

[5] Our data suggest two possible explanations of this phenomenon. As we will show, heparin significantly increases the amount of inhibitor inactivated by thrombin-Sepharose columns. If heparin were to effect similarly the inactivation of the inhibitor in solution, a decrease in the final amount of neutralized enzyme would occur. Alternately, the dramatic increase in the proteolysis of the antithrombin-thrombin complex by thrombin when heparin is present suggests that thrombin-antithrombin-thrombin complexes may be more rapidly formed under these conditions. These tertiary complexes might protect the "loosely bound" thrombin from undissociable binary complex formation. If this higher order interaction product had an appropriate lifetime and if substrate could compete effectively with the antithrombin-thrombin complex for the second molecule of "loosely bound" enzyme, more esterase or clotting activity would be measured in the presence of heparin than in its absence.

Under the same conditions of pH, ionic strength, and temperature, DIP-thrombin and native thrombin were allowed to compete for antithrombin in the presence and absence of heparin.

Antithrombin and heparin were incubated for 30 s at final concentrations of 0.216 absorbance unit per ml and 25 units per ml, respectively. This incubation mixture was added to an equal volume of DIP-thrombin at a concentration of 0.880 absorbance unit per ml, or native thrombin at a concentration of 0.216 absorbance unit per ml, or buffer. The concentration of native thrombin was chosen carefully to neutralize virtually all of the antithrombin without any free thrombin being detected under these experimental conditions. After 5 min of incubation at 37°, thrombin was added at a concentration of 0.324 absorbance unit per ml in a volume equivalent to the intermediate incubation mixture, and the esterolytic activity of residual thrombin was measured at 10 s. Five separate incubation mixtures were employed for each combination studied. As expected, if native thrombin were initially added to almost saturate antithrombin, 96% ± 3% of the final thrombin addition was present at the time of the esterolytic assay. However, if DIP-thrombin or buffer were initially admixed, only 64% ± 2% or 61% ± 3% of the final thrombin addition remained at 10 s by esterolytic assay.

A statistical analysis, similar to that previously employed, demonstrated that DIP-thrombin could not be distinguished from a buffer addition. However, both of these bland additives significantly differ from the initial native thrombin addition. Since the concentration of DIP-thrombin was approximately four times that of the native thrombin initially added, we could easily have detected binding, provided that 25% or more of the alkylphosphorylated enzyme had successfully competed for sites on antithrombin.

Similar experiments have been conducted in the absence of heparin using 10-min initial and final periods of incubation. Under these conditions, the addition of DIP-thrombin was again indistinguishable from buffer (50% ± 5% *versus* 44% ± 5%). Therefore the active center serine of thrombin is of major importance for interaction with the inhibitor in the presence or absence of heparin.

Interaction of Inhibitor with Thrombin-Sepharose, DIP-Thrombin-Sepharose, and Ethanolamine-Sepharose—To examine further the interaction of antithrombin and thrombin, the purified inhibitor was filtered through columns (0.63 × 17 cm) of thrombin-Sepharose, DIP-thrombin-Sepharose, or ethanolamine-Sepharose at 37°. Prior to each experiment, 2 column volumes of 0.15 M NaCl in 0.01 M Tris-HCl (pH 7.5) were filtered through the matrix at 10 ml per hour. To initiate an experiment, 1.5 ml of antithrombin were applied whose absorbance averaged 0.252 absorbance unit per ml. The flow of equilibrating buffer was re-established at 10 ml per hour. The initial 9 ml were collected and concentrated to 1.0 to 1.5 ml by ultrafiltration with PM-10 membranes. Antithrombin, heparin cofactor, and immunologic assays were utilized to study the concentrated column effluents. These results are summarized in Table II. Both the DIP-thrombin-Sepharose column and the ethanolamine-Sepharose column showed virtually no adsorption of inhibitor. As expected, little antithrombin or heparin cofactor activity emerged from the thrombin-Sepharose column. However, fluorescence measurements demonstrated that 35% of the sample had passed through these columns. This was in reasonable agreement with the immunologic assays, which showed that 25% of the inhibitor was present in the column effluents. When this inactive inhibitor was compared by Ouchterlony double diffusion

TABLE II

Percentage of applied inhibitor in column effluents[a]

Assay	Thrombin-Sepharose		DIP-thrombin-Sepharose		Ethanolamine-Sepharose	
	Heparin	No Heparin	Heparin	No Heparin	Heparin	No Heparin
Heparin cofactor	0	5.9	70	95	66	85
Antithrombin[b]		6.4		92		90
Immunological	46	25.5	66	90	72	87

[a] Each value represents the average of two column filtrations.

[b] Antithrombin measurements are not valid in the presence of heparin.

to purified native inhibitor, a reaction of immunological identity resulted. The inactive inhibitor was also examined by disc gel and sodium dodecyl sulfate gel electrophoresis. Disc gel electrophoresis revealed a major component which moved rapidly toward the anode and a minor component with an anodic mobility only slightly greater than that of native antithrombin. Sodium dodecyl sulfate gel electrophoresis showed major and minor components with apparent molecular weights of 68,000 and 62,000, respectively. The antithrombin filtered through the DIP-thrombin-Sepharose columns or the ethanolamine-Sepharose columns exhibited no alteration in mobility when examined by either electrophoretic method.

To study thrombin-antithrombin-heparin interaction, the above experiments were repeated with 7.5 units per ml of heparin added to the sample prior to filtration. As shown in Table II, filtration of these samples through the DIP-thrombin-Sepharose columns gave results similar to those obtained with the bland ethanolamine-Sepharose columns. However the recovery of inhibitor was considerably less than that obtained with similar filtrations conducted in the absence of heparin. This appears to be due to increased adsorption of inhibitor-heparin complexes by PM-10 membranes. With thrombin-Sepharose columns, no heparin cofactor activity emerged. However, the concentrated column effluents contained 57% of the applied sample by fluorescence measurement and 46% of the applied sample by immunoassay. The inactive derivative gave a reaction of immunological identity with purified antithrombin when examined with Ouchterlony double diffusion. Sodium dodecyl sulfate gel electrophoresis of the inactive inhibitor gave results similar to those obtained when antithrombin was filtered through thrombin-Sepharose columns in the absence of heparin. However disc gel electrophoresis revealed two new components whose mobilities differed from those observed in the absence of heparin. The inhibitor filtered through the DIP-thrombin-Sepharose columns or the ethanolamine-Sepharose columns showed no change in mobility in either electrophoretic system.

Due to the large amount of inactive derivatives produced in the presence of heparin, one might question whether binding of the inhibitor to thrombin-Sepharose actually occurred. Therefore, an attempt was made to saturate small columns of thrombin-Sepharose (0.63 × 5 cm) with purified inhibitor both in the presence and absence of heparin. At a concentration of 0.200 absorbance unit per ml, the inhibitor was filtered through each column in 0.5-ml aliquots. After each filtration, the initial 3 ml of effluent were collected and its heparin cofactor activity determined. Column saturation was defined as the point at which the inhibitory activity of the effluent became equivalent to that of the applied sample. As expected, 1.5 ml of inhibitor were required to saturate the thrombin-Sepharose columns in the absence of heparin, but 5.0 ml of inhibitor were needed to achieve the same result if 7.5 units per ml of heparin were added to samples prior to their filtration. These data confirmed the binding of inhibitor to thrombin-Sepharose in the presence of heparin and emphasized the lack of interaction of inhibitor with DIP-thrombin-Sepharose under similar conditions. In addition, these observations are in excellent agreement with results obtained by single filtrations under "nonsaturating" conditions (Table II).

Heat-defibrinated plasma has also been filtered through columns (0.63 × 17 cm) of thrombin-Sepharose, DIP-thrombin-Sepharose, and ethanolamine-Sepharose, both in the presence and absence of heparin. After concentration of the column effluents, their inhibitor content was measured with immunological and inhibitory activity assays. The results obtained were virtually identical with those previously seen with purified protein. This demonstrated that production of inactive derivatives occurs with this minimally manipulated source of inhibitor as well as with our purified material.

Sodium Dodecyl Sulfate Gel Electrophoretic Analysis of Thrombin-Inhibitor Interactions—When disc gel electrophoresis was used to study the thrombin-antithrombin interaction, the development of a complex was observed. This complex was stable after treatment with varying combinations of 8 M urea, 2% sodium dodecyl sulfate, 0.1 M β-mercaptoethanol, and 6 M guanidine hydrochloride. However, if either protein were initially denatured, an antithrombin-thrombin complex was not seen. These findings suggested that this interaction could be analyzed by serially denaturing samples and observing the molecular species with sodium dodecyl sulfate gel electrophoresis.

Therefore, antithrombin and thrombin were mixed at final concentrations of 0.106 absorbance unit per ml with 0.15 M NaCl in 0.01 M Tris-HCl (pH 7.5) at 37°. This concentration ratio was chosen such that a measurable excess of proteolytic enzyme (~10%) would be present at completion of the reaction. Aliquots of 0.2 ml, which were sequentially removed, were immediately denatured and treated as described in Fig. 5 prior to their analysis by sodium dodecyl sulfate gel electrophoresis.

Both antithrombin and thrombin migrate as single bands in this electrophoretic system with apparent molecular weights of 62,300 ± 1,500 and 33,800 ± 900, respectively. When these proteins are incubated together, both bands disappear over 5 min as a complex of apparent molecular weight 88,700 ± 700 (C_I) is formed (Fig. 5). A minor band (~5%) of apparent molecular weight 68,000 ± 1,000 is also noted. If the interaction is allowed to proceed for 60 min, C_I is half converted to a molecular species of apparent molecular weight 81,000 ± 1,000 (C_{II} in Fig. 5). However, thrombin is not released during this transition.

The interaction of antithrombin and thrombin was also studied in the presence of heparin (5 units per ml). The protein concentrations, conditions of incubation, and analytical methods employed were identical with those described above. Fig. 6 shows the immediate formation of C_I. Densitometric analysis of these and other sodium dodecyl sulfate gels (not shown) suggested that a 15-s period of incubation in the presence of heparin yielded an amount of C_I equivalent to that observed at 3½ min of incubation without heparin. After 15 s of interaction in the presence of heparin, the formation of a minor component of apparent molecular weight 68,000 is also observed. During the next 10 min of interaction, C_I is completely converted to C_{II} (Fig. 6).

To determine whether small amounts of free uncomplexed

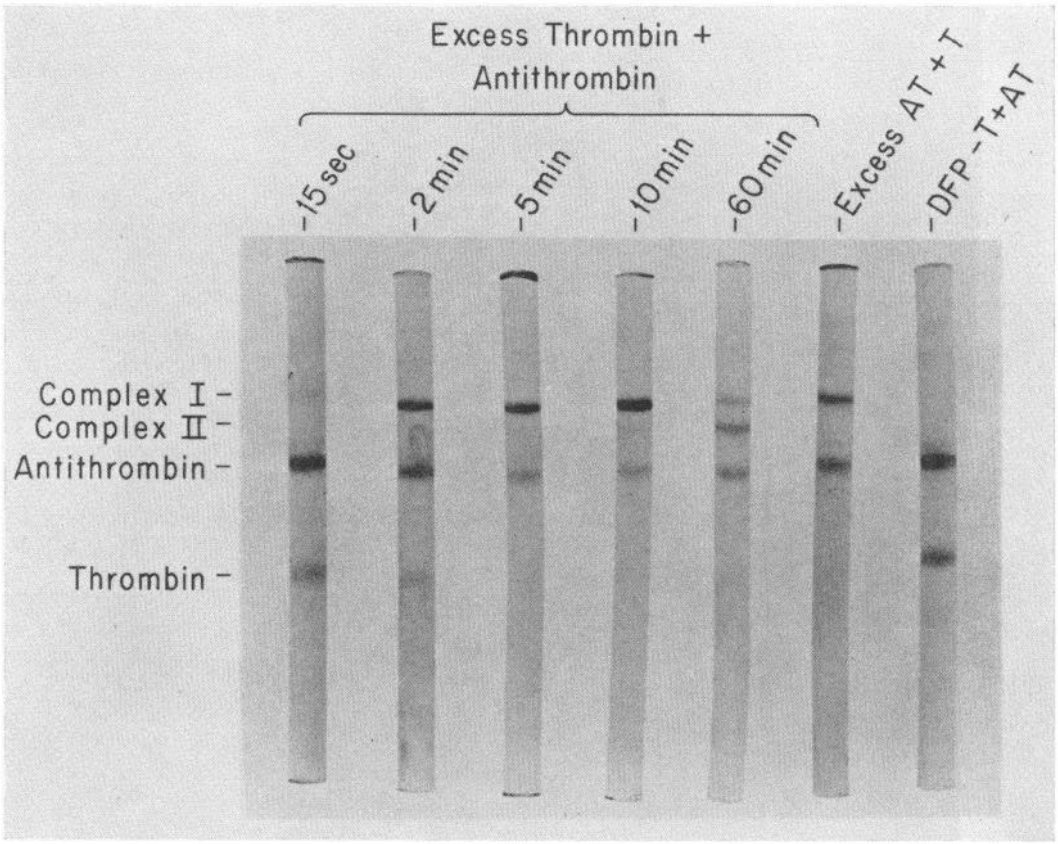

FIG. 5. Sodium dodecyl sulfate gel electrophoretic analysis of thrombin-antithrombin and DFP-treated thrombin-antithrombin interactions. Prior to electrophoretic analysis, samples were heated to 100° for 1 min in 0.01 M sodium phosphate (pH 7.0) containing 20 mM DTT, 1% β-mercaptoethanol, and 0.1% sodium dodecyl sulfate. Samples of 0.15 ml were then applied to gels (5 × 85 mm) in 10% (v/v) glycerol and were run for 3 hours at 5 mA per tube. See text for other experimental details.

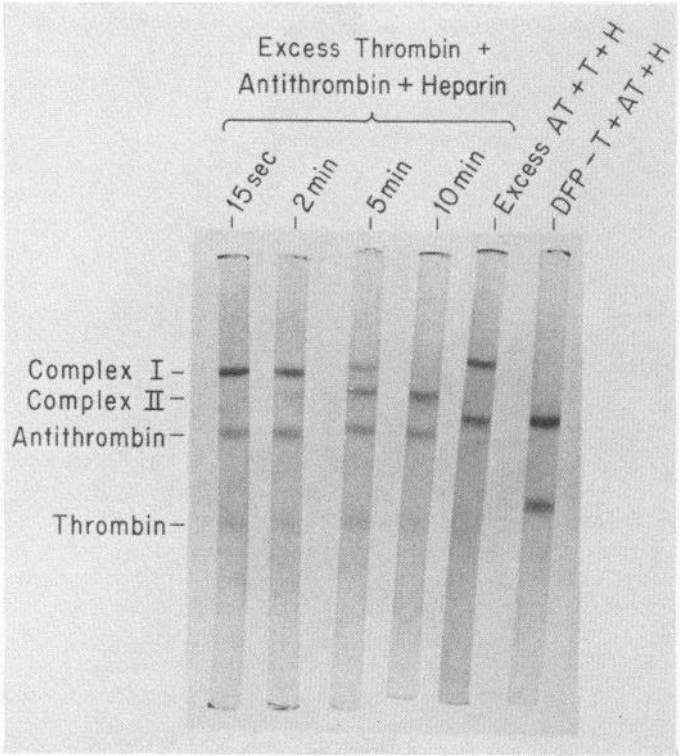

FIG. 6. Sodium dodecyl sulfate gel electrophoretic analysis of thrombin-antithrombin-heparin and DFP-treated thrombin-antithrombin-heparin interactions. See Fig. 5 legend.

thrombin might be responsible for the C_I to C_{II} transition, the final concentration of antithrombin was increased to 0.142 absorbance units per ml and that of thrombin was decreased to 0.050 absorbance unit per ml. No free thrombin could be detected by fibrin end point assay in the presence of this large excess of inhibitor and 5 units per ml of heparin. An examination with sodium dodecyl sulfate gel electrophoresis of the reaction products at 10 min revealed that, indeed, only antithrombin and C_I are present (Fig. 6). In parallel with these experiments, similar studies conducted with a slight excess of thrombin showed the customary C_I to C_{II} conversion. In identical fashion, the slower transition of C_I to C_{II} which occurs in the absence of heparin may also be suppressed by the same excess of antithrombin (Fig. 5).

Sodium dodecyl sulfate gel electrophoresis has also been used to study the interaction of DFP-treated thrombin with antithrombin. In the presence of heparin (5 units per ml) or in its absence, DFP-treated thrombin and antithrombin at final concentrations of 0.075 absorbance unit per ml and 0.106 absorbance unit per ml were incubated for 10 min. Sodium dodecyl sulfate gel electrophoretic analysis of the reaction products demonstrated only DIP-thrombin and antithrombin bands without formation of C_I or C_{II} (Figs. 5 and 6). Therefore, the active center serine of thrombin is essential for the development of this enzyme-inhibitor complex.

Modification of Arginine and Lysine Residues in Antithrombin

Modification of Antithrombin with O-Methylisourea—Antithrombin was dialyzes at 24° against either 0.5 M *O*-methyliso-

TABLE III
Guanidination of inhibitor with O-methylisourea

Samples[a]	Assay employed	
	Antithrombin	Heparin cofactor
	%	%
Inhibitor stored at 4°	100[b]	100[b]
Inhibitor dialyzed against the control solution at 24°	91 ± 4	84 ± 6
Inhibitor dialyzed against the modification solution at 24°	75 ± 6	10 ± 0.8

[a] Aliquots of antithrombin (0.6 ml to 1.5 ml) were dialyzed at 24° against either 200 ml of a solution composed of 0.5 M *O*-methylisourea in 0.01 M EDTA (pH 9.5), or 200 ml of a control solution of identical conductivity composed of 0.55 M NaCl in 0.01 M EDTA (pH 9.5). After 62 hours, all samples were further dialyzed against 1 liter of 0.15 M NaCl in 0.01 M Tris-HCl (pH 8.3) for 16 hours at 4°, and assayed for antithrombin and heparin cofactor activity. The values given represent the mean and standard error of four separate experiments with different preparations of inhibitor. In each experiment single samples were dialyzed against the appropriate buffer.

[b] The inhibitor stored at 4° is arbitrarily set at 100% activity.

TABLE IV
Basic amino acid analysis of the control and modified inhibitors

With respect to the modified inhibitors, the values given represent the mean obtained for two separate 20-hour hydrolysates with two determinations per sample. Since the control samples for both modifications were indistinguishable, the data were combined and the values given represent four separate 20-hour hydrolysates with two determinations per sample. No significant changes were noted for other amino acids and the values obtained for aspartic acid were employed to normalize this data.

Samples	Basic amino acids			
	Lysine	Histidine	Arginine	Homoarginine[a]
	μmoles residue/μmole aspartic acid			
Inhibitor dialyzed against control solution	0.740	0.073	0.401	None
Inhibitor dialyzed against 0.5 M *O*-methylisourea	0.257	0.072	0.400	0.436
Inhibitor dialyzed against 1,2-cyclohexanedione	0.738	0.070	0.317	None

[a] Homoarginine was purchased from Calbiochem, La Jolla, California, and employed as a standard in these determinations.

urea in 0.01 M EDTA (pH 9.5) or a control solution at the same ionic strength and pH. The results of four separate experiments are presented in Table III and are compared to samples of the inhibitor stored at 4°.

Dialysis against the control solution has a relatively small effect upon the antithrombin and heparin cofactor activity. Dialysis against 0.5 M *O*-methylisourea virtually eliminates heparin cofactor activity, while antithrombin activity is only slightly reduced. To determine whether the assay conditions might be responsible for these disproportionate effects, the incubation time of the assay was varied from 3 min to 10 min and the antithrombin and heparin cofactor assays were conducted at varying pH values from 7.5 to 8.7. The results obtained were identical with those previously observed. In addition, sodium dodecyl sulfate gel electrophoretic analysis of thrombin-guanidinated inhibitor interactions revealed a qualitatively similar result. When compared to inhibitor dialyzed against the control solution, the guanidinated species formed little protease-protease inhibitor complex at 10 s in the presence of heparin, but an equivalent amount of protease-protease inhibitor complex at 10 min in the presence or absence of heparin.

When the *O*-methylisourea modified inhibitor and its control sample were subjected to amino acid analysis, the only change observed in the modified inhibitor was a 63% decrease in the lysine content and the appearance of an equivalent amount of homoarginine (Table IV). Other basic amino acids, as well as neutral and acidic amino acids, were unchanged.

To determine whether heparin bound to antithrombin would protect critical lysine residues from modification, experiments were conducted utilizing varying concentrations of *O*-methylisourea in the presence and absence of this sulfated mucopolysaccharide. These experiments employed antithrombin and heparin at final concentrations of 0.3 absorbance unit per ml and 500 units per ml, respectively. Samples of the inhibitor, with and without heparin, were dialyzed at 24° against either varying concentrations of *O*-methylisourea in 0.01 M EDTA (pH 9.5) or a control solution of matched conductivity with varying concentrations of NaCl in 0.01 M EDTA (pH 9.5).

Preliminary protection experiments were conducted at 0.1 M, 0.2 M, 0.25 M, 0.30 M, and 0.5 M *O*-methylisourea. The lowest and highest concentrations of *O*-methylisourea were of little value. At 0.1 M *O*-methylisourea, no loss of heparin cofactor activity was observed and at 0.5 M *O*-methylisourea heparin did not protect the inhibitor against loss of activity. However, the three intermediate concentrations of *O*-methylisourea produced a significant decline in heparin cofactor activity in the absence of heparin and gave clear evidence for the protection of critical lysine residues in the presence of heparin.

To examine quantitatively this phenomenon, the concentration of *O*-methylisourea was fixed at 0.3 M and four separate experiments were performed (Table V). The data clearly indicate that little or no loss of activity occurs during dialysis at 24° in the presence and absence of heparin.

Furthermore, treatment with 0.3 M *O*-methylisourea inactivated 71% of the inhibitor as compared to the control dialysate in the absence of heparin and 24% as compared to the control dialysate in the presence of heparin. Therefore, heparin protected 65% of the inhibitor against inactivation due to guanidination. Similar experiments have also been conducted at a higher pH and lower heparin concentration. It is of interest that heparin does not protect the inhibitor against modification of critical lysine residues at pH 10.5 and above or at heparin concentrations of 50 units per ml.

Interaction of Native and Guanidinated Inhibitor with Heparin-Sepharose—To determine whether the guanidinated inhibitor binds heparin, both native and modified inhibitor were mixed with heparin-Sepharose and the amount of unadsorbed protein was determined immunologically.

The guanidinated and control preparations of inhibitor were obtained as described earlier. Both preparations were then dialyzed against 0.15 M NaCl in 0.01 M Tris-HCl buffer (pH 7.5) for 16 hours. Appropriate assays revealed the customary disproportionate reduction in heparin cofactor activity and the essentially normal antithrombin activity of the guanidinated inhibitor. Only minimal reductions in either activity were observed with the control sample.

A dilute heparin-Sepharose slurry was prepared with 0.15 M

TABLE V

Guanidination of inhibitor in presence of heparin

Samples[a]	Heparin cofactor assay
	%
Inhibitor stored at 4°	100[b]
Inhibitor dialyzed against the control solution at 24°	86 ± 0.7
Inhibitor dialyzed against the modification solution at 24°	25 ± 1
Inhibitor and heparin dialyzed against the control solution at 24°	98 ± 3
Inhibitor and heparin dialyzed against the modification solution at 24°	74 ± 3

[a] These experiments employed antithrombin and heparin at final concentrations of 0.3 absorbance unit per ml and 500 units per ml, respectively. Samples of the inhibitor, with and without heparin, were dialyzed for 62 hours at 24° against either 200 ml of solution consisting of 0.3 M *O*-methylisourea in 0.01 M EDTA (pH 9.5) or 200 ml of control solution of matched conductivity with 0.39 M NaCl in 0.01 M EDTA (pH 9.5). After a subsequent 16-hour dialysis at 4° against 1 liter of 0.15 M NaCl in 0.01 M Tris-HCl (pH 8.3), aliquots were assayed for heparin cofactor activity. The values given represent the mean and standard error of four separate experiments with different preparations of inhibitor. In each experiment single samples were dialyzed appropriately.

[b] The inhibitor stored at 4° is arbitrarily set at 100% activity.

NaCl in 0.01 M Tris-HCl (pH 7.5) so that it was equivalent to 10% by weight of packed gel and buffer. In each experiment, three sets of guanidinated and control samples were mixed in 0.2-ml aliquots with 0.2 ml of the heparin-Sepharose slurry. The final protein concentrations were 0.2 absorbance unit per ml and the final concentration of bound heparin was estimated to be 3 units per ml (see "Methods and Materials"). The solutions were incubated at 37° for 3 min, centrifuged at 5000 × *g* for 5 min, and the supernatants were examined immunologically for inhibitor. In two separate experiments, 37% ± 3% of the control sample and 100% ± 6% of the guanidinated sample were found in the supernatant solution.

Thus, under conditions where 63% of the native inhibitor binds to heparin-Sepharose, little of the guanidinated species is adsorbed. To demonstrate that heparin was responsible for this phenomenon, the experiments were repeated with Sepharose 4B in place of heparin-Sepharose. Now 100% ± 5% of both inhibitor samples were found in the supernatant solution.

Modification of Antithrombin with 1,2-Cyclohexanedione and 2,3-Butanedione—Antithrombin was dialyzed at 24° against either 0.015 M 1,2-cyclohexanedione with 0.01 M EDTA in 0.1 M triethylamine (pH 11.0) or a control solution at the same ionic strength and pH. The results of four separate experiments are given in Table VI.

The data indicate that dialysis against the control solution results in only small losses of antithrombin and heparin cofactor activity. However, the addition of 1,2-cyclohexanedione to the solution virtually eliminates both activities. Identical results were obtained when antithrombin and heparin cofactor activity assays were conducted at varying pH from 7.5 to 8.7 and when the incubation time of the antithrombin assay was prolonged from 3 min to 10 min. In addition, a sodium dodecyl sulfate gel electrophoretic analysis of thrombin-1,2-cyclohexanedione-treated inhibitor interactions revealed a qualitatively similar result. When compared to inhibitor dialyzed against

TABLE VI

Modification of inhibitor with 1,2-cyclohexanedione

Samples[a]	Assay	
	Antithrombin	Heparin cofactor
	%	
Inhibitor stored at 4°	100[b]	100[b]
Inhibitor dialyzed against the control solution at 24°	86 ± 5	88 ± 5
Inhibitor dialyzed against the modification solution at 24°	5 ± 3	7 ± 3

[a] Aliquots of antithrombin (0.6 ml to 1.5 ml) were dialyzed in the dark at 24° against either 200 ml of a solution composed of 0.015 M 1,2-cyclohexanedione with 0.01 M EDTA in 0.10 M triethylamine (pH 11.0) or 200 ml of a control solution which did not contain 1,2-cyclohexanedione. After 6 hours of contact with either solution, the samples were dialyzed against 0.15 M NaCl in 0.01 M Tris-HCl (pH 8.3) at 4° for 16 hours, and assayed for antithrombin and heparin cofactor activity. The values given represent the mean and standard error of four separate experiments with different preparations of inhibitor. In each experiment single samples were dialyzed against the appropriate buffer.

[b] The inhibitor stored at 4° is arbitrarily set at 100% activity.

the control solution, the 1,2-cyclohexanedione-treated inhibitor formed little protease-protease inhibitor complex at 10 to 15 s and 10 min with and without optimal amounts of heparin.

When the 1,2-cyclohexanedione-modified inhibitor and the control sample were subjected to amino acid analysis, the only change noted was a 21% reduction in the arginine content of the modified inhibitor (Table IV). Other basic amino acid residues as well as neutral and acidic amino acid residues were unchanged. However measurements at 440 nm demonstrated that the modified inhibitor had appreciable absorbance in this spectral region

$$\left(\frac{\text{absorbance at 440 nm}}{\text{absorbance at 280 nm}} = 0.2\right)$$

while the control sample exhibited essentially no absorbance at this wave length.

Liu *et al.* (37) have studied the inactivation of many protease inhibitors with 1,2-cyclohexanedione. They have observed the emergence of this absorbance band when some degree of lysine modification has occurred. However, these products appear unstable during acid hydrolysis. Therefore, the loss of heparin cofactor activity observed with 1,2-cyclohexanedione could be partially due to lysine alterations undetected by amino acid analysis rather than arginine modification. However, the loss of antithrombin activity noted with this reagent can be ascribed to arginine modification, since guanidination has no effect upon this inhibitory function.

To demonstrate that arginine residues are essential for heparin cofactor function, a more stringently controlled modification was utilized. Heparin has already been shown to protect critical lysine residues against guanidination. Therefore, this sulfated mucopolysaccharide was used to protect these lysine groups during arginine modification. Since 1,2-cyclohexanedione had little effect below pH 10.5, while heparin-dependent protection of lysine residues is minimal at or above this pH, we employed an alternate method for modifying arginine residues. The reagent 2,3-butanedione was chosen since it has a specificity identical with 1,2-cyclohexanedione and is active at more acidic pH.

TABLE VII

Modification of inhibitor with 2,3-butanedione in the presence and absence of heparin

Samples[a]	Heparin cofactor assay
	%
Inhibitor stored at 4°	100[b]
Inhibitor dialyzed against the control solution at 24°	93 ± 8
Inhibitor dialyzed against the modification solution at 24°	14 ± 7
Inhibitor and heparin dialyzed against the control solution at 24°	107 ± 8
Inhibitor and heparin dialyzed against the modification solution at 24°	12 ± 8

[a] The inhibitor and heparin were employed at final concentrations of 0.3 absorbance unit per ml and 500 units per ml, respectively. Aliquots of inhibitor (0.6 ml to 1.0 ml), with and without heparin, were dialyzed for 6 hours at 24° against either 200 ml of a solution consisting of 0.115 M 2,3-butanedione and 0.39 M NaCl in 0.01 M EDTA (pH 9.5) or 200 ml of a control solution of 0.39 M NaCl in 0.01 M EDTA (pH 9.5) without 2,3-butanedione. After a subsequent 16-hour dialysis at 4° against 0.15 M NaCl in 0.01 M Tris-HCl (pH 8.3), aliquots of the samples, if indicated, were assayed for heparin cofactor activity. The values given represent the mean and standard error of three separate experiments with different preparations of inhibitor. In each experiment single samples were dialyzed appropriately.

[b] The inhibitor stored at 4° is arbitrarily set at 100% activity.

The conditions of pH and ionic strength were chosen to correspond to those previously employed for the protection of critical lysyl residues by heparin. In these modification studies the inhibitor was dialyzed at 24° against either 0.115 M 2,3-butanedione and 0.39 M NaCl in 0.01 M EDTA (pH 9.5) or a control solution at the same pH and ionic strength. The results of three separate experiments are given in Table VII. Dialysis of the inhibitor against the control solution, with or without heparin, resulted in little or no loss of inhibitor activity. However, exposure of the inhibitor to 2,3-butanedione produced the same significant loss of heparin cofactor activity in the presence or absence of heparin.

Effect of Protamine Sulfate on Heparin-accelerated Inhibition of Thrombin by Antithrombin—Thrombin and antithrombin were incubated together at 37°. At varying times aliquots were removed, 0.1 ml of buffer was added, and the samples were immediately tested for thrombin activity by clotting assay. *Curve A* of Fig. 7 demonstrates that a gradual neutralization of activity occurs. When heparin was added at a final concentration of 0.125 unit per ml and the experiment was repeated, an immediate inhibition of thrombin was observed (*Curve B*). Variation of the heparin concentration from 0.0125 unit per ml to 1.25 units per ml revealed that the heparin concentration previously employed was the minimal amount necessary to accelerate the inhibitor optimally. To study the effect of protamine sulfate on this interaction, reaction mixtures similar to those of *Curve B* were prepared. After varying times of incubation, aliquots were removed, 0.1 ml of protamine sulfate was added (previously determined to neutralize 0.193 unit per ml of heparin), and the samples were immediately tested for thrombin activity (*Curve C*). No instantaneous increase in thrombin activity was detected. Similar experiments were conducted at pH 7.5 to 8.5 and with varying concentrations of thrombin, antithrombin, heparin, and protamine. Release of native thrombin from the thrombin-antithrombin-heparin complex was never observed. To determine whether inactive thrombin might be released with protamine addition, sodium dodecyl sulfate gel electrophoresis was employed. Two reaction mixtures of enzyme, inhibitor, and heparin were prepared and incubated for 30 s at 37°. Protamine sulfate was added to one sample in an amount sufficient to neutralize twice the original concentration of heparin, and an equivalent volume of buffer was added to the other sample. Each sample was denatured in the fashion previously described and examined by sodium dodecyl sulfate gel electrophoresis. As shown in the *inset* to Fig. 7, breakdown of the enzyme-inhibitor complex was not observed.

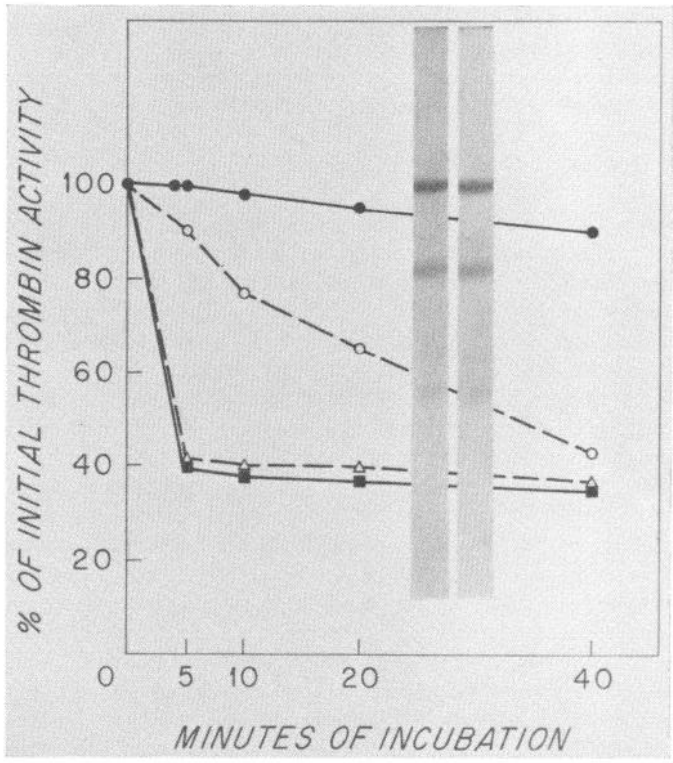

FIG. 7. The effect of protamine sulfate on the thrombin-antithrombin-heparin complex. Thrombin and antithrombin, after an extensive dilution with 0.15 M NaCl in 0.01 Tris-HCl (pH 7.5) were incubated together, with or without heparin at final concentrations of 0.017 absorbance unit per ml and 0.010 absorbance unit per ml, respectively. ○- - -○, *Curve A* represents the inactivation of thrombin by antithrombin; △- - -△, *Curve B* represents the acceleration of the thrombin-antithrombin interaction by 0.125 unit per ml of heparin; ■——■, *Curve C* is identical with *Curve B* except that protamine sulfate was added prior to the assay; ●——●, *Curve D* represents thrombin incubated with buffer. Each *point* represents the average of three separate incubation mixtures. To prevent loss of thrombin on vessel surfaces, all plastic containers were presaturated with a 0.01% solution of bovine albumin and rinsed with buffer prior to use. The *inset* shows sodium dodecyl sulfate gels of the thrombin-antithrombin-heparin interaction with (*left*) and without (*right*) protamine sulfate. Identical conditions of pH, ionic strength, and temperature were employed but the enzyme, inhibitor, and heparin were utilized at final concentrations of 0.07 absorbance unit per ml, 0.106 absorbance unit per ml, and 5 units per ml, respectively. See text for additional experimental details.

DISCUSSION

Human antithrombin was purified by aluminum hydroxide adsorption-elution, Sephadex G-200 filtration, DEAE-Sephadex

fractionation, DEAE-cellulose chromatography, and isoelectric focusing in sucrose density gradients. The final yield of this procedure averages 12% and the final product is homogeneous by disc gel electrophoresis, sodium dodecyl sulfate gel electrophoresis, and immunoelectrophoresis.

Human antithrombin has previously been purified by Abildgaard (9, 28, 38). With his method approximately 200 ml of plasma can be processed, and yields average 1%. The superiority of our purification method is due, in large measure, to the significant increase in yield attained during aluminum hydroxide adsorption-elution as well as gel filtration, improved means for storing and concentrating products of intermediate purity, the substitution of a DEAE-cellulose chromatography for rechromatography on DEAE-Sephadex and Sephadex G-200, the use of isoelectric focusing rather than preparative disc gel electrophoresis, and our over-all ability to process five to eight times more protein with each fractionation step. Thus our technique represents the only currently available method for obtaining this pure human protein in amounts sufficient for biochemical studies.

In addition, our investigations provide convincing evidence that antithrombin activity and heparin cofactor activity are properties of a single molecular species. Both activities are present in our homogeneous preparations, co-isoelectric focus, adsorb to thrombin-Sepharose together, and interact with heparin-Sepharose together. Furthermore, an antibody directed against the purified inhibitor precipitated an equal amount of each activity from defibrinated plasma. Therefore, we can substantiate and considerably strengthen the contentions of Waugh *et al.* (7), Monkhouse *et al.* (8), Abildgaard (9), and Yin *et al.* (39) that this unique protein is responsible for most, if not all, of the antithrombin and the heparin cofactor activities of defibrinated plasma.[6]

In a highly specific fashion, DFP alkylphosphorylates a unique serine residue in thrombin (40). Employing the native enzyme and this specific modification, we have shown that the active center serine of thrombin is essential for interaction with antithrombin.

Firstly, the enzyme's active center is responsible for a remarkably stable enzyme-inhibitor complex. Mixtures of thrombin and inhibitor as well as DIP-thrombin and inhibitor were examined by sodium dodecyl sulfate gel electrophoresis. The inhibitor formed an undissociable complex with native enzyme but not with DFP-treated protease. Similar results were obtained in the presence of optimal concentrations of heparin.

Secondly, we have demonstrated that the modified enzyme does not compete with the native enzyme for a site on the inhibitor. In these experiments, after the DIP-thrombin was admixed with inhibitor, native enzyme was added. The esterolytic activity of the native enzyme is preserved to a greater or lesser extent, depending upon the competition between DIP-thrombin and native thrombin for inhibitor. Using this type of assay, in the presence and absence of heparin,[7] we have established that less than 25% of the DIP-thrombin binds to the inhibitor site occupied by the native enzyme. Therefore, we can assume that residues other than those constituting the enzyme's active center are of minimal importance in initiating the customary enzyme-inhibitor complex.

Thirdly, we have shown that interaction of the inhibitor with thrombin is greatly reduced if the active serine residue is blocked. To obtain this result we have filtered the inhibitor, with and without heparin, through columns of thrombin-Sepharose, DIP-thrombin-Sepharose, and ethanolamine-Sepharose. While the inhibitor is almost completely adsorbed by the thrombin-Sepharose columns, the binding of this protein to the DIP-thrombin-Sepharose columns was equivalent to that observed with the control ethanolamine-Sepharose column. Thus, with three separate methods, we have demonstrated that the active center serine of thrombin is required for antithrombin-thrombin as well as heparin-antithrombin-thrombin interactions.[8] These findings are remarkably similar to the results obtained with trypsin-trypsin inhibitor systems. Green (41) has shown that DIP-trypsin does not interact with several trypsin inhibitors. Indeed, the mechanism of action of the trypsin inhibitors is a prototype model for our more complex system.

On the basis of their extensive studies with trypsin and soybean trypsin inhibitor (Kunitz), Finkenstadt and Laskowski, Jr. (42), have postulated that this protease-protease inhibitor interaction results in the cleavage of a unique peptide bond (reactive site of the inhibitor) with formation of an acyl complex between the newly formed COOH-terminal residue of the inhibitor and the active site serine of the enzyme. Laskowski, Jr., and his collaborators have suggested that most, if not all, protease inhibitors function in this fashion. Objections to this mechanism have been raised (43), but the majority of critics accept the abundant evidence that the active site serine of the enzyme and a unique arginine or lysine residue of the inhibitor are essential for the formation of almost all protease-protease inhibitor complexes.

Feeney, one of the most effective critics of the Laskowski model, has, in fact, developed an elegant method which determines whether a trypsin inhibitor has an arginine-x or a lysine-x reactive site. He and his co-workers have employed *O*-methylisourea to inactivate inhibitors with lysine-x reactive sites (44) and have utilized 2,3-butanedione or 1,2-cyclohexanedione to inactivate inhibitors with arginine-x reactive sites (37).

Since thrombin is known to have a remarkably narrow specificity for unique arginine-x bonds (45), we suspected that our inhibitor employed this residue as a reactive site. Therefore, we treated the inhibitor with 1,2-cyclohexanedione and were able to demonstrate an almost complete destruction of heparin cofactor as well as antithrombin activity. The apparent specificity of this chemical modification was shown by amino acid analysis. However, the absorbance spectrum of the modified inhibitor suggested that some lysine modification may also have occurred.

[6] The determination of plasma antithrombin activity requires defibrination of samples. Therefore the adsorption of thrombin to fibrin does not contribute to our estimates of inhibitory capacity. In addition, this assay employs low concentrations of thrombin and thus minimizes the inactivation of this enzyme by α_1-trypsin inhibitor and α_2-macroglobulin. However, this method most closely approximates the *in vivo* situation, where small amounts of thrombin must be neutralized prior to the appearance of fibrin if thrombus formation is to be avoided.

[7] Yin *et al.* (50) have shown that heparin accelerates the inhibition of partially purified bovine thrombin by rabbit antithrombin when fibrinogen, but not tosyl-L-arginine methyl ester is employed as a substrate. We have found the same degree of enzymatic inhibition employing either tosyl-L-lysine or tosylarginine methyl ester. Therefore, it is not clear whether this disparate result is due to their poorly characterized enzyme preparations or species differences.

[8] The heparin-dependent acceleration of thrombin-inhibitor interaction might be partially accomplished by a heparin-induced conformational change in the active center of the enzyme. However, the esterolytic and clotting activities of thrombin are not altered by 10 to 15 units per ml of heparin. In addition, the heparin effect is absent if this molecule can not bind to the inhibitor. This suggests that the enzyme is not an important target for heparin action.

This minor degree of lysine modification induced by 1,2-cyclohexanedione has been shown to have minimal effect upon the activity of protease inhibitors with lysine-x reactive sites (37). But our inhibitor is unlike all other protease inhibitors since heparin dramatically accelerates its action. In view of the acidic nature of heparin, we thought it likely that lysine residues of the inhibitor might function as a binding site for this highly charged species. Thus, alteration of lysine residues might be expected to effect the activity of the inhibitor independent of reactive site modification.

Indeed, treatment of the inhibitor with 0.5 M *O*-methylisourea virtually abolishes heparin cofactor activity with only a minimal effect upon antithrombin activity. Under these conditions, this chemical modification has been shown to guanidinate lysyl ε-amino groups only (46). As expected, amino acid analysis revealed the specificity of this chemical modification.

The physical interaction of heparin with native and guanidinated inhibitor was examined to determine whether a loss of heparin cofactor activity was due to a reduced ability of the protein to bind this acidic mucopolysaccharide. Both native and modified inhibitor were mixed with heparin-Sepharose, the solutions were centrifuged, and the amount of inhibitor present in the supernatant was determined immunologically. Under conditions where 63% of the native inhibitor adsorbed to this matrix, no measurable amount of the guanidinated inhibitor was bound.

Of course, one might question whether guanidinated ε-amino groups are at the binding site and thus directly involved in the interaction with heparin, or whether the inhibition of heparin binding results from the modification of ε-amino groups located elsewhere in the molecule with a resultant change in the conformation of the inhibitor. Since the guanidinated inhibitor losses little, if any, antithrombin activity and can still form an undissociable complex with thrombin, a major conformational alteration of the inhibitor seems unlikely. But in an attempt to determine whether heparin binds to lysine residues, a 100-fold molar excess of heparin was mixed with inhibitor and the resulting solution was exposed to 0.3 M *O*-methylisourea. Approximately 65% of the heparin cofactor function could be protected against activity loss.

The protection afforded by heparin against inactivation by *O*-methylisourea suggests that lysyl groups are directly involved in heparin binding. However a detailed knowledge of the mechanism of heparin binding will be required to substantiate this tentative conclusion.

Since the guanidinated inhibitor retains its antithrombin activity, modification of reactive site arginine residue(s) by 1,2-cyclohexanedione seems certain. However, the inhibition of heparin cofactor activity by this agent might be ascribed to altered lysine residue(s) of the heparin-binding site. As we have shown, heparin can protect the binding site against lysine modification for 62 hours at pH 9.5, ionic strength of 0.39, at 24°. Employing these same conditions, we studied the inactivation of the inhibitor by 2,3-butanedione, a reagent of specificity identical with 1,2-cyclohexanedione (47), and were able to show that heparin cofactor activity virtually disappeared within 6 hours. Therefore arginine residue(s) are of critical importance for this inhibitor function. This conclusion is strengthened by the known specificity of the thrombin active site for arginine-x bonds and our demonstration that this active site is required for the formation of a protease-protease inhibitor complex in the presence of heparin.

Whether 1 or more arginine residue(s) are required for inhibitor function and whether the same residue(s) are critical for both antithrombin and heparin cofactor activity remains undetermined. However, in analogous fashion to other protease inhibitors, we suggest that a unique arginine residue forms the reactive site of the inhibitor. Furthermore, we believe that the simplest and most attractive hypothesis of inhibitor function would require this reactive site to be employed for both antithrombin and heparin cofactor action. Within the framework of this hypothesis, we suggest that binding of heparin to the inhibitor induces a conformation which positions the reactive site more favorably for interaction with serine proteases.[9] Indirect evidence for this altered conformation has been obtained during our studies of the thrombin-dependent proteolysis of free inhibitor and the enzyme-inhibitor complex (see below).

The formation of the enzyme-inhibitor complex was studied by incubating thrombin with antithrombin, in the presence and absence of heparin, for varying lengths of time, rapidly denaturing the proteins, and examining them with sodium dodecyl sulfate gel electrophoresis.

The molecular weight of the complex is approximately equal to the sum of the molecular weights of thrombin and antithrombin. Although sodium dodecyl sulfate gel electrophoresis is usually employed with noninteracting polypeptide chains, it has yielded valid molecular weight estimates when the subunits of oligomeric proteins are covalently cross-linked through ε-amino groups (48). Of course, the molecular weight of the complex would indicate a 1:1 stoichiometry for the thrombin-inhibitor interaction. An independent estimate of this stoichiometry can be obtained from our experimental data. Using results obtained with both the esterolytic assay as well as the fibrin end point assay, and averaging over four different preparations of thrombin and antithrombin, we calculate that the equivalence point of this reaction occurs at a thrombin to antithrombin absorbance ratio of 1.15 without heparin and 1.30 with heparin. Employing the known molecular weight and extinction coefficient of human thrombin as well as our estimates of the extinction coefficient and molecular weight of human antithrombin, we find that a thrombin to antithrombin absorbance ratio of 1.1 would represent 1:1 stoichiometry. Thus our tentative estimate of the molecular weight of the enzyme-inhibitor complex seems valid.

Since our complex remains intact after exposure to various combinations of denaturing and reducing agents, the formation of a covalent acyl bond, between a newly created COOH-terminal residue of the inhibitor and the active center serine of the enzyme, was considered. Attempts by many investigators to trap the postulated acyl intermediate formed during trypsin-trypsin inhibitor interactions have proven fruitless (43). Indeed, denaturation of these complexes inevitably results in the regeneration of the original reactants (49). Therefore, some property other than the arginine-serine interaction may be responsible for the remarkable stability of the thrombin-antithrombin complex. Perhaps a large number of electrostatic and hydrophobic interactions are brought into play after initiation of the complex and aid it in resisting denaturing agents. Given our knowledge of the tenacity of the thrombin-antithrombin interaction, we were intrigued by the claims of Yin *et al.* (50) that thrombin can be released from the thrombin-anti-

[9] Alternately, the binding of heparin to the inhibitor could lead to the exposure of a new reactive site which allows a more rapid interaction with serine proteases. Thus different arginine residues might be employed for antithrombin and heparin cofactor function.

thrombin-heparin complex with the addition of protamine. Employing conditions similar to those utilized by these investigators, we have never observed a protamine-dependent regeneration of thrombin activity.

Although the complex is resistant to protamine addition and denaturation, we have observed a time-dependent reduction in its molecular weight when a slight excess of thrombin is present (~10%). Using sodium dodecyl sulfate gel electrophoresis, this transition of the complex from an apparent molecular weight of 88,000 to one of 81,000 is complete at 5 min in the presence of heparin. In the absence of heparin, this transition is only half complete at 60 min of incubation. When the interaction was studied with a large excess of inhibitor, no reduction in apparent molecular weight of the complex was observed. Therefore, a cleavage of the complex by free thrombin appears likely. Absence of the (C_I-C_{II}) peptide of molecular weight ~7000 from the sodium dodecyl sulfate gel electrophoretic patterns may be due to loses during dialysis or to our inability to detect low molecular weight components with the system presently employed.

Whether the thrombin or antithrombin portion of the complex is being proteolyzed remains unknown. However, the dramatic increase in rate of reaction with heparin suggests that the complex has a different conformation when this sulfated mucopolysaccharide is bound to it. Free uncomplexed inhibitor may also be cleaved by thrombin. Filtration of antithrombin through thrombin-Sepharose columns, with and without heparin, results in both adsorption and inactivation of the inhibitor. The creation of inactive derivatives is probably not an artifact of the purification procedure, since filtration of heat-defibrinated plasma results in a similar amount of inactivated inhibitor. The amount of inactive derivative formed appears related to the binding of thrombin to Sepharose. When thrombin-antithrombin interactions, in the presence or absence of heparin, are studied in solution by sodium dodecyl sulfate gel electrophoresis, only small amounts (~5%) of the inactive derivatives were observed. Thus the linking of thrombin to Sepharose accentuates inhibitor inactivation, possibly by sterically hindering the formation of a protease-protease inhibitor complex.[10]

Tschesche and Klein (52) have filtered porcine pancreatic trypsin inhibitor through columns of insoluble trypsin and have noted a conversion to inactive derivatives. This temporary inhibitor is modified by multiple cleavages of lysine-x and arginine-x bonds at some distance from the reactive binding site.

Since our data demonstrated that the active center serine of thrombin was required to produce inactive inhibitor, a similar proteolytic process seemed likely. Therefore, an apparent increase in the molecular weight of the major new component was unexpected. Inappropriate behavior in sodium dodecyl sulfate gel electrophoresis is known to occur when the carbohydrate content of proteins is significant (53) or when an unusual charge or conformation of a polypeptide chain exists (54). If human antithrombin has as high a carbohydrate content as rabbit antithrombin (12), cleavage of a carbohydrate-poor fragment might result in an apparent increase in the molecular weight of the remaining portion of the molecule. If the inactive derivatives have an unusual charge distribution, an abnormal conformation in sodium dodecyl sulfate might result, and thus molecular weight estimates would be erroneously high. Therefore, it is probable that a proteolytic process takes place when antithrombin is filtered through thrombin-Sepharose columns and that increased cleavage of the inhibitor in the presence of heparin is due to an altered conformation of the molecule induced by the bound acidic mucopolysaccharide.

In summary, antithrombin functions as a trypsin-like protease inhibitor neutralizing the activity of thrombin by complex formation via a reactive site-active center interaction. Heparin binds to lysyl residues of the inhibitor and accelerates this reaction. The dramatic increase in the rate of interaction is probably due to a heparin-dependent conformational alteration of the inhibitor which renders the reactive site arginine more accessible to the active center serine of thrombin. This mechanism suggests that other serine proteases may be neutralized in a similar manner and that heparin may accelerate these interactions. Indeed, we have recently found several biologically critical serine proteases, other than Factor X_a, which are inactivated in this heretofore unsuspected fashion in the presence of heparin and its cofactor. We, therefore, believe that antithrombin-heparin cofactor will, in due time, be recognized as an important physiological regulator.

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[10] Coupled enzymes often retain much less activity towards large substrates than towards smaller synthetic ones (51). These observations suggest that matrix-bound enzyme is hindered sterically by its support and interacts in a less specific fashion with other macromolecules.

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