

## Interaction of antithrombin III with bovine aortic segments. Role of heparin in binding and enhanced anticoagulant activity.

D Stern, P Nawroth, J Marcum, D Handley, W Kiesel, R Rosenberg, K Stern

*J Clin Invest.* 1985;75(1):272-279. <https://doi.org/10.1172/JCI111685>.

### Research Article

Bovine antithrombin III (AT III) interaction with the luminal surface of bovine aortic segments with a continuous layer of endothelium was examined. Incubation of 125I-AT III with vessel segments, previously washed free of endogenous AT III, demonstrated specific, time-dependent binding to the protease inhibitor to the endothelium. Half-maximal binding was observed at an added AT III concentration of 14 nM. Binding of 125I-AT III to the vessel wall was reversible (50% dissociated in 4 min), and addition of either heparin or Factor Xa accelerated displacement of 125I-AT III from the vessel segment. Dissociation of 125I-AT III from the vessel segment in the presence of factor Xa coincided with the formation of a Factor Xa-125I-AT III complex. Inactivation of Factor IXa and Factor Xa by AT III was facilitated in the presence of vessel segments. Pretreatment of vessel segments with highly purified *Flavobacterium heparinase* precluded the vessel-dependent augmentation of AT III anticoagulant activity as well as specific binding of 125I-AT III to the vessel endothelium. In contrast, pretreatment of the vessel segments with chondroitinases (ABC or AC) had no detectable effect on 125I-AT III binding or on AT III anticoagulant activity. AT III binding to vessel segments was competitively inhibited by increasing concentration of platelet factor 4. Binding of the protease inhibitor to vessel segments was inhibited by chemical modification of [...]

Find the latest version:

<https://jci.me/111685/pdf>



# Interaction of Antithrombin III with Bovine Aortic Segments

## Role of Heparin in Binding and Enhanced Anticoagulant Activity

D. Stern, P. Nawroth, J. Marcum, D. Handley, W. Kiesel, R. Rosenberg, and K. Stern

Columbia University College of Physicians and Surgeons, New York, 10032; Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; Sandoz, Inc., East Hanover, New Jersey 07936; University of Washington, Seattle, Washington 98195; Manhattan Eye, Ear and Throat Hospital, New York 10021

### Abstract

Bovine antithrombin III (AT III) interaction with the luminal surface of bovine aortic segments with a continuous layer of endothelium was examined. Incubation of  $^{125}\text{I}$ -AT III with vessel segments, previously washed free of endogenous AT III, demonstrated specific, time-dependent binding of the protease inhibitor to the endothelium. Half-maximal binding was observed at an added AT III concentration of 14 nM. Binding of  $^{125}\text{I}$ -AT III to the vessel wall was reversible (50% dissociated in 4 min), and addition of either heparin or Factor  $\text{X}_a$  accelerated displacement of  $^{125}\text{I}$ -AT III from the vessel segment. Dissociation of  $^{125}\text{I}$ -AT III from the vessel segment in the presence of factor  $\text{X}_a$  coincided with the formation of a Factor  $\text{X}_a$ - $^{125}\text{I}$ -AT III complex. Inactivation of Factor  $\text{IX}_a$  and Factor  $\text{X}_a$  by AT III was facilitated in the presence of vessel segments. Pretreatment of vessel segments with highly purified *Flavobacterium heparinase* precluded the vessel-dependent augmentation of AT III anticoagulant activity as well as specific binding of  $^{125}\text{I}$ -AT III to the vessel endothelium. In contrast, pretreatment of the vessel segments with chondroitinases (ABC or AC) had no detectable effect on  $^{125}\text{I}$ -AT III binding or on AT III anticoagulant activity. AT III binding to vessel segments was competitively inhibited by increasing concentration of platelet factor 4. Binding of the protease inhibitor to vessel segments was inhibited by chemical modification of AT III lysyl or tryptophan residues. These AT III derivatives retained progressive inhibitory activity. These data suggest that heparin-like molecules are present on the aortic vessel wall and mediate binding of AT III to the vessel surface, as well as enhancing the anticoagulant activity of AT III at these sites.

### Introduction

Antithrombin III is a plasma glycoprotein that inhibits a number of serine proteases including those involved in blood coagulation (1-6). Congenital deficiencies or abnormalities of antithrombin III generally result in increased frequency of thrombotic episodes and underlines the importance of this inhibitor in hemostasis (7). Several studies have detailed the

reaction of antithrombin III in vitro with purified coagulation proteases (1-6). In these reactions, antithrombin III forms a stoichiometric covalent complex with the proteases. The rate of these reactions is accelerated by the presence of catalytic amounts of heparin (4, 8).

Heparan sulfate proteoglycans have been isolated from cultured aortic endothelial cells (9). Recent studies have suggested that these molecular species may be involved in the regulation of the coagulation cascade. Buonassisi and Colbern (10, 11) have demonstrated that heparan sulfate proteoglycans from cultured rabbit endothelial cells inhibit the generation of thrombin but in an antithrombin-independent fashion. Busch and Owen (12) observed a small reduction in thrombin activity and a concomitant augmentation in thrombin-antithrombin complex formation when the enzyme and protease inhibitor were perfused through an isolated heart preparation or across a bed of cultured endothelial cells. However, these investigators could not unambiguously identify the molecular nature of the endothelial cell receptor which accelerated the above interaction and have claimed that it is not a heparin-like substance (13). Rosenberg and colleagues (14, 15) have isolated heparin-like molecules from a variety of vascular tissues including those which have no mast cells and have demonstrated that these complex carbohydrates enhance the rate of inhibition of thrombin and Factor  $\text{X}_a$  by antithrombin in a fashion similar to commercial heparin. This group has also perfused the rat hindlimb preparation with thrombin and antithrombin and have shown that heparin-like molecules associated with the surface of the vasculature are able to accelerate enzyme-inhibitor complex formation by as much as ~20-fold (16). Our previous study concerning the interaction of coagulation Factor  $\text{X}_a$  with the native endothelium of bovine aortic segments showed that endogenous antithrombin III present on the vessel wall prevented Factor  $\text{X}_a$  binding and subsequent activation of prothrombin through the formation of a Factor  $\text{X}_a$ -antithrombin III complex (17). This finding suggested that the intact vessel wall facilitated antithrombin III binding and the inhibitory activity of this protein. In this report, we demonstrate for the first time specific binding of antithrombin III to bovine aortic vessel with a continuous layer of endothelium, involving heparin or heparin-like mucopolysaccharides on the surface of the vessel. The importance of this binding in understanding the protective function of the vessel wall becomes evident from the finding that heparin-like compounds on the endothelial cell surface mediate antithrombin III binding to the endothelium and enhance its anticoagulant activity.

### Methods

**Coagulation factors.** Bovine coagulation factors were used throughout these studies. Bovine antithrombin III was prepared according to

This work was presented at the Annual Meeting of the American Society of Clinical Investigation, Washington, DC, 5 May 1984. Address correspondence to Dr. Stern, Department of Medicine, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 10032.

Received for publication 25 June 1984 and in revised form 13 September 1984.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/85/01/0272/08 \$1.00

Volume 75, January 1985, 272-279

Mahoney et al. (18). The final product was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)<sup>1</sup> with a molecular weight of 52,000 D and exhibited an inhibitory activity of 1.0 U thrombin/ $\mu$ g. Heparin cofactor activity was measured by the method of Abildgaard et al. (19) using Tos-Gly-Pro-Arg-pNA (Boehringer Mannheim, Inc., Indianapolis, IN). Bovine  $\alpha$ -thrombin (2.5 U/ $\mu$ g) was prepared as previously described (20). Progressive antithrombin activity was also measured by the method of Abildgaard et al. (21) using Tos-Gly-Pro-Arg-pNA. Radiolabeling of antithrombin III was accomplished by the lactoperoxidase method (22) using the Enzymobead Reagent (Bio-Rad, Richmond, CA) according to the manufacturer's protocol. The specific radioactivity was  $4-8 \times 10^3$  cpm/ng. Iodinated antithrombin III retained full anticoagulant activity and comigrated on SDS-PAGE with unlabeled antithrombin III. All studies using <sup>125</sup>I-antithrombin III were performed within 24 h of the radiolabeling procedure.

Modification of antithrombin III lysyl residues with *O*-methylisourea (K + K Labs, Plainview, NY) was carried out by the method of Haynes and Feeney (23) as modified by Rosenberg and Damus (1). Following *O*-methylisourea treatment, antithrombin III had <8% of the initial heparin cofactor activity while progressive antithrombin III activity was >80% of the starting material. Tryptophan modification was carried out as previously described (24).

Bovine Factor IX was purified to homogeneity (260 U/mg) by the method of Fujikawa et al. (25) and was activated by incubation with Factor XI<sub>a</sub> coupled to CNBr-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously (26). Factor IX activation was complete as judged by coagulant assay and release of tritiated activation peptide (27). Bovine Factor X was purified to homogeneity by the method of Fujikawa et al. (28) and was activated with the Factor X activator from Russell's viper venom (29) insolubilized on CNBr-Sepharose (Pharmacia Fine Chemicals) in Tris-buffered saline (20 mM Tris, pH 7.5; 0.13 M NaCl) containing 5 mM CaCl<sub>2</sub> (26). Activation was complete as judged by coagulant assay (100 U/mg) performed by the method of Bajaj and Mann (30) and by SDS-PAGE.

Monospecific antisera against antithrombin III were raised in rabbits by standard methods (31). Immunoglobulin G from rabbit serum was purified using protein A-Sepharose CL-6B (Pharmacia Fine Chemicals) according to the manufacturer's recommendations. Protein concentrations were determined colorimetrically (32) and by active site titration with *p*-nitrophenyl-*p*'-guanidinobenzoate (33). SDS-PAGE was carried out by the method of Laemmli (34). Samples of vessel-bound <sup>125</sup>I-antithrombin III were prepared for SDS-PAGE by incubating the tissue segments for 20 min at 4°C with Tris-buffered saline containing 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO), 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and 0.3 mM leupeptin (Boehringer Mannheim, Inc.). The eluate was then prepared for electrophoresis (34).

Elution of endogenously bound antithrombin III from calf aortas was carried out on aortas excised immediately after slaughter. Vessels were washed four times with Tris-buffered saline and then incubated for 2 min at 21°C with Tris-buffered saline containing sodium heparin (Sigma Chemical Co.) at a concentration of 5 U. S. Pharmacopeia units/ml (150 U/mg). Heparin eluates were subjected to nonreduced SDS-PAGE (10%) by the method of Laemmli (34) and material from the gel electrophoretically transferred to nitrocellulose paper, 0.45  $\mu$ m pore size (Schleicher and Schuell, Inc., Keene, NH), by a modification of the method of Towbin et al. (35). Electrophoretic transfer was for 5 h at 17°C and at constant power (13 W). Excess binding sites on the nitrocellulose membrane were blocked by a 1-h incubation at 40°C with Tris-buffered saline containing 5% bovine serum albumin (fraction V, Sigma Chemical Co.). The membrane was then reacted for 18 h at 21°C with 15 ml of a 1:200 dilution of rabbit anti-bovine antithrombin

III IgG dissolved in Tris-buffered saline containing 5% bovine serum albumin. The nitrocellulose membrane was extensively washed with Tris-buffered saline and then incubated for 3 h at 21°C with 15 ml of a 1:100 dilution peroxidase-labeled swine anti-rabbit immunoglobulin (Dakopatts, Accurate Scientific, Westbury, NY) dissolved in Tris-buffered saline containing 5% bovine serum albumin and 0.05% Tween-20 (Fisher Scientific Co., Pittsburgh, PA). Immunoreactive material on the nitrocellulose membrane was visualized with 4-chloro-1-naphthol-containing substrate (HRP-Color Development Reagent, Bio-Rad) following the manufacturer's instructions. Purified platelet factor 4 was generously provided by Dr. Henry Slayter (Harvard Medical School, Boston, MA). The protein was homogeneous on SDS-PAGE and when titrated with heparin yielded a molar stoichiometry of 0.85–1.0 (36). Heparin titrations were carried out as described (16). Bovine prothrombin and protein C were purified as described previously (37, 38).

**Bovine aortas.** Thoracic aortas, a generous gift of the Great American Veal Co. (Newark, NJ), were obtained from 1-yr-old calves within 5 min postmortem. Sacrifice and exsanguination of the animals occurred simultaneously. This procedure was critical for obtaining intact aortic endothelium, as histological studies revealed a considerable number of adherent platelets as well as fibrin deposition on endothelium obtained from animals exsanguinated several minutes after sacrifice. Experiments were either performed immediately at the abattoir or after transport to the laboratory (~40 min later). During transport, aortas were stored at 21°C immersed in Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY) containing 25 mg/ml bovine serum albumin-fatty acid free. For binding and activation studies, the surrounding fat was removed and aortas were cut longitudinally between the intercostal vessels into 4.5  $\times$  21-cm segments, spread flat, and placed within a lucite template. The lucite template consisted of two sheets of 0.5 in. thick lucite. The lower sheet was solid, and the upper sheet was prepared by drilling 0.79 cm<sup>2</sup> holes at regularly spaced intervals. Aortic segments were placed on the lower sheet and covered with the upper sheet. The sheets fit closely together so that wells were formed by the holes in the upper sheet. No leakage from the wells was observed during the incubation period as >98% of the added radioactivity could be recovered. Control experiments were carried out in natural depressions in the vessel formed by resting the aorta on top of the upper sheet of the lucite template. Natural depressions of roughly the same volume as those generated by the 0.79 cm<sup>2</sup> well area were selected. Additional experiments were performed directly on lucite sheets without aortas to exclude possible artifacts due to lucite-coagulation factor interaction.

Morphologic studies of the vessels were performed by scanning electron microscopy using standard procedures (37).

**Binding and inactivation studies on aortic segments.** After the aorta was opened and placed beneath the template, each well was washed five times with incubation buffer over a total of 60 min to elute endogenously bound antithrombin III. Progressive loss of the protease inhibitor from the vessel by the wash procedure was assessed by radioimmunoassay (see below). The integrity of the endothelium was determined microscopically. In the next step, 0.5 ml incubation buffer was added to each 0.79 cm<sup>2</sup> well along with tracer and other components of the reaction mixture, each in a volume of 10–25  $\mu$ l. Aorta was then incubated at 21°C for the indicated time on an orbital shaker at a shaking frequency of 30 oscillations/min. At the end of the incubation period, wells were washed five times with 1 ml of incubation buffer for a total of 10 s/well. Bound <sup>125</sup>I-antithrombin III was eluted by adding 0.5 ml of 0.2 M NaOH:1% SDS:0.01 M EDTA for 5 min. Treatment of vessel segments with enzyme preparations was carried out by incubating the vessel for 30 min at 25°C with either purified Flavobacterium heparinase (5 U/ml) in 0.1 M NaCl, 0.05 M Na acetate, pH 6.5, or chondroitinase ABC or AC (each at 0.5 U/ml) in 0.1 M NaCl, 0.05 M Tris/HCl, pH 8.0. Controls were incubated with the same buffer in the absence of the enzyme. Purified Flavobacterium heparinase (1,219 U/mg) was prepared as described (16) and chondroitinases (ABC and AC) were purchased from Sigma Chemical Co.

1. Abbreviations used in this paper: S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Where indicated, vessel segments were preincubated with dextran sulfate (1 mM; molecular weight, 5,000 D; Sigma Chemical Co.) for 40 min, and washed four times, and then, binding experiments were carried out.

Inactivation of Factor IX<sub>a</sub> and Factor X<sub>a</sub> by antithrombin III in the presence of vessel segments was carried out by incubating Factor IX<sub>a</sub> (4 nM) or Factor X<sub>a</sub> (2 nM) in a reaction well together with antithrombin III (4 μM) for 0 to 15 min at 21°C. Residual Factor X<sub>a</sub> was determined as described by Odegaard et al. (40) by removing 0.1 ml of supernatant and incubating it with 0.5 ml of 40 mM Tris/HCl, pH 7.8, 175 mM NaCl, 10 nM EDTA, 0.5 mg/ml ovalbumin, and 0.1 ml Bz-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) (Helena Laboratories, Beaumont, TX), at a final S-2222 concentration of 1.5 mM for 120 s at 37°C. Amidolysis was stopped by addition of 0.3 ml acetic acid and the absorbance read at 405 nm. Factor X<sub>a</sub> concentrations were determined from the linear portion of a standard curve in which known amounts of Factor X<sub>a</sub> were assayed under conditions identical to the experimental samples. Residual Factor IX<sub>a</sub> activity was determined by clotting assay, as previously described (41), using Factor IX-deficient human plasma. Factor IX<sub>a</sub> concentrations were determined from a standard curve made by determining the clotting times of known amounts of Factor IX<sub>a</sub>.

**Radioimmunoassay for antithrombin III.** Radioimmunoassay for antithrombin III was carried out by a modification of the method described for the radioimmunoassay of human Factor IX (42). The assay was conducted in siliconized (Surfasil, Pierce Chemical Co., Rockford, IL) 1.5 ml microcentrifuge tubes by adding <sup>125</sup>I-antithrombin III (5 μl), antithrombin III antiserum (10 μl), incubation buffer (20 μl), and samples from aortas (20 μl), or antithrombin III standard dilutions (20 μl). All dilutions were made with incubation buffer. Tubes were incubated overnight at 4°C after which 50 μl of a 10% suspension of *Staphylococcus* protein A (IgGSorb, Enzyme Center, Inc., Walden, MA) was added to each tube for 30 min at 21°C.

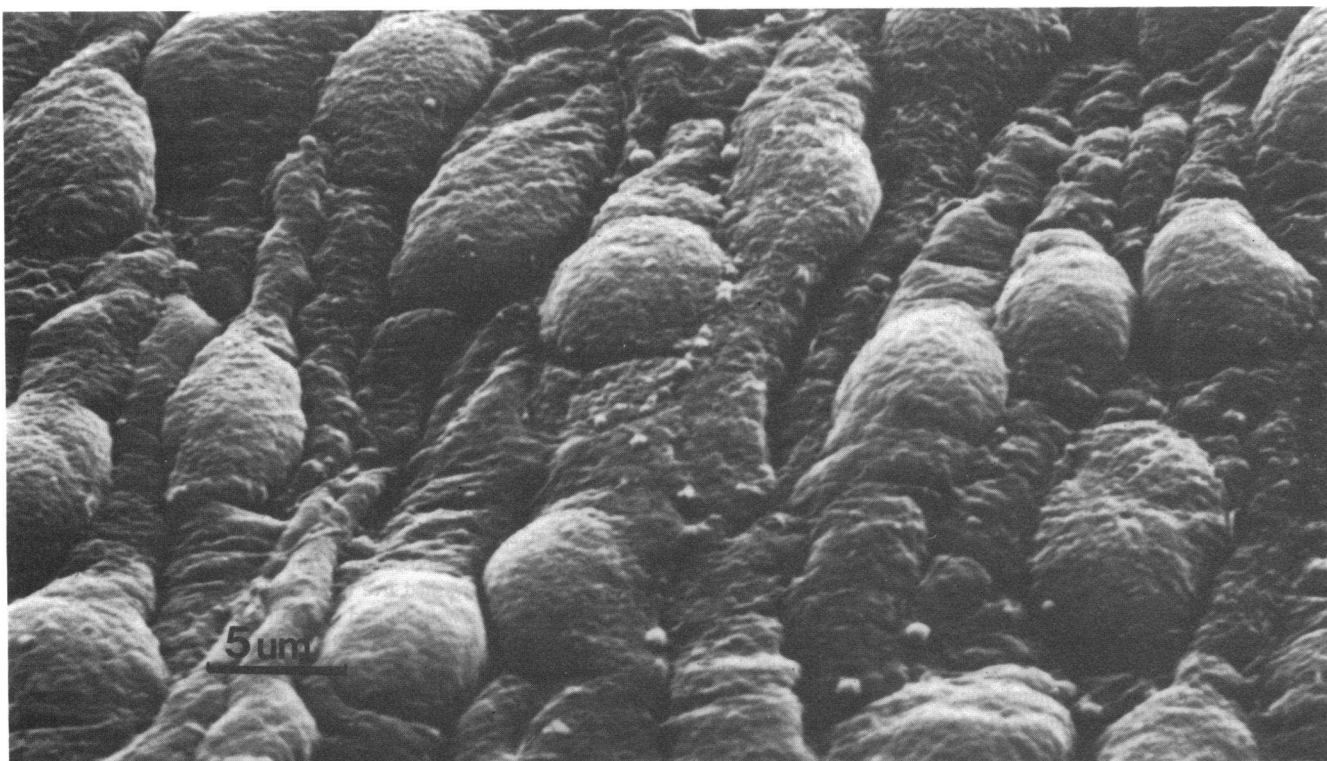
Incubation buffer (500 μl) was added next, and the tubes were centrifuged for 3 min in an Eppendorf microcentrifuge. The supernatant was aspirated, and the pellets counted in a gamma counter. The limit of detection in this assay was 0.2 nM antithrombin III antigen which corresponded to 80% binding on the standard curve.

## Results

**Microscopic study of calf aortas.** Examination of the aortic segments by scanning electron microscopy revealed no evidence of cytoplasmic rupture, endothelial loss, exposure of the internal elastic laminae or adherent platelets (Fig. 1). However, the cells did exhibit a protuberant character presumably resulting from lack of transmural pressure. Direct cell counts showed  $1 \pm 0.04 \times 10^6$  cells/cm<sup>2</sup> (mean ± 1 SD) vessel surface area.

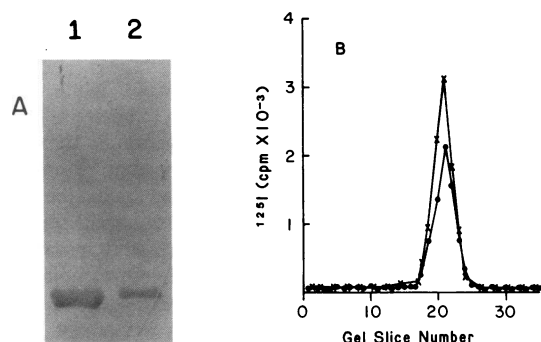
**Binding studies.** Antithrombin III was eluted from vessel segments (Fig. 2 A) employing commercial heparin which dissociates vessel-associated inhibitor. Western blotting using a monospecific antibody to bovine antithrombin III indicated that the vessel-eluted material migrated identically to purified plasma antithrombin III on SDS-PAGE.

Binding of <sup>125</sup>I-antithrombin III to the luminal surface of aortic segments was studied after extensive washing to remove endogenous protease inhibitor. Washing was considered complete when no antithrombin III was detectable by radioimmunoassay in eluates from representative wells incubated with heparin-containing (5 U/ml) incubation buffer (see below). Incubation of <sup>125</sup>I-antithrombin III with the vessel segments demonstrated time-dependent binding (Fig. 3 A). The level of nonspecific binding was determined in the presence of a 200-



**Figure 1.** Scanning electron micrograph of calf aorta showing luminal surface after a binding assay for 35 min at 21°C was carried out as described in Methods. This is a representative picture taken at 0° tilt

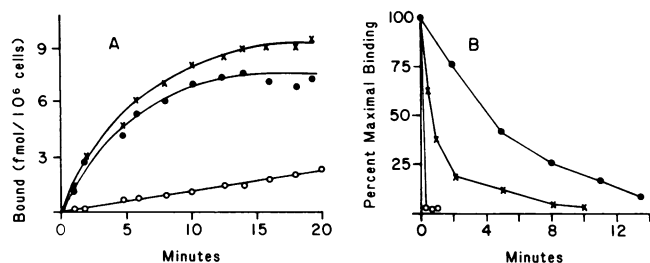
to prevent distortion. The endothelium is observed as an intact monolayer.



**Figure 2.** SDS-PAGE of antithrombin III. (A) Western blots after nonreduced SDS-PAGE (10%). The sample in lane 1 was purified antithrombin III (1  $\mu$ g), and in lane 2, the eluate of an aorta ( $3 \times 5$  cm of vessel surface area) obtained immediately after slaughter, washed, and incubated with heparin (5 U/ml) to remove endogenously bound antithrombin III. Each sample contained heparin at a final concentration of 5 U/ml and the protocol is described in Methods. (B) SDS-PAGE of  $^{125}$ I-antithrombin III before incubation with vessel (●) and after elution of bound  $^{125}$ I-antithrombin III with 1% Nonidet P-40 from bovine aortic vessel segments (×). Binding was accomplished by incubating  $^{125}$ I-antithrombin III (4 nM) for 20 min at 21°C with vessel segments, washing, and then solubilizing the vessel-bound tracer as described in Methods.

fold molar excess of unlabeled antithrombin III. This amount of unlabeled antithrombin III completely eliminated specific binding since addition of more unlabeled antithrombin III had no effect. Vessel-bound  $^{125}$ I-antithrombin III did not appear to be internalized under these conditions, as brief trypsin treatment (2 mg/ml for 4 min at 21°C) resulted in quantitative dissociation of bound radioactivity without any apparent effect on endothelial cell viability as judged by trypan blue exclusion.

Experiments in which increasing concentrations of  $^{125}$ I-antithrombin III were incubated with the vessels demonstrated that specific binding was saturable. Semilogarithmic plots (Fig.



**Figure 3.** Binding of  $^{125}$ I-antithrombin III to bovine aortic segments. (A) Time course of antithrombin III binding. Bovine aortic segments were incubated with  $^{125}$ I-antithrombin III (3 nM) alone (×), or with tracer along with unlabeled antithrombin III (650  $\mu$ M) (○). Specific binding (●) is the total minus the nonspecific binding. (B) Reversibility of  $^{125}$ I-antithrombin III binding. Bovine aortic segments were incubated with  $^{125}$ I-antithrombin III (4 nM). To one set of wells, unlabeled antithrombin III (2  $\mu$ M) was added after 30 min (●). To another set of wells, unlabeled Factor X<sub>a</sub> (10 nM) was added after 30 min (×), and heparin (1 U/ml) was added to a final set of wells after 30 min (○). Nonspecific binding was determined from wells in which  $^{125}$ I-antithrombin III (4 nM) and unlabeled antithrombin III (2  $\mu$ M) were added simultaneously. Percent maximal specific binding is plotted. The experiments were repeated three times and the mean of duplicates is shown.

4) revealed antithrombin III binding was half-maximal at an added  $^{125}$ I-antithrombin III concentration of 14 nM. Thus, at saturation,  $\sim 90$  fmol of antithrombin III bound/ $10^6$  endothelial cells, corresponding to  $5.4 \times 10^4$  molecules bound/cell. Experiments in which  $^{125}$ I-antithrombin III was diluted with unlabeled antithrombin III showed identical binding parameters indicating that unlabeled and labeled antithrombin III interacted with the vessel segments similarly (data not shown). Experiments carried out in natural depressions of the vessel showed similar binding to that observed in the template wells. Lucite did not bind  $^{125}$ I-antithrombin III specifically (data not shown).

Vessel-bound  $^{125}$ I-antithrombin III that eluted after 20 min with 1% Nonidet P-40 was identical to the initial tracer by SDS-PAGE (Fig. 2 B). Neither  $^{125}$ I-antithrombin III in the supernatant following incubation with vessel segments nor vessel-bound antithrombin III exhibited any significant degradation after 40 min of incubation as judged by SDS-PAGE and precipitability in 5% trichloroacetic acid. The binding of  $^{125}$ I-antithrombin III to vessels was reversible (Fig. 3 B), with 50% dissociation of the bound ligand in 4 min. Addition of Factor X<sub>a</sub> (2 nM) accelerated dissociation of vessel-bound  $^{125}$ I-antithrombin III and resulted in the formation of the Factor X<sub>a</sub>- $^{125}$ I-antithrombin III complex that was detected in the supernatants by SDS-PAGE. Heparin markedly accelerated dissociation of vessel-bound  $^{125}$ I-antithrombin III (Fig. 3 B).

Competitive binding studies (Fig. 5) showed inhibition of  $^{125}$ I-antithrombin III-vessel wall binding in the presence of increasing concentrations of unlabeled platelet factor 4 and antithrombin III. In addition, endogenous antithrombin III obtained from vessel washings after slaughter inhibited  $^{125}$ I-antithrombin III-vessel wall binding identically to that observed with purified plasma antithrombin III. Prothrombin, Factor X, Factor IX, and protein C, each up to 1  $\mu$ M concentration, had no effect on specific  $^{125}$ I-antithrombin III binding to vessel segments.

Inhibition of  $^{125}$ I-antithrombin III binding by platelet factor 4 suggested the possible involvement of heparin-like molecules in the binding of antithrombin III to the vessel. This was demonstrated by pretreatment of the vessel with purified *Flavobacterium heparinase*. Treatment of vessel segments with the bacterial enzyme followed by extensive washing prevented subsequent  $^{125}$ I-antithrombin III-vessel wall binding, whereas vessels incubated with buffer alone or chondroitinases showed identical binding (Fig. 4). When the concentration of chondroitinases was increased to 3 U/ml, the incubation time 1 h, and the temperature to 37°C, the results were unchanged. Modification of antithrombin III lysine residues by treatment with *O*-methylisourea also prevented subsequent  $^{125}$ I-antithrombin III binding to vessels (Fig. 4). Control antithrombin III samples dialyzed against the same buffers used in the *O*-methylisourea modification, but in the absence of the modifying reagent, showed vessel wall binding comparable with untreated antithrombin III. Tryptophan-modified antithrombin III also did not bind to aortic segments. The level of nonspecific binding of lysine-modified and tryptophan-modified antithrombin III was comparable with that seen with unmodified antithrombin III. Vessel segments preincubated with dextran sulfate showed identical antithrombin III binding compared with controls.

**Inactivation studies.** Inactivation of Factor IX<sub>a</sub> (Fig. 6 A) by antithrombin III was more rapid in the presence of vessel segments than in their absence. Treatment of the vessels with purified *Flavobacterium heparinase* greatly diminished the

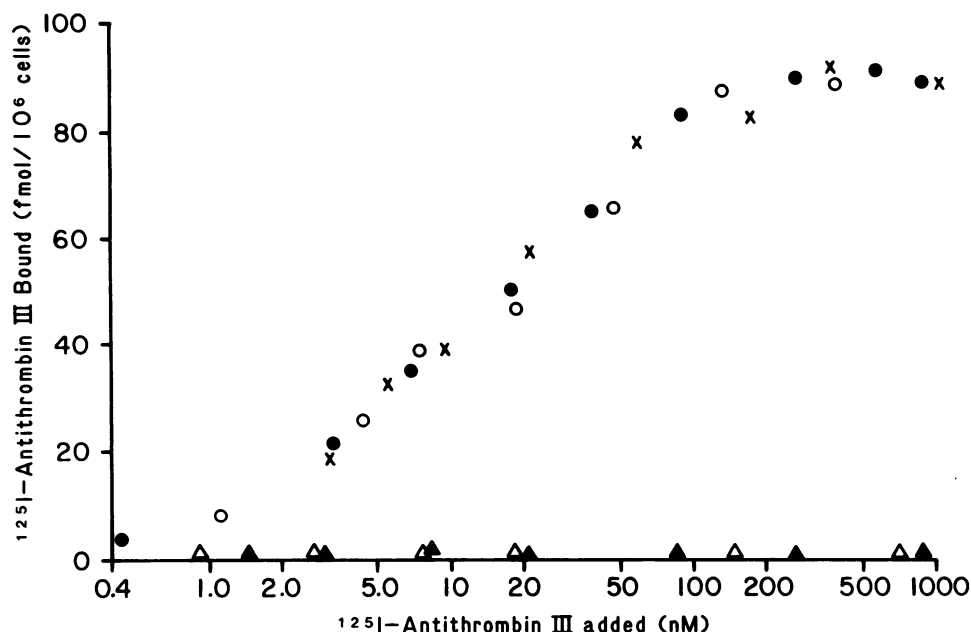


Figure 4. Saturability of  $^{125}\text{I}$ -antithrombin III binding to bovine aortic segments; semilogarithmic graph in which specifically bound  $^{125}\text{I}$ -antithrombin III is plotted against the concentration of added tracer. Aortic segments were washed to remove endogenously bound antithrombin III (as described in Methods), and then, a binding assay was carried out (●). Wells were washed and then preincubated with heparinase (5 U/ml) (Δ), chondroitinase ABC (0.5 U/ml) (○), chondroitinase AC (0.5 U/ml) (×), or  $^{125}\text{I}$ -antithrombin III modified by treatment with *O*-methylisourea was used in place of untreated antithrombin III (▲). The experiment was repeated four times and the mean of triplicates is shown.

vessel-mediated acceleration of Factor  $\text{IX}_a$  inactivation. A similar acceleration of Factor  $\text{X}_a$  inactivation by antithrombin III in the presence of vessels was observed (Fig. 6 B). The cofactor effect of the vessel, however, appeared to be far less pronounced for Factor  $\text{X}_a$  inactivation than that observed with Factor  $\text{IX}_a$ . Controls in which Factor  $\text{IX}_a$  and Factor  $\text{X}_a$  were incubated with vessels under identical conditions to those employed in the experiments in Fig. 6, but without exogenous antithrombin III, demonstrated no loss of Factor  $\text{IX}_a$  activity and a 2–3% loss of Factor  $\text{X}_a$  activity after 15 min of incubation.

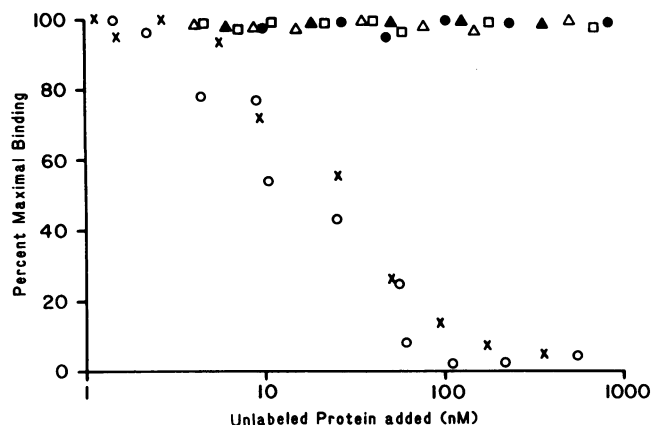
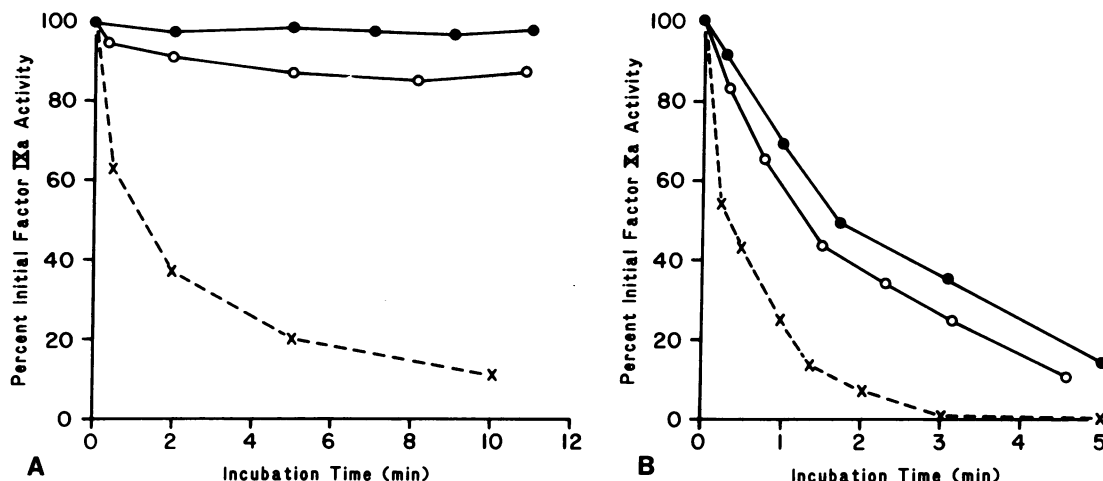


Figure 5. Competitive binding studies. Vessels were incubated with  $^{125}\text{I}$ -antithrombin III (3 nM) for 30 min in the presence of increasing concentrations of unlabeled antithrombin III (×), platelet factor 4 (○), Factor IX (Δ), Factor X (▲), protein C (□), and prothrombin (●). Wells were then washed and solubilized as described. Maximal binding (21 fmol/ $10^6$  cells) was defined as the amount of  $^{125}\text{I}$ -antithrombin III bound when  $^{125}\text{I}$ -antithrombin III was incubated with aortic segments alone minus the amount of  $^{125}\text{I}$ -antithrombin III bound in the presence of a 100-fold molar excess of unlabeled antithrombin III. The mean of duplicates is plotted and the experiment was repeated four times.

## Discussion

These studies demonstrate that bovine aortic vessel segments with a continuous layer of endothelium bind antithrombin III and enhance its anticoagulant activity. Antithrombin III was detected on calf aortas obtained immediately after sacrifice and this vessel-associated antithrombin III was identical to isolated plasma antithrombin III based on SDS-PAGE and immunoreactivity. Furthermore, competitive binding studies demonstrated comparable inhibition of  $^{125}\text{I}$ -antithrombin III-vessel wall binding by the vessel-eluted inhibitor and antithrombin III purified from bovine plasma.

After washing vessel segments to remove endogenously bound antithrombin III, incubation of aortas with  $^{125}\text{I}$ -antithrombin III resulted in specific, time-dependent binding of the inhibitor (Figs. 3 and 4). This binding appears to be slower than the very rapid observed, antithrombin III-heparin binding in solution (36). Although the reason for this difference is unclear, structural differences in the heparin-like molecules or the cellular microenvironment may be involved. Antithrombin III binding sites were half-maximally occupied at a total inhibitor concentration of 14 nM and completely occupied at 1  $\mu\text{M}$ . Thus, it is highly probable that these binding sites are occupied at the plasma concentration of antithrombin III (2.5  $\mu\text{M}$ ). Vessel-binding of antithrombin III was reversible (Fig. 3) and addition of Factor  $\text{X}_a$  accelerated dissociation of antithrombin III. This resulted in Factor  $\text{X}_a$ -antithrombin III complex detectable in the supernatants by SDS-PAGE, indicating that the vessel-bound inhibitor retained its anticoagulant activity. Further studies demonstrated enhancement of antithrombin III anticoagulant activity in the presence of vessels (Fig. 6). Antithrombin III, which is not a potent inhibitor of Factor  $\text{IX}_a$  in the absence of heparin, inactivated 50% of the added Factor  $\text{IX}_a$  in 90 s in the presence of a vessel (Fig. 6). Factor  $\text{X}_a$  inactivation was also enhanced in the presence of aortas with 50% inhibition of the added Factor  $\text{X}_a$  in 20 s compared with 2 min for the same degree of inactivation in the absence of vessels (Fig. 6). Factor  $\text{X}_a$  inactivation by



**Figure 6.** Inactivation of Factor IX<sub>a</sub> and Factor X<sub>a</sub> by antithrombin III in the presence of bovine aortic segments. (A) Inactivation of Factor IX<sub>a</sub>. Factor IX<sub>a</sub> (4 nM) and antithrombin III (2 μM) in 0.5 ml incubation buffer were incubated in a testtube (●) in the presence of a washed bovine aortic vessel segment (0.79 cm<sup>2</sup>) (×) or a vessel segment pretreated with heparinase (5 U/ml) (○) as described in Methods. Aliquots of supernatant were removed at the indicated

times and assayed for Factor IX<sub>a</sub> as described in Methods. (B) Inactivation of Factor X<sub>a</sub>. The protocol was identical to A, except that Factor X<sub>a</sub> at 1 nM was added and aliquots of supernatant were removed at the indicated times for the S2222 chromogenic substrate assay. The experiments were repeated three times and the mean of triplicates is shown.

antithrombin III observed in our reaction mixtures in the absence of vessels was slower than that reported by Odegaard and colleagues (40) for bovine Factor X<sub>a</sub>. The reason for this difference is unknown, although their studies utilized human antithrombin III while our studies have employed bovine antithrombin III.

Competitive binding studies demonstrated inhibition of <sup>125</sup>I-antithrombin III-vessel wall binding in the presence of unlabeled platelet factor 4. This finding raised the possibility that heparin-like molecules were involved in the vessel wall-antithrombin III binding site (Fig. 5). Chemical modification of antithrombin III lysine and tryptophan residues, which blocks its heparin cofactor activity (1), prevented subsequent antithrombin III-vessel wall binding (Fig. 5). Similarly, purified flavobacterium heparinase treatment of vessel segments precluded antithrombin III binding to these vessels. These results strongly suggest that heparin-like molecules are instrumental for antithrombin III binding to vessel segments. Both heparin and heparin-like glycosaminoglycans with anticoagulant activity have been isolated from calf vessel preparations and appear to be localized to the intima (14, 15). On the other hand, Schiffman and Pizzo (43) were unable to obtain evidence for the involvement of vascular heparin-like molecules as binding sites in their studies of antithrombin III clearance in the mouse. Furthermore, Lollar et al. (13) reported that glycosaminoglycans having antithrombin III cofactor activity do not contribute significantly to binding and inhibition of bovine thrombin in a perfused rabbit heart preparation. Although the reason for the differences between the conclusions from these studies and ours is unclear, the experiments done were quite different. We have examined the interaction of bovine antithrombin III with native bovine aortic endothelium. The two other studies cited above have employed more complex vascular beds, have examined clearance, and have used antithrombin III of a different species than that of the endothelium. We find that these heparinase-sensitive glycosaminoglycans on the surface of native endothelium do promote inhibition of Factor

X<sub>a</sub> and Factor IX<sub>a</sub> by antithrombin III in freshly isolated bovine aortic segments. Our findings imply that antithrombin III binding to these endothelial cell heparin-like molecules provide an important link in understanding the enhanced inactivation of Factor IX<sub>a</sub> and Factor X<sub>a</sub> by antithrombin III in the presence of native endothelium. This leads us to hypothesize that antithrombin III binding to heparin-like molecules on the vasculature might also be the underlying mechanism in the enhancement of thrombin inactivation by antithrombin III as seen by Marcum and colleagues in an animal model (16).

Comparable inhibition of <sup>125</sup>I-antithrombin III-vessel wall binding by unlabeled antithrombin III and platelet factor 4 were observed. Since the concentration of antithrombin III in plasma (2.5 μM) is much greater than platelet factor 4 (100 pM) (44), one would expect that the antithrombin III-vessel wall interaction would predominate. During activation of coagulation when platelet release reaction occurs, however, it is possible that high local concentrations of platelet factor 4 may be achieved. In this circumstance, platelet factor 4 would then saturate the vessel wall heparin-like binding sites and prevent antithrombin III from interacting with the vessel surface.

The origin of this heparin-like material participating in antithrombin III binding to the vessel surface is uncertain. Either this heparin-like material exists as an integral endothelial cell surface component, or is adsorbed to the endothelium following its release from other cells, particularly mast cells, associated with the intima of the vessel. Marcum and Rosenberg (14) suggested that intimal heparin-like material from calf aortas may originate from mast cells. Heparin adsorbed to vascular matrices is known to be dissociable from the matrix by relatively high concentrations of dextran sulfate (14). In our studies, preincubation of vessel segments with dextran sulfate produced no detectable change in subsequent antithrombin III binding compared with untreated vessels, indicating a tight association of the heparin-like binding sites for



the cellular surface. Furthermore, we examined Factor IX<sub>a</sub> inactivation in aortas of mice deficient in mast cells (w/w<sup>v</sup> strain [45], Jackson Laboratory, Bar Harbor, ME), and found that Factor IX<sub>a</sub> inactivation in these animals was only slightly less in comparison to control mice (data not shown). This suggested that, in the aorta, mast cells provide only marginal amounts of heparin-like glycosaminoglycans promoting antithrombin III anticoagulant activity.

The physiologic significance of the heparin-antithrombin III interaction is implied by the thrombotic diathesis observed in kindreds with antithrombin III molecules interacting abnormally with heparin (46–50). One possible mechanism by which this defect may lead to localized thrombotic events is the impaired ability of antithrombin III to bind to the vessel wall and failure to accelerate inactivation of the coagulation factors. The ability of antithrombin III to bind to heparin-like molecules is particularly important in Factor IX<sub>a</sub> inhibition which proceeds slowly in the absence of heparin. Thus, vessel wall-antithrombin III interaction may be an important component of the antithrombotic nature of the vascular endothelium. Furthermore, our data suggest that pathophysiological states leading to abnormalities of vessel wall heparin-like glycosaminoglycans may predispose an area of the vessel to thrombosis by impairing antithrombin III binding.

## Acknowledgments

We would like to gratefully acknowledge the advice and encouragement of Dr. S. Silverstein. The expert technical assistance of M. Drillings, J. McKenney, J. Harris, and J. Bartos was invaluable.

This work was supported by National Institutes of Health grants HL-15486, HL-16919, HL-06615, HL-21006, and HL-28625. This work was done during the tenure of Dr. Stern as a recipient of a Clinician-Scientist Award from the American Heart Association and with funds contributed in part by the New York Affiliate.

## References

- Rosenberg, R., and P. Damus. 1973. The purification and mechanism of action of human antithrombin-heparin cofactor. *J. Biol. Chem.* 248:6490–6503.
- Rosenberg, J., P. McKenna, and R. Rosenberg. 1975. Inhibition of human factor IX<sub>a</sub> by human antithrombin. *J. Biol. Chem.* 250:8883–8891.
- Yin, E., S. Wessler, and P. Stoll. 1971. Biological properties of the naturally occurring plasma inhibitor of activated factor X. *J. Biol. Chem.* 246:3703–3710.
- Damus, P., M. Hicks, and R. Rosenberg. 1973. Anticoagulant action of heparin. *Nature (Lond.)* 246:355–356.
- Stead, N., A. Kaplan, and R. Rosenberg. 1976. Inhibition of activated factor XII by antithrombin-heparin cofactor. *J. Biol. Chem.* 251:6481–6485.
- Kurachi, K., K. Fujikawa, G. Schmer, and E. Davie. 1976. Inhibition of bovine factor IX<sub>a</sub> and factor X<sub>a</sub> by antithrombin III. *Biochemistry* 15:373–377.
- Hirsh, J. 1982. Laboratory diagnosis of thrombosis. In *Hemostasis and Thrombosis*. Colman, R., J. Hirsh, Y. Marder, and E. Salzman, editors. Lippincott Co., Philadelphia, PA. 795–796.
- Jordan, R., D. Beeler, and R. Rosenberg. 1979. Fractionation of low molecular weight heparin species and their interaction with antithrombin. *J. Biol. Chem.* 254:2902–2913.
- Oohira, A., T. Wight, and P. Bornstein. 1983. Sulfated proteoglycans synthesized by vascular endothelial cells in culture. *J. Biol. Chem.* 258:2014–2021.
- Buonassisi, V., and P. Colburn. 1983. Antibodies to the heparan sulfate proteoglycans synthesized by endothelial cell cultures. *Biochim. Biophys. Acta* 760:1–12.
- Colburn, P., and V. Buonassisi. 1982. Anti-clotting activity of endothelial cell cultures and heparan sulfate proteoglycans. *Biochem. Biophys. Res. Commun.* 104:220–227.
- Busch, C., and W. Owen. 1982. Identification in vitro of an endothelial cell surface cofactor for antithrombin III. *J. Clin. Invest.* 69:726–729.
- Lollar, P., S. MacIntosh, and W. Owen. 1984. Reaction of antithrombin III with thrombin bound to the vascular endothelium. *J. Biol. Chem.* 259:4335–4338.
- Marcum, J., and R. Rosenberg. 1984. Anticoagulant active heparin-like molecules from vascular tissue. *Biochemistry* 23:1730–1737.
- Marcum, J., L. Fritze, S. Galli, G. Karp, and R. Rosenberg. 1983. Microvascular heparin-like species with anticoagulant activity. *Am. J. Physiol.* 245:H725–H733.
- Marcum, J., J. McKenney, and R. Rosenberg. 1984. The acceleration of thrombin-antithrombin complex formation in rat hind-quarters via heparin-like molecules bound to endothelium. *J. Clin. Invest.* 74:341–350.
- Stern, D., P. Nawroth, W. Kiesel, M. Drillings, J. Bartos, and H. Nessel. 1983. A pathway of coagulation from factor IX<sub>a</sub> to thrombin formation on the surface of endothelial cells. *Blood* 62(Suppl. 1):311a.
- Mahoney, W. C., K. Kurachi, and M. A. Hermodson. 1980. Formation and dissociation of the covalent complexes between trypsin and two homologous inhibitors,  $\alpha_1$ -antitrypsin and antithrombin III. *Eur. J. Biochem.* 105:545–552.
- Abildgaard, U., M. Lie, and O. R. Odegaard. 1977. Antithrombin (heparin cofactor) assay with "new" chromogenic substrates (S-2238 and Chromozym TH). *Thromb. Res.* 11:549–558.
- Lundblad, R. L., L. C. Uhteg, C. N. Vogel, H. S. Kingdon, and K. G. Mann. 1975. Preparation and partial characterization of two forms of bovine thrombin. *Biochem. Biophys. Res. Commun.* 66:482–286.
- Abildgaard, U., M. Lie, and O. R. Odegaard. 1976. A simple amidolytic method for the determination of functionally active antithrombin III. *Scand. J. Clin. Lab. Invest.* 36:109–120.
- David, G., and R. Reisfeld. 1974. Protein iodination with solid state lactoperoxidase. *Biochemistry* 13:1014–10231.
- Haynes, R. and R. Feeney. 1968. Transformation of active-site lysine in naturally occurring trypsin inhibitors. A basis for a general mechanism for inhibition of proteolytic enzymes. *Biochemistry* 7:2879–2885.
- Blackburn, M., and C. Sibley. 1980. The heparin binding site of antithrombin III. *J. Biol. Chem.* 255:824–826.
- Fujikawa, K., A. R. Thompson, M. Legaz, R. Meyer, and E. Davie. 1973. Isolation and characterization of bovine factor IX. *Biochemistry* 12:4938–4944.
- Stern, D., M. Drillings, W. Kiesel, P. Nawroth, H. Nessel, and K. LaGamma. 1983. Activation of factor IX bound to cultured bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* 81:913–917.
- Zur, M., and Y. Nemerson. 1980. Kinetics of factor IX activation via the extrinsic pathway. *J. Biol. Chem.* 255:5703–5707.
- Fujikawa, K., M. Legaz, and E. Davie. 1972. Bovine factors X<sub>1</sub> and X<sub>2</sub>. Isolation and characterization. *Biochemistry* 11:4882–4891.
- Kiesel, W., H. Hermodson, and E. Davie. 1976. Factor X activating enzyme from Russell's viper venom. Isolation and characterization. *Biochemistry* 15:4901–4906.
- Bajaj, P., and K. Mann. 1973. Simultaneous purification of bovine prothrombin and factor X. *J. Biol. Chem.* 248:7729–7741.
- Harboe, N., and A. Ingild. 1973. Immunization, isolation of immunoglobulins, and estimation of antibody titre. In *Manual of Quantitative Immunoelectrophoresis*. N. H. Axelson, J. Kroll, and B. Weeke, editors. Universitetsforlaget, Oslo. 161–164.
- Lowry, O., N. Rosebrough, L. Farr, and R. Randall. 1951. Protein measurement with Folin reagent. *J. Biol. Chem.* 193:265–275.



33. Chase, T., and E. Shaw. 1969. Comparison of the esterase activities of trypsin, plasmin, and thrombin on guanidinobenzoate esters. Titration of enzymes. *Biochemistry*. 8:2212-2224.
34. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature (Lond.)*. 227:680-685.
35. Towbin, H., T. Strachelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350-4354.
36. Jordan, R., L. Favreau, E. Braswell, and R. Rosenberg. 1982. Heparin with two binding sites antithrombin or platelet factor 4. *J. Biol. Chem.* 257:400-406.
37. Mann, K. 1976. Prothrombin. *Methods Enzymol.* 45:123-156.
38. Kisiel, W., L. Ericsson, and E. Davie. 1976. Proteolytic activation of protein C from bovine plasma. *Biochemistry*. 15:4893-4900.
39. Hayat, A. M. 1972. Principles and Techniques of Electron Microscopy. Van Nostrand Reinhold Co., New York. 350 pp.
40. Odegaard, O., A. Abildgaard, M. Lie, and M. Miller-Andersson. 1977. Inactivation of bovine and human thrombin and factor X<sub>a</sub> by antithrombin III studied with amidolytic methods. *Thromb. Res.* 11: 205-216.
41. Biggs, R., and R. McFarland. Human Blood Coagulation. Blackwell Scientific Publishers, Oxford. Third ed. 465 pp.
42. Suzuki, L., and A. Thompson. 1982. Factor IX antigen by a rapid *Staphylococcal* protein A-membrane binding radioimmunoassay results in haemophilia B patients and carriers in fetal samples. *Br. J. Haematol.* 50:673-682.
43. Shiffman, M., and S. Pizzo. 1982. The in vivo metabolism of antithrombin III and antithrombin III complexes. *J. Biol. Chem.* 257:3243-3248.
44. Kaplan, K., and J. Owen. 1982. Radioimmunoassay of platelet factor 4. *Methods Enzymol.* 84:83-92.
45. Kitamura, Y., S. Go, and K. Hatanaka. 1978. Decrease of mast cells in w/w, mice and their increase by bone marrow transplantation. *Blood*. 52:447-454.
46. Sas, G., G. Blasko, D. Banhegyi, J. Kajo, and L. Palos. 1974. Abnormal antithrombin III (antithrombin III "Budapest") as a cause of familial thrombophilia. *Thromb. Diath. Haemorrh.* 32:105-113.
47. Tran, T., H. Bounameaux, C. Bondeli, H. Honkanen, G. Marbet, and F. Duckert. 1980. Purification and partial characterization of a hereditary abnormal antithrombin III fraction of a patient with recurrent thrombophlebitis. *Thromb. Haemostasis*. 44:87-96.
48. Sorensen, P., J. Dyerberg, E. Stoffersen, and M. Jensen. 1980. Familial functional antithrombin III deficiency. *Scand. J. Haematol.* 24:105-113.
49. Wolf, M., C. Boyer, J. Lavergne, and M. Larrieu. 1982. A new familial variant of antithrombin III: "Antithrombin III Paris." *Br. J. Haematol.* 51:285-292.
50. Bauer, K., J. Ashenhurst, J. Chedrak, and R. Rosenberg. 1983. Antithrombin "Chicago": a functionally abnormal molecule with increased heparin affinity causing familial thrombophilia. *Blood*. 62:1242-1250.