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CANINE ANTITHROMBIN-III:

SOME BIOCHEMICAL AND BIOLOGIC PROPERTIES

by

Hilary Diane Sigmon

Dissertation submitted to the Faculty of the Department of Physiology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1987

DEDICATION

To Adam for daily joy,

To Brian for "coaxing" me on,

To Andy for everything!

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I. INTRODUCTION

Coagulopathies have been reported to occur after many forms of stress and trauma. These thrombohemorrhagic responses are likely consequent to disturbances of the delicate interplay of the hemostatic compartments (vasculature, platelets, and plasma proteins), resulting in the systemic activation of the clotting-fibrinolytic (and other) systems with eventual depletion of critical hemostatic components. This syndrome* of generalized intravascular proteolysis or disseminated intravascular coagulation (DIC), an intermediary mechanism of disease, is usually (but not always) associated with well-defined clinical entities. It can manifest as a wide clinical spectrum.

For example, if the intravascular clotting process is dominant and secondary fibrino(geno)lysis minimal, DIC may be expressed primarily as diffuse thromboses, as in malignancy. Alternatively, if the secondary fibrinolysis which occurs with DIC is dominant, and the drive toward procoagulant activity minimal, the clinical manifestations will be hemorrhage, by far the more common expression of DIC (1-4). Patients often demonstrate combinations of these two clinical manifestations. Thus, DIC represents a wide spectrum of clinical findings with patients presenting anywhere in the continuum between diffuse thromboses and/or hemorrhage (1,4,5).

Table I lists conditions associated with DIC. In each instance, the systemic response relates to either platelet damage, vessel wall damage, direct plasma protein (clotting factors) activation, or any combination thereof.

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*It is labeled as a "syndrome," and not a "disease" since it is manifested as a consequence of other disease processes.

TABLE I

CONDITIONS ASSOCIATED WITH DIC (6)

A. Obstetrical Accidents

1. Amniotic fluid embolism
2. Placental abruption
3. Retained fetus syndrome

B. Intravascular Hemolysis

1. Hemolytic transfusion reaction
2. Multiple transfusions - Banked whole blood

C. Septicemia

1. Gram-negative (endotoxin)
2. Gram-positive (mucopolysaccharides?)

D. Viremia (Varicella)

E. Solid Malignancy

F. Leukemias

1. Promyelocytic
2. Other

G. Acidosis/Alkalosis

H. Burns

I. Crush Injury and Tissue Necrosis

J. Vascular Disorders

A. PHYSIOLOGY AND PATHOPHYSIOLOGY OF DIC

As summarized in Figure 1, the net result of any triggering mechanism is the ultimate generation of two potent enzymes: thrombin and plasmin, systemically. As thrombin circulates, it enzymatically attacks fibrinogen, systemically rather than locally. This results in fibrin deposition primarily in the microvasculature, leading to impedance of blood flow, tissue hypoxia, and resultant ischemia and necrosis in multiple end organs. In addition, polymerized fibrin deposited in the microvasculature leads to entrapment of platelets, attendant thrombocytopenia, and may cause microangiopathic hemolytic anemia from fibrin-red cell contact (7). The microhemolysis can provide more triggering material for continued intravascular coagulation (ADP and red cell membrane phospholipid), thus creating a pathologic cycle.

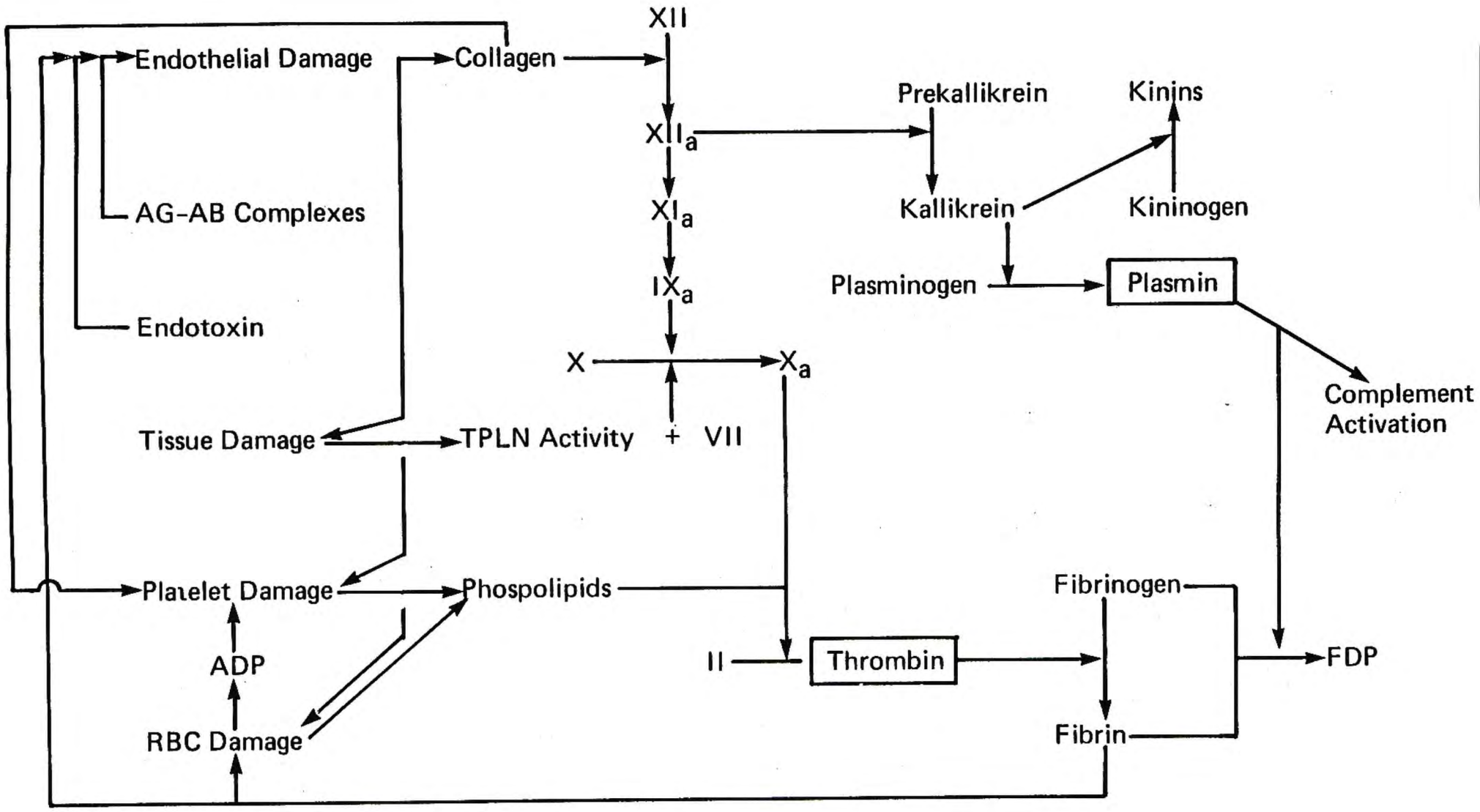
Another important consideration in appreciating the pathophysiology of DIC is the interrelationship between the clotting process and other plasma protein enzyme systems. During an intravascular clotting episode, the activation of Factor XII occurs. Factor XII_a via the kallikrein system indirectly activates the fibrinolytic system, which may serve as an alternate route of secondary fibrinolysis in DIC (8).

In the presence of plasmin most plasma proteins are hydrolyzed, including fibrinogen resulting in fibrinogen fibrin degradation products (FDP) the X, Y, D, and E fragments (9), some of which interfere with fibrin polymerization and cause platelet dysfunction by coating the membrane surface.

Plasmin also activates the first and third component of complement, thus initiating the complement sequence with potential subsequent cell lysis, immunoadherence, and other immune phenomena (10). Kallikrein generates kinins from kininogens, leading to hypotension, increased vascular permeability, pain and other manifestations (11).

Figure 1

Pathophysiology of Disseminated Intravascular Coagulation (6). Endothelial damage, antigen-antibody complexes, endotoxin, tissue damage, platelet and red cell injury, by a variety of interrelated mechanisms (noted by arrows) have the potential for serving as "triggers" for activation of the coagulation and fibrinolytic systems resulting in the generation of two key enzymes: thrombin and plasmin. Abbreviations: AG-AB = antigen-antibody, ADP = adenosine diphosphate, RBC = red blod cell, TPLN = tissue plasminogen, a = active, FDP = fibrin/fibrinogen degradation (split) products.


XII
Endothelial Damage
Collagen
Prekallikrein
Kinins
XIIa
AG-AB Complexes
XIa
Kallikrein
Kininogen
IXa
Plasminogen
Plasmin
Endotoxin
X
Xa
Complement Activation
Tissue Damage
TPLN Activity
+ VII
Platelet Damage
Phospolipids
Fibrinogen
ADP
II
Thrombin
FDP
RBC Damage
Fibrin

Patients with DIC have numerous abnormal laboratory tests of hemostasis. In subacute or chronic DIC, many laboratory parameters may be difficult to interpret. One laboratory test which is helpful in confirming a diagnosis of DIC is an Antithrombin III (AT-III, heparin-cofactor) determination (12). Consumption of this naturally occurring inhibitor (an α_2-globulin) due to generation of thrombin and other serine proteases - of plasma and/or cellular origin - occurs early on and to a significant degree, in most instances of acute DIC.

B. PHYSIOLOGIC IMPORTANCE OF AT-III

The clotting system is modulated by three inhibitor systems: protein C, heparin cofactor II (HC II) and AT-III. Briefly, protein Ca* regulates the amount of Factor Xa and thrombin formed in that it destroys Factors VIIIa and Va (13). Heparin Cofactor II (HC II), recently identified in human plasma by Tollefsen and others (14-16), is a heparin-dependent inhibitor of thrombin. It blocks the proteolytic and amidolytic activities of thrombin by forming a covalent, 1:1 molar complex with the protease (16). In the presence of dermatan sulfate (a proteoglycan found in the intima and media of large arteries as well as in skin, heart valves, and tendons), HC II binds thrombin as it is being generated in plasma during coagulation (16). In experiments with purified proteases, HC II does not significantly inhibit other coagulation factors (15).

Antithrombin III (AT-III), in contrast, regulates the activity of several enzymes formed during clotting and, therefore, governs the effect that these enzymes exert on their respective substrates. It not only inactivates thrombin, as the name suggests, but also (to varying degrees) Factors Xa (17), IXa (18), XIa

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*Ca = active protein C (accepted nomenclature)

(19), VII (17,18,19), XIIa (20), kallikrein (21), trypsin (22), plasmin (22) and urokinase (23). In all documented instances, AT-III neutralizes the enzymes in a progressive manner forming a 1:1 stoichiometric complex (24).

With most of the enzymes studied, and especially with thrombin, AT-III activity is markedly enhanced in the presence of heparin (24). This interaction seems to bring about a change in the molecular configuration of AT-III resulting in an instantaneous interaction with the enzymes (25). At the endothelial surface, AT-III interacts with bound acid aminoglycans, such as heparan sulfate and dermatan sulfate (26), resulting in enhancement of its neutralizing activity (27).

The amount of AT-III in normal adult human plasma is approximately 150 ug/ml (28). Normal, healthy newborn infants have levels of about 50% of the adult levels at birth (29), but their levels increase to normal within the first six months of life.

Decreased levels of protein Ca, HC II and AT-III have been associated with thromboembolic disease.

C. BIOCHEMICAL CHARACTERIZATION OF AT-III AND MECHANISMS OF ACTION

1. General Properties

Antithrombin III (AT-III) is an α-2 glycoprotein found in plasma. Human and rabbit AT-III (and, to some extent, bovine and canine AT-III), have been biologically and biochemically characterized (29,30,31,32,33). The four molecular species have been purified using a heparin-resin affinity matrix, validated by gel electrophoresis (30,32,33).

Molecular heterogeneity for both human and rabbit AT-III has been documented. Different fractions have different molecular weights (between 62,000 and 67,000) and are separable by crossed immunoelectrophoresis (C.I.E.).

The two main molecular species of rabbit AT-III have identical heparin cofactor and progressive AT-III activities when assayed by chromogenic substrates (31). Multiple AT-III molecular species in normal human subjects and in patients suffering from an acquired or congenital AT-III deficiency also have been documented. Two distinct heparin cofactors have been described (34). The first has marked heparin cofactor activity, and little progressive activity; the second has marked progressive AT-III activity and little heparin cofactor activity. This molecular heterogeneity of the human AT-III may correlate with the same heterogeneity in AT-III disease states observed by C.I.E. (32).

Human AT-III contains three disulfide bridges and four points of carbohydrate attachment (35). Its complete primary structure has been reported (35). Neither amino acid composition nor sequencing data have been reported on canine or rabbit AT-III.

2. The AT-III/Thrombin/Heparin Axis

The complex between AT-III and thrombin is remarkably stable and cannot be disrupted by a variety of denaturing agents. Thus it is possible to follow the AT-III-thrombin interaction using polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). As shown by Rosenberg and Damus (24), when purified human thrombin and human AT-III were incubated together, the bands representing the individual proteins disappeared over a period of a few minutes, and a complex with an estimated molecular size of about 90,000 was formed. In the presence of heparin, complex formation was virtually instantaneous.

When thrombin was modified by diisopropylphosphofluoridate (DFP), AT-III-thrombin complex formation was prevented in both the presence and

absence of heparin (36). Thus the active center serine of thrombin is required for the interaction.

Thrombin is known to have a remarkably narrow specificity for unique arginyl-X bonds (37). Therefore, this residue might be expected to be involved in the AT-III reactive site. When AT-III was treated with 1,2-cyclohexanedione, which complexes with arginyl residues, there was almost complete destruction of heparin cofactor as well as progressive antithrombin activities (36). Subsequent amino acid analysis of the inhibitor confirmed that the major modification involved alteration of 32% of the inhibitor's arginine residues.

In view of the acidic nature of heparin, it had been theorized that the lysine residues of the inhibitor might function as a binding site for the highly charged mucopolysaccharide. Treatment of the inhibitor with o-methylisourea (which complexes with lysine residues) practically abolished heparin cofactor activity with only a minimal effect on progressive antithrombin activity (36). Heparin cofactor activity could be protected against loss of activity by preincubation of the inhibitor with excess heparin prior to exposure to o-methylisourea.

Antithrombin-III (AT-III) thus appears to neutralize the activity of thrombin by complex formation via an inhibitor reactive site (Arg) - enzyme center (Ser) interaction.

Rosenberg and coworkers hypothesized a model for the heparin acceleration of the AT-III-thrombin interaction (38). They proposed that heparin, binding to the inhibitor via lysyl residues, invoked a "heparin-dependent" conformation change in the inhibitor, rendering the reactive site (Arg) more accessible to the active center (Ser) of thrombin.

An alternative model for the interaction between heparin, AT-III and thrombin has been proposed by other investigators (39,40,41). Hatton and Rogoeczi (39) studied the inhibition of thrombin by AT-III in the presence of

heparin conjugated to Sepharose. When the enzyme was adsorbed to the heparin conjugate in quantities insufficient to occupy all the heparin sites, subsequent passage of AT-III through the matrix produced complete inhibition of thrombin activity, as measured by the response to a chromogenic substrate. When the loading sequence was reversed (i.e., adsorbing nonsaturating quantities of AT-III before thrombin), thrombin activity was only insignificantly inhibited, indicating that the thrombin preferentially adsorbed to a free heparin binding site rather than a heparin-AT-III binding site. The authors proposed that if the relative affinities of free heparin and the heparin-thrombin complex or the heparin-AT-III complex for the third component of the inactivation system is an indicator of the order in which the components interact, heparin promotes the inactivation of thrombin by direct thrombin interaction, thereby augmenting its reaction with AT-III.

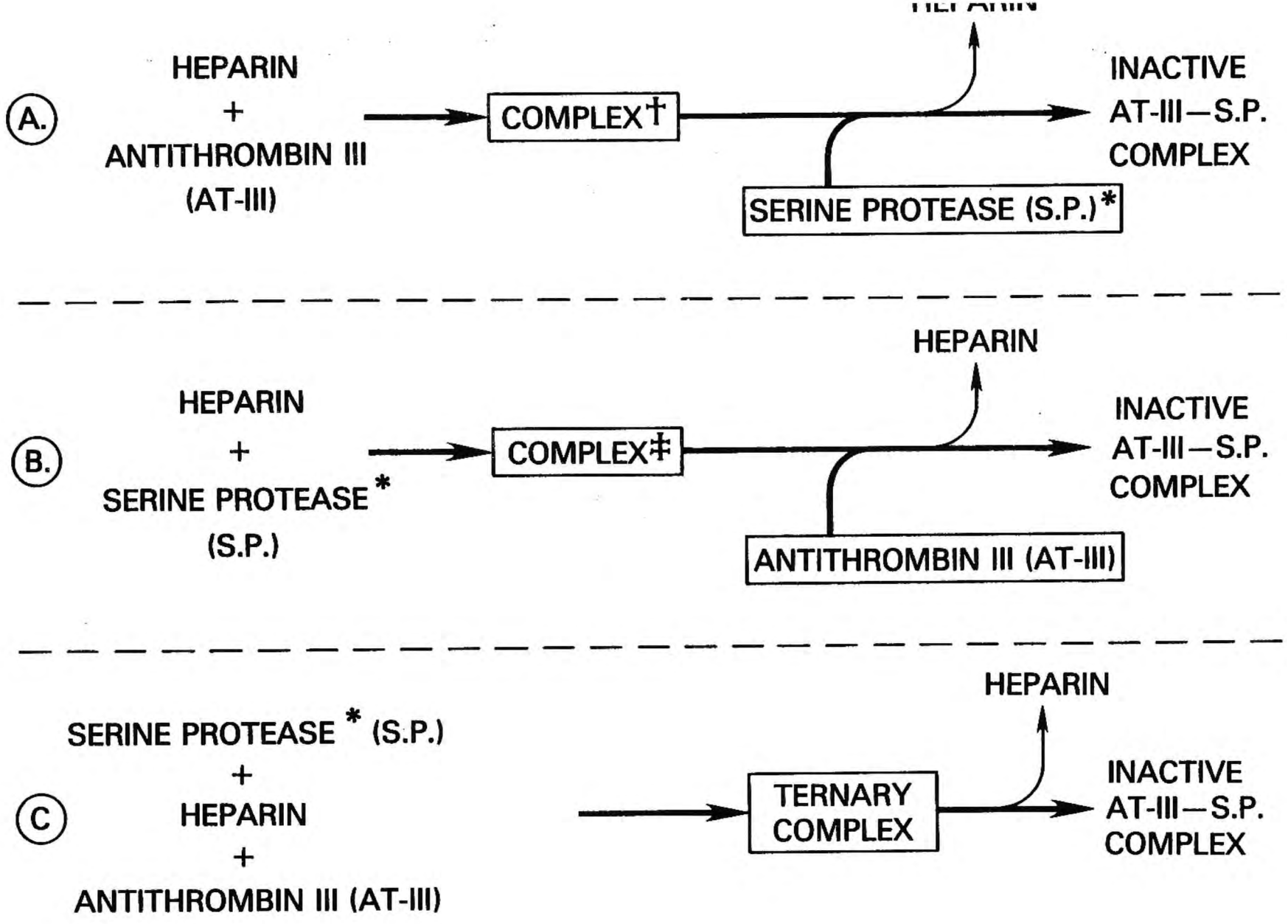
Thus, two seemingly opposite models have been proposed describing the nature of heparin's acceleration of the AT-III-thrombin interaction. The relative importance of either and/or both, in vivo, remains to be determined. Figure 2 summarizes the proposed mechanisms. In addition, a third hypothesis is illustrated. This depicts heparin as a "catalyst" which acts simultaneously on the enzyme and the inhibitor in a fashion which accelerates the formation of an irreversible complex. Once the complex is formed, the heparin is released and is free to mediate another sequence in complex formation.

D. AT-III DEFICIENCIES - CONGENITAL AND ACQUIRED

Congenital AT-III deficiency was first described in 1965 (42). Since then, a large number of families with this deficiency have been documented (43,44). The pattern of heredity is autosomal dominant and, so far, only

Figure 2

Proposed models (A, B, C) for the inhibition of serine proteases by antithrombin-III in the presence of heparin (6). Heparin either modifies (allosterically) the antithrombin-III molecule (A) or the serine protease (B) or acts catalytically by mediating complexing of the inhibitor with the enzyme, forming a ternary complex (C). Most likely, in vivo, mechanism C is operative.



* THROMBIN, KALLIKREIN, PLASMIN, FACTORS IXa, Xa, XIa, XIIa

† STERICALLY MODIFIED AT-III

‡ STERICALLY MODIFIED S.P.

heterozygotes have been identified. Their AT-III levels range between 40 and 65% of normal. Patients with decreased AT-III levels and patients with biologically abnormal AT-III have been described (43). Venous thromboembolism is the most commonly encountered abnormality associated with congenital AT-III deficiency (44).

Acquired AT-III deficiencies have been described (45) in conjunction with a number of disease entities, such as massive deep vein thrombosis, pulmonary embolisms, liver and renal diseases, sepsis, DIC, burns, and shock of various etiology (1,2,11,45,46,47,48). It is assumed that this decrease is due to "intravascular consumption" (45) and thus is not only a valuable tool in diagnosis (45), but apparently of prognostic value, especially in patients with septic shock (11).

E. THERAPEUTIC EXPERIENCE WITH PURIFIED HUMAN AT-III

In 789 patients with septic shock, Lammle (11) found that AT-III levels less than 70% were associated with a 90% mortality, whereas AT-III levels of less than 60% were associated with a 100% mortality.

Since AT-III plays a key role in the defense of the organism against DIC, theoretically it should be possible to use purified human AT-III in patients with low levels to influence a potentially disastrous outcome (49). Since this product has been available in Europe for a few years, several investigators have studied it as a potentially useful therapeutic agent for a variety of clinical conditions associated with acquired decreases in AT-III. Most of these clinical studies have been uncontrolled. A number of reports describe the "successful" use of purified AT-III in patients with acute liver failure (50,51), severe preeclampsia and DIC (52), postpartum hemolytic uremic syndrome (53), and in infants with respiratory distress syndrome (54). Also, patients with severe renal failure, with or without

DIC, whose hemodialysis was jeopardized by low AT-III levels, could be dialyzed again after AT-III infusions (55). In another series of studies, patients with DIC of various etiology were treated with purified AT-III (47,56-59), with the response being "generally favorable," although most patients were treated with other therapeutic modalities as well, rendering evaluation of therapeutic efficacy essentially impossible.

In animal models, purified human AT-III has been studied in an endotoxin shock dog and rabbit model with promising results (60-62). In the rabbit, human AT-III decreased mortality, although little changes were noted in the animals' endogenous AT-III levels (62). Also, improved results were described with a burn model (rabbits) when heparin and purified AT-III were jointly administered (63).

In a limited series, Mammen and others (64) documented increased survival of dogs (in which DIC was induced by the infusion of lactic acid) when purified human AT-III was infused intravenously. Other investigators have documented decreased levels of AT-III in dogs, consequent to various forms of injury (65-67).

Based on the limited clinical (uncontrolled) experience in humans, and on the paucity of data generated in animal models (none utilizing specie-specific material), it is evident that a clear need exists for further development of (a) appropriate animal models and (b) the purification and biochemical characterization of specie-specific AT-III to render results of therapeutic studies interpretable.

II. SPECIFIC AIMS

The purpose of this project was to isolate and biochemically characterize canine AT-III. Specifically:

1. Purification from canine plasma in high yields, utilizing a minimum of maneuvers and standard chromatographic procedures involving ion exchange and affinity matrices as practiced in, or similar to, the isolation of the human analogue.

2. Characterization in terms of (a) yield, (b) specific activity (biologic and immunochemical), (c) stability, (d) general mechanism of action: AT-III-thrombin complex formation in the presence and absence of heparin, (e) amino acid analysis, (f) partial amino acid sequence, and (g) molecular size.

In its pure form, canine AT-III could be used in controlled specie-specific studies to assess its therapeutic usefulness in acute DIC in one or more of the models described (33,64-67). In turn, these animal studies would serve as a basis for future limited but controlled human studies in which the therapeutic efficacy of purified human AT-III could be assessed in a controlled, randomized, double-blind mode.

III. METHODS AND MATERIALS

All reagents, unless specifically qualified, were analytical grade. Also, all containers, unless specifically stated, were plasticware.

A. PREPARATION OF CANINE AT-III

1. Affinity Matrix and Buffer Systems

Immobilized heparin (heparin-conjugated Sepharose, CL-6B, Pharmacia Fine Chemicals) served as the affinity matrix in these studies. Seven buffer systems were explored for their ability to serve as equilibrating, washing and eluting media.

a. Buffers

Buffer I = TRIS=0.05M, sodium citrate=0.01M, NaCl=0.15M, pH=8.5

Buffer II = TRIS=0.10M, sodium citrate=0.01M, NaCl=0.15M, pH=7.4

Buffer III = TRIS=0.10M, sodium citrate=0.01M, NaCl=2.0M, pH=7.4

Buffer IV = TRIS=0.05M, sodium citrate=0.01M, NaCl=0.43M, pH=7.4

Buffer V = TRIS=0.05M, sodium citrate=0.01M, NaCl=0.71M, pH=7.4

Buffer VI = TRIS=0.05M, sodium citrate=0.01M, NaCl=1.07M, pH=7.4

Buffer VII = TRIS=0.05M, sodium citrate=0.01M, NaCl=3.00M, pH=7.4

b. Resin Preparation/Equilibration

In a syntered glass funnel, 500 ml of settled resin were rinsed four times with 500 ml distilled water, four times with 500 ml 2.0M NaCl, and four

times with 500 ml distilled water. The resin was then washed twice with 500 ml of Buffer I and filtered. The matrix was suspended in 300 ml of Buffer I, stirred and stored at 4°C until ready for use.

2. Collection of Blood/Plasma

All blood collected for study, regardless of specie, was collected in buffered sodium citrate solutions. It was centrifuged to remove cellular components, the plasma siphoned off and stored in plastic containers at -70°C. Samples for study (whether analytical or preparative) were thawed only once.

Canine pooled blood was procured through the National Institutes of Health, Poolesville, Maryland, animal farm. Each 450 ml of blood was collected with 67.5 ml of the anticoagulant, citrate dextrose (ACD solution). Each 67.5 ml of ACD contained 540 mg citric acid (hydrous), U.S.P., 1.49 g sodium citrate (hydrous), U.S.P., and 1.65 g dextrose (hydrous), U.S.P. The blood was centrifuged at 4000 x g for 30 minutes at 4°C.

3. Defibrinogenation of Plasma

In preparation for affinity chromatography, plasma was defibrinogenated with the thrombin-like enzyme Reptilase.* With stirring, the enzyme was added to plasma at a concentration of 0.5 units (thrombin-like)/ml. The mixture was incubated at 37°C with continuous stirring for 60 minutes, at which time the fibrin clot was removed, placed in gauze and all entrapped liquid expressed and collected in a plastic container.

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*This enzyme is isolated from Mayalan pit viper (Bothrops Atrox) venom (68). It has the unique properties of converting fibrinogen to fibrin, by removing fibrinopeptide A only (and not B). Furthermore, it is not inhibited by AT-III, for example, it does not complex with it.

4. Affinity Chromatography

In preparation for large-scale purification, a number of experiments (batch style) were performed to establish optimal conditions (i.e., ionic strength of buffers, complexing time, matrix/plasma ratios, influence of high salt concentrations on test reagents, etc.) for best yields and quality of product. Once these conditions were selected, all subsequent preparative procedures were executed in two major steps: (a) a batch adsorption/elution step and (b) a gradient chromatography step. For the most part, volumes of about 500 ml of plasma were processed for each complete run.

a. Product I

° **Batch adsorption and elution**

A matrix/plasma (optimal) ratio of 1:10 was used in this step of purification. The mixture was stirred gently at 22°C for 30 minutes (optimal complexing time), centrifuged at 1000 x g for 6 minutes, and the resin cake washed three times with an equivalent volume of Buffer I. It was subsequently reequilibrated in Buffer II (three times resin volume). The product (AT-III) was batch eluted with Buffer VII (three times resin volume).

° **Dialysis**

Antithrombin-III (AT-III) solutions in Buffer VII were placed in Visking Tubing dialysis membranes and dialyzed overnight at 4°C against 20 volumes of Buffer I. This material was designated: Product I. This procedure is essential for the removal of the high salt in preparation for re-adsorption on immobilized heparin.

b. Product II

° **Adsorption on Immobilized Heparin**

Product I was mixed with the matrix (immobilized heparin) at a ratio of 1:10, stirred at 22°C for one hour and the entire slurry poured into a glass column (5 cm in diameter) containing a volume of matrix equivalent to the initial amount.

° **Chromatography**

The settled matrix was washed with one column volume of Buffer I and three column volumes of Buffer II. The column was developed with a linear NaCl gradient generated automatically with an LKB 11300 Ultrograd gradient mixer, with a 16-hour scanning rate, mixing the equilibrating Buffer II with the limiting Buffer VII. Elution was executed at a flow rate of 1 ml/min. Fractions of 10 ml were collected using an LKB Ultrorac fraction collector, and monitored automatically at 280 nm with an LKB 8300 Uvicord II. Once AT-III activity was identified (by assaying every fifth fraction), appropriate peaks were pooled, dialyzed against 20 volumes of Buffer I, concentrated by ultrafiltration using a PM-10 semipermeable membrane (Amicon Company), and frozen in aliquots at -70°C. This material was designated Product II.

B. ANALYTICAL PROCEDURES

1. Biologic Activity of AT-III

Determination of the biologic activity of canine AT-III was done, according to the method of Bick, et al. (12), using commercially available reagents. In principle, this assay procedure is configured as follows: A precise quantity of AT-III (using normal, pooled, defibrinogenated plasma as a source) is incubated at 37°C for a defined period of time with a precise quantity of purified thrombin. At the end of this incubation period, an aliquot of this incubate is

removed, added to purified fibrinogen at 37°C, and the clotting time recorded. To generate a standard curve, correlating clotting time with AT-III concentration, the plasma (serving as the source for AT-III) is serially diluted in a neutral buffer, containing heparin at 3 units/ml. Generally, four dilutions are prepared (1:60, 1:40, 1:30, and 1:24). The clotting time is directly correlated to the residual (unneutralized) thrombin in the incubate. One unit of activity is defined by international standards as that amount of activity contained in one ml of normal pooled plasma. In the assay procedure described, the 1:30 dilution is operationally defined as the 100% value (AT-III activity). This procedure was used to assess plasma concentrations, determine yields during purification and determine stability of the purified product. In most instances, the sample in question was assayed at a dilution of 1:30 (appropriately prediluted if necessary) and values interpolated from the standard curve, which was prepared fresh on each work day. In those instances where the activity was (suspected) to be less than one unit/ml, the sample was diluted less than 1:30 (for example, 1:10 or 1:20) to insure that the clotting time generated fell within the linear portion of the curve.

2. Protein Determination

The protein concentration of various purified fractions was spectrophotometrically determined by routine absorbancy measurements at 280 nm, utilizing a value of 7.72 as the absorbance coefficient. The latter, defined as the optical density (OD) value of a 1 g/dl solution at 280 nm, was determined by analyzing solutions (in 0.1 M Tris-chloride, pH 7.6 buffer), the concentrations of which had been previously determined by differential refractometry at 546 nm, with a value of 0.186 ml/g for dn/dc (refractive index increment) (69).

3. Immunodiffusion

Immunodiffusion tests were performed by the method of Ouchterlony (70) using a 1% agarose matrix. Volumes of samples tested were limited to 7 ul/well. Plates were incubated overnight in a moisture chamber, and dried by blotting with absorbant filter paper. Precipitin arcs were visualized by staining for two hours at ambient temperature with the following solution: 0.5% Coomassie Brilliant Blue, 45% ethanol, 10% glacial acetic acid. Destaining was accomplished by allowing each gel to remain for 72 hours in the following solution: 45% ethanol, 10% glacial acetic acid.

4. Radial Immunodiffusion

Immunologic quantitation of AT-III in normal dog plasma was assessed by radial immunodiffusion as described by Mancini, et al. (71). In this technique, one measures the equilibrium point of an antigen-antibody reaction. This point is visualized by a circular precipitin line, the diameter of which is dependent on the concentration of the antigen being tested. In all instances, various dilutions of the sample being tested are placed in a well and allowed to diffuse into the matrix which contains the antibody. Agar templates (1% solutions, SEAKEM Agarose, in 0.1M sodium-barbital buffer, pH 8.6) were prepared by mixing goat antidog AT-III serum (see METHODS AND MATERIALS) at a serum/agar ratio of 0.8/10. Liquid agar was allowed to cool to 45°C prior to the addition of serum, poured into templates, allowed to cool at 4°C, and stored overnight prior to punching 2 mm wells. Samples (10 ul) to be analyzed were applied to each well, and allowed to diffuse into the antibody - containing matrix for 48 hours at 4°C in a moisture chamber. The readily visible precipitin circles were measured and recorded. A standard curve correlating diameter squared $(d)^2$ (precipitin diameter) with

concentration of purified AT-III was prepared and used to interpolate values from normal (unknown) plasma.

5. Crossed Immunoelectrophoresis (C.I.E.)

Crossed immunoelectrophoresis (C.I.E.) involves the electrophoretic separation (migration) of a protein(s) in an agarose matrix, followed by a second electrophoresis, perpendicular to the first dimension. In the second electrophoresis, the matrix is impregnated with antiserum, thereby permitting the visualization of a precipitin arc (peak), the size, homogeneity, and sharpness of which are dependent on the purity and concentration of the antigens.

Crossed immunoelectrophoresis (C.I.E.) was performed by the procedure of Sas, et al. (72). A 1% Agarose Solution (Medium Electroendosmosis, Marine Colloids, Inc.), heated to 56°C, was poured onto a glass plate (90 x 110 x 1.5 mm). Seven microliters (7 ul) of canine plasma were applied in each well. Electrophoresis in the first dimension was run at 8 v/cm for 60 minutes. The segment of Agarose not containing electrophoresed sample was discarded and replaced by 10 ml of 1% Agarose containing 100 ul of goat antidog AT-III serum. Electrophoresis in the second dimension was performed at 15 v/cm for four hours perpendicular to the direction of the first run. Plates were washed in 0.1 M NaCl overnight, dried, and stained with 0.5% Coomassie Brilliant Blue, 45% ethanol, and 10% glacial acetic acid and destained with 45% ethanol and 10% glacial acetic acid.

6. Immuno-Blotting

Immuno-blotting was used to detect potential cross-reactivities of antisera to human, rabbit, and canine AT-III. In principle, this technique involves (a) electrophoresis of the antigen in polyacrylamide gels; (b) transferal of the migrated protein to nitrocellulose; (c) reaction of the migrated antigen with

specific (homologous or heterologous) antiserum; and (d) visualization of the interaction (Ag-Ab Complex) by reaction with a second antibody, conjugated with an enzyme which will generate a specific color with a specific low molecular weight substrate.

The immuno-blot method of Mondrell and Zollinger (73) was used. Electrophoresis of AT-III antigen (human piasma, rabbit plasma, canine plasma), transfer to nitrocellulose membrane, complexing with specific (first) antibody (goat antidog/human/rabbit AT-III), complexing with second antibody (rabbit antigoat IgG, coupled to horseradish peroxidase (HRP)*), and color development were accomplished as per manufacturers' recommendations using a commercially available kit (Bio-Rad Immuno-Blot Protein A Horseradish Peroxidase - HRP - Conjugate).

7. Polyacrylamide Gel Electrophoresis (PAGE)

Purity of various AT-III fractions, estimation of the molecular size, and complex formation, were assessed by the sodium dodecyl sulfate (SDS-PAGE) system described by Weber and Osborn (74). In this system, samples applied to polyacrylamide gel matrices (60 ug) were electrophoresed in a buffer system containing an anionic detergent (SDS) thereby negating the net electrical charge on each protein. As a consequence, each protein migrates to the anode, and the rate of migration is logarithmically related to the molecular size. Gels were stained with 0.5% Coomassie Brilliant Blue, 45% ethanol, 10% glacial acetic acid and destained with 45% ethanol, 10% glacial acetic acid.

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*Compliments of Dr. S. L. Chung.

8. High Resolution Liquid Chromatography (HPLC)

To confirm the purity of the AT-III products prepared by the described procedure, to document the formation of complexes (with and without heparin) and to prepare fractions of ultrahigh purity in preparation for amino acid composition and sequencing analysis, a high resolution chromatographic system (75,76) utilizing a TSK-3000 column, instrumented in an automated Beckman 450 data system (controller) was used. In this system, the principle of separation of multiple components is based on molecular size, as the matrix is inert and permits molecular filtration in a fashion similar to that exhibited to matrices such as Sephadex.

Flow rates were fixed at 0.5 ml/min. Samples were applied in either 0.02 M ammonium acetate, pH 7.4 or in Tris-chloride, 0.1M, pH 7.4 and eluted in the same buffer. Profiles were monitored by automated measurements at 280 nm.

9. Amino Acid Analysis

Samples to be analyzed for amino acid composition were obtained by the HPLC preparation procedure (see METHODS AND MATERIALS), lyophilized, resuspended in 6N HCl and hydrolyzed in a nitrogen atmosphere at 110°C for 22 hours. Hydrolysates (approximately 1 n mole of protein) were analyzed (77) on a Beckman System 6300 (High Performance Analyzer).

10. Amino Acid Sequencing

Samples to be analyzed for primary structure (partial N-Terminal) were prepared by HPLC (see METHODS AND MATERIALS). Sequence analysis was based on the chemical process originated by Edman (78,79) utilizing an automated system (Applied Biosystems, Model 470A Gas Phase Sequencer). Briefly, in this system, approximately 1 n mole of intact protein is applied. Each N-Terminal

amino acid is coupled with phenylisothiocyanate, cleaved, harvested and the anilinothiazolinone derivative converted to the more stable thiohydantoin derivative with trifluoroacetic acid. At the end of each cycle, the hydantoin derivative is automatically transferred to an on-line reverse phase HPLC (Applied Biosystems, Model 120A) for quantitative analysis and identification - the latter accomplished by comparing elution profiles to a series of standards. All procedures were executed as per manufacturer's recommendations.

C. STABILITY STUDIES

The stability of purified canine AT-III was tested at four temperatures: -70°C, -40°C, 4°C, 22°C, at prescribed time intervals, over the course of three months. Residual biologic activity (12) was determined (see METHODS AND MATERIALS).

D. PREPARATION OF ANTISERA (ANTI-AT-III)

In preparation for studies to examine the potential cross-reactivites of antisera against purified AT-III from canine, human, and rabbit plasma, appropriate sera were prepared. Antithrombin-III (AT-III) (canine, human, rabbit) specific antiserum was obtained from goats subsequent to repeated weekly intradermal injections of 0.5 mg specific antigen (1:1 with complete Freunds adjuvant). At the end of four weeks (after the fourth injection), animals were plasmapheresed, serum prepared by recalcification (by adding calcium chloride to a final concentration of 25 mmolar) and stored in aliquots at -70°C.

IV. EXPERIMENTAL RESULTS

A. STANDARDIZATION OF AT-III (BIOLOGIC ACTIVITY) ASSAY

In order to establish a degree of confidence in the assay system described (12), a number of assays were performed (standard curves generated) establishing acceptable variations (within day and day to day). Tables II and III summarize these data.

B. PURIFICATION OF CANINE AT-III

1. Preliminary Studies

In preparation for large-scale purification, a number of studies designed to document the efficiency and economy of the proposed isolation procedure were performed. Data representing the rate of AT-III adsorption onto immobilized heparin at various plasma/matrix ratios, and the efficiency of batch AT-III elution at varying buffer ionic strengths are summarized in Figures 3 and 4. It is apparent (Figure 3) that, while canine AT-III (activity) binds in 120 minutes at all matrix/plasma ratios tested, a ratio of 1:10 represents a practical combination with respect to economy of material and time. It was chosen for the large-scale studies and, indeed, proved successful and reproducible.

In Figure 4, the recovery profile indicates that, once bound, canine AT-III remains firmly attached to the matrix and that buffers of substantial ionic strength (see METHODS AND MATERIALS) are required to elute it. The differential elution profile with Buffers III and VII may reflect heterogeneous populations of AT-III molecules or heparin molecules (or both) with varying mutual affinities. To insure maximum yields of AT-III from the affinity matrix, Buffer VII was chosen as the limiting gradient buffer for the large-scale preparative work.

TABLE II

WITHIN DAY VARIATIONS OF AT-III ASSAY

(n = 6)

Standard	Mean Clotting Time	S.D.	Var.
Blank	14.9	0.25	0.06
50% AT-III	19.4	0.14	0.04
75% AT-III	23.9	0.70	0.49
100% AT-III	30.1	1.51	2.27
125% AT-III	39.6	1.73	3.01

TABLE III

DAY TO DAY VARIATIONS OF AT-III ASSAY

(n = 10)

Standard	Mean Clotting Time	S.D.	Var.
Blank	14.2	0.92	0.76
50% AT-III	19.2	2.09	3.96
75% AT-III	27.7	1.40	1.80
100% AT-III	32.3	1.50	2.20
125% AT-III	38.6	1.80	3.00

Figure 3

Rate of adsorption of canine AT-III activity onto heparin-Sepharose at the volume ratios indicated in insert. Plasma from various pools was added to the appropriate volume of resin and stirred gently at ambient temperature. At convenient time intervals, samples were centrifuged (5,000 x g) for 1 minute, and the fluid phase was sampled for residual biologic activity (12) (see METHODS AND MATERIALS). All samples were tested at one single dilution, 1:30, which is defined as the 100% value in the assay system described (see METHODS AND MATERIALS).

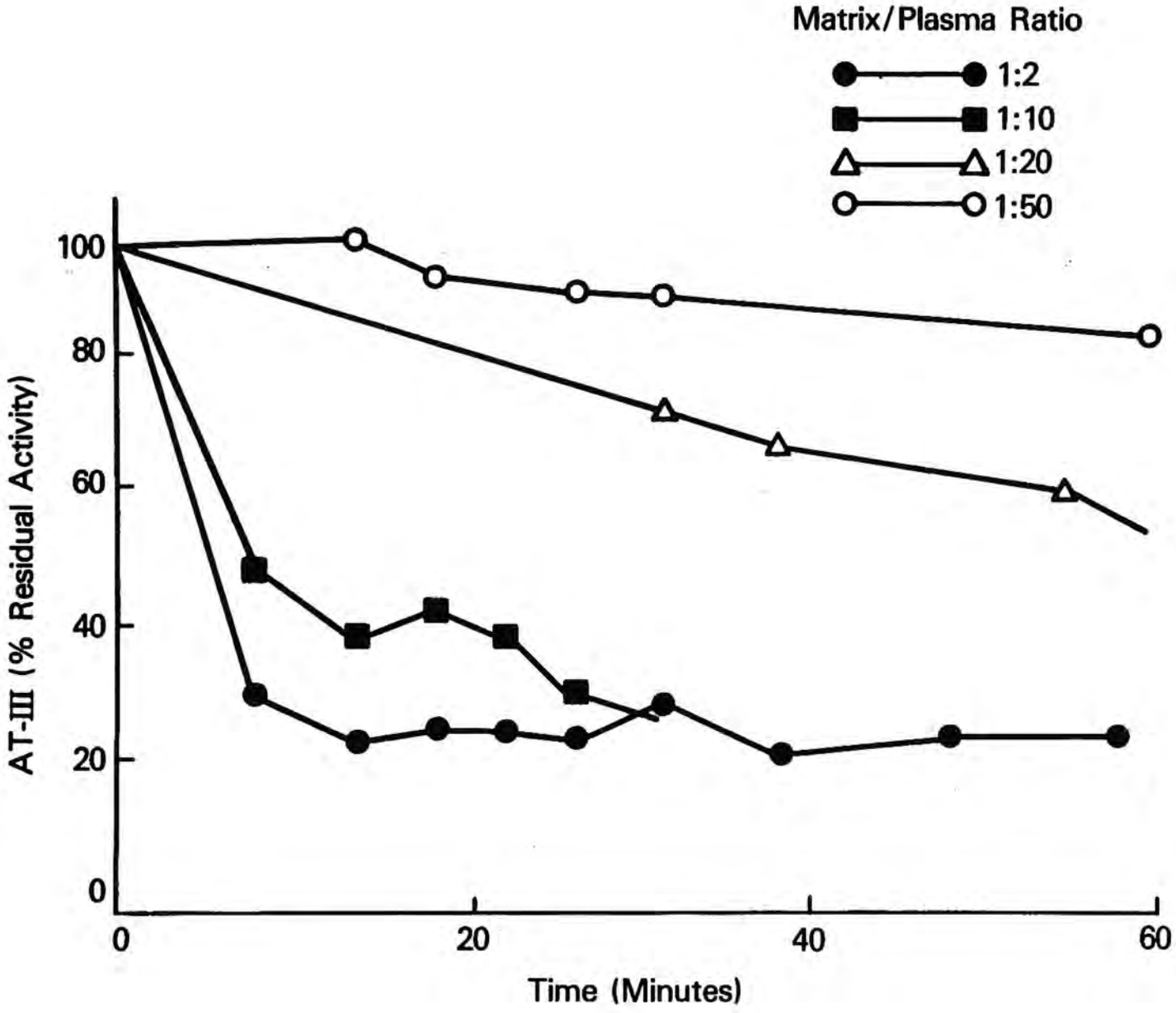
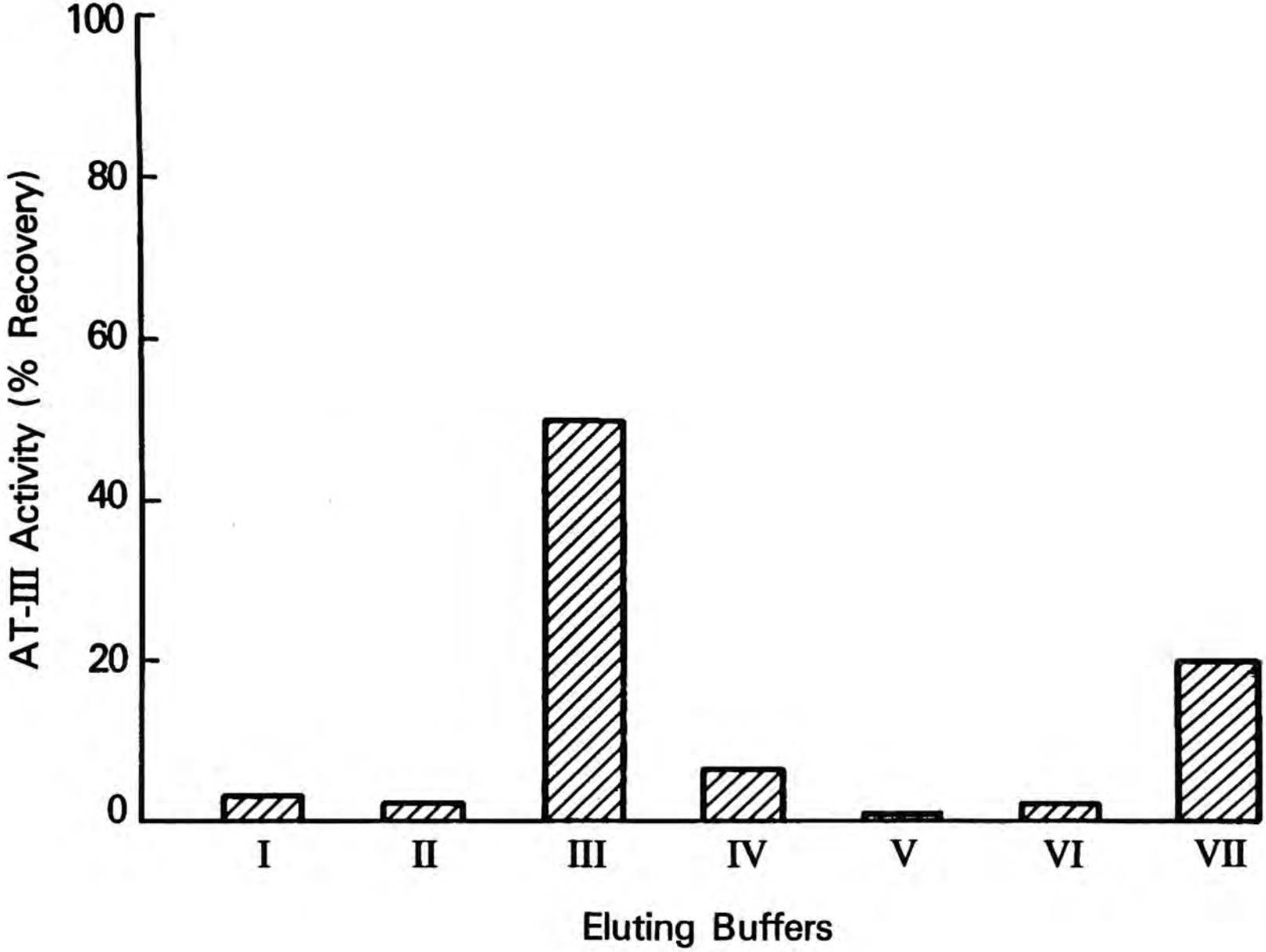

Matrix/Plasma Ratio
1:2
1:10
1:20
1:50
AT-III (% Residual Activity)
100
80
60
40
20
0
0
20
40
60
Time (Minutes)

Figure 4

Recovery of canine AT-III activity from heparin-Sepharose with buffers at varying ionic strengths (see METHODS AND MATERIALS). The matrix containing bound AT-III was washed with three volumes (in sequence) of respective buffer, centrifigued at 5000 x g for 1 minute, and the supernatant assayed for AT-III biologic activity as described (12). Appropriate control studies were performed to ascertain that the high salt concentrations did not influence the assay procedure. Also, to insure sensitivity, special precautions were taken to dilute the test sample(s) less than 1:30 during testing. Yields (% recovery) are plotted as the mean of four studies. SEM values for yields obtained with Buffer I through VII were: 2.2, 2.0, 5.0, 1.0, 0.3, 0.8 and 4.0, respectively.


100
80
60
40
20
0
AT-III Activity (% Recovery)
I
II
III
IV
V
VI
VII
Eluting Buffers

2. Large-Scale Preparative Studies

Figure 5 summarizes the purification procedure. The recovery of biologically active canine AT-III from normal (pooled) canine plasma is summarized in Tables IV, V, and VI. Based on a series of seven preparations of Product-I and two pooled preparations of Product-II, a product of high purity could be prepared with an average yield of about 28%.

Figure 6 illustrates a typical chromatographic profile of Product-I eluted from heparin-Sepharose as Product-II with the salt gradient indicated. In accord with data obtained in preliminary studies, canine AT-III binds strongly to the matrix (as evidenced by the late elution) and elutes as a polydisperse protein peak. Biologic activity measurements reveal the presence of multiple activity peaks (Fractions II, III, and IV), with similar specific activities and electrophoretic (SDS-PAGE) profiles.

C. BIOCHEMICAL/BIOPHYSICAL/IMMUNOLOGIC CHARACTERIZATION OF CANINE AT-III

Unless otherwise indicated, Fraction II or Fraction III (Figure 6) served as the primary source for biochemical and biophysical characterization.

1. Absorbance Coefficient

The absorbance coefficient of canine AT-III (defined as: $A^{1\%}_{280\ nm}$) was determined to be 7.72. It was obtained by determining the optical density (OD) value of a solution (previously determined to have a concentration of 8.29 mg/ml). The following values were used for the calculations:

Figure 5

Summary of purification procedure for canine AT-III from citrated plasma.

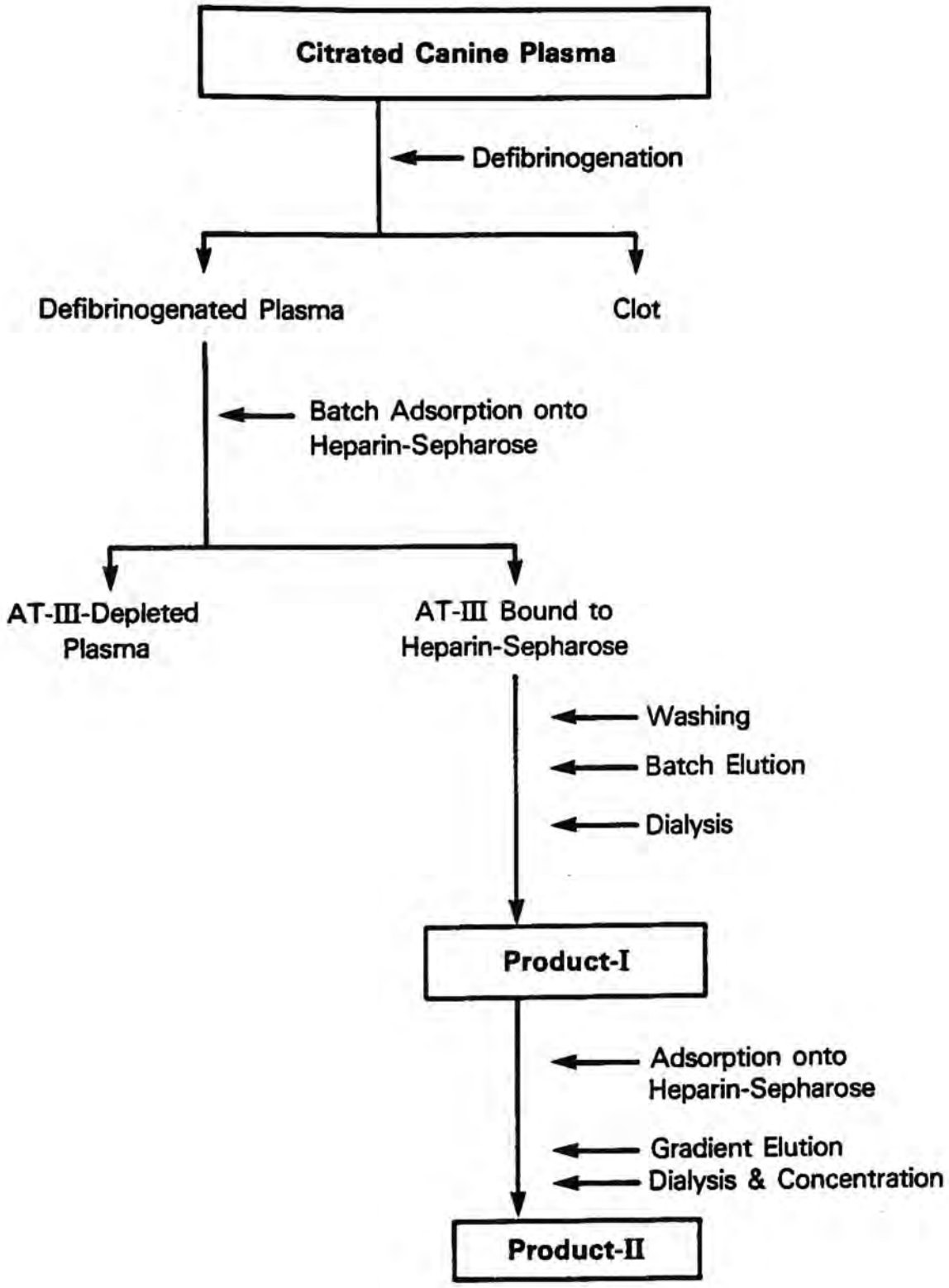

Citrated Canine Plasma
Defibrinogenation
Defibrinogenated Plasma
Clot
Batch Adsorption onto
Heparin-Sepharose
AT-III-Depleted
Plasma
AT-III Bound to
Heparin-Sepharose
Washing
Batch Elution
Dialysis
Product-I
Adsorption onto
Heparin-Sepharose
Gradient Elution
Dialysis & Concentration
Product-II

TABLE IV

PURIFICATION OF CANINE AT-III. PRODUCT-I YIELDS FOR SEVEN PREPARATIONS

		Starting Plasma (defib.)				Product-I				
	Preparation	Vol.	U/ml	Total Units	Expected Yield (%)#	Vol.	U/ml	Total Units	Yield (%)+	
	1	250	1.07	268	107	370	0.50	185	69	
	2	470	1.23	578	123	317	0.68	216	37	
	3	563	1.24	698	124	275	0.96	264	38	Pool*
	4	500	1.34	670	134	245	1.10	269	40	
Average		446	1.22	544	122	302	0.81	233	46	
	5	500	1.04	520	104	295	1.24	366	70	
	6	500	1.34	670	134	295	1.10	324	48	Pool**
	7	500	1.27	635	127	280	1.65	462	73	
Average		500	1.22	608	122	290	1.33	384	64	

Based on the definition that 1 unit is that amount contained in 1 ml of normal plasma.
\+ Relative to starting (expected yield) plasma.
* This pool served as source for Product-II (Study 1).
** This pool served as source for Product-II (Study 2).

TABLE V

PURIFICATION OF CANINE AT-III.
PRODUCT-II YIELDS FOR VARIOUS FRACTIONS IN TWO STUDIES

	Product	Total Volume (ml)	Units/ml	Total Units		Yield %
	Starting Plasma	1783	1.21	2157		121#
	Product-I	1207	0.77	929		43+
	Product-II					
STUDY 1	° Fraction I	70	0.45	31		1.4+
	° Fraction II	115	2.14	246		11.3+
	° Fraction III	63	2.04	128		6.0+
	° Fraction IV	142	0.70	99		4.5+
			Total	473	Total	21.8+
	Starting Plasma	1500	1.22	1830		122#
	Product-I	870	1.32	1148		63+
	Product-II					
STUDY 2	° Fraction I	107	0.27	29		1.5+
	° Fraction II	125	2.30	287		15.7+
	° Fraction III	65	2.54	165		9.0+
	° Fraction IV	137	1.15	157		8.6+
			Total	609	Total	33.3+

\# Expected yield.
\+ Actual yields relative to starting plasma.

TABLE VI

PURIFICATION OF CANINE AT-III.
SUMMARY OF PRODUCT-I AND PRODUCT-II YIELDS

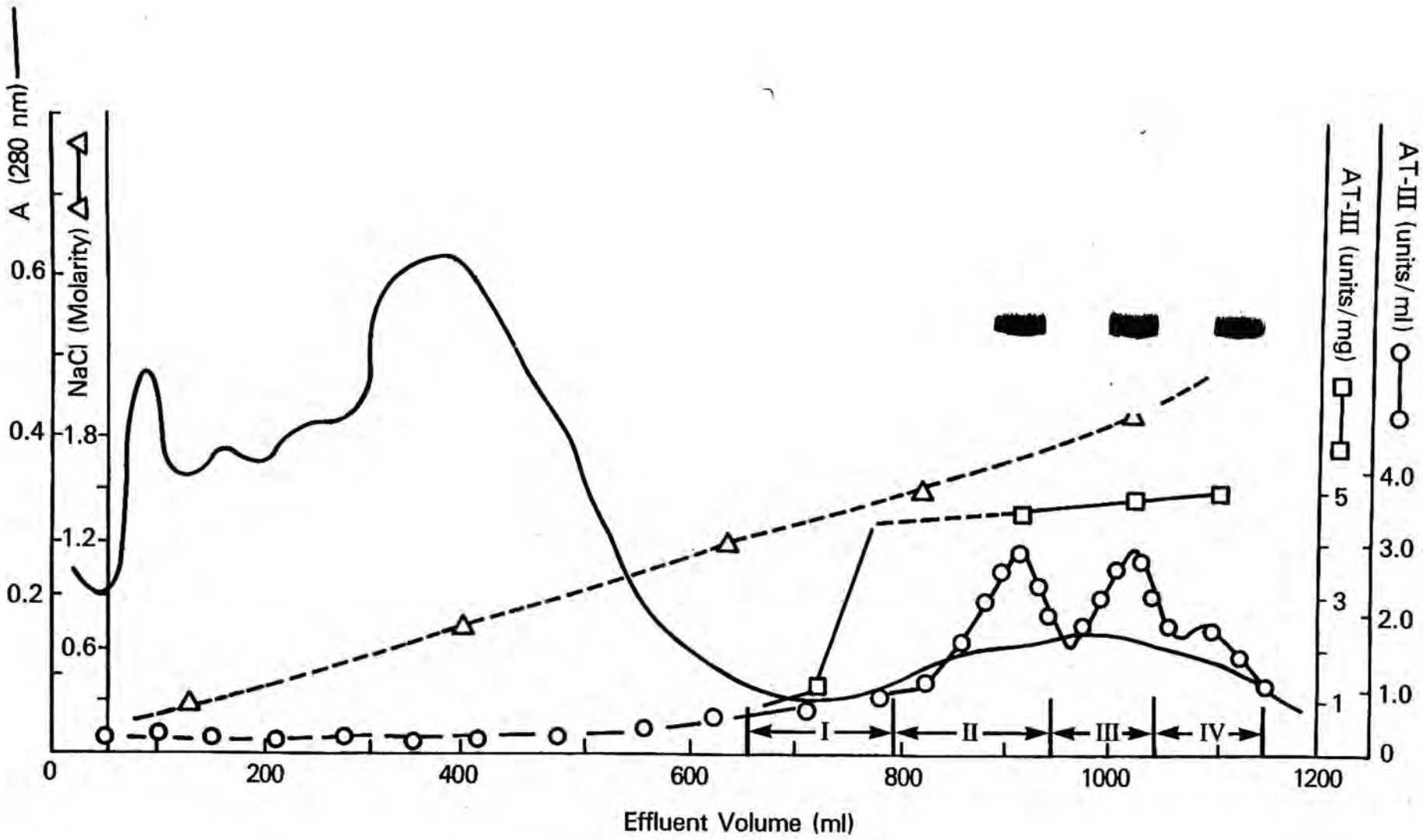
(N = 7)

	Product-I	Product-II*	
	Relative to Plasma	Relative to Plasma	Relative to Product-I
Study 1	43%	21.8%	50.7%
Study 2	63%	33.3%	53.0%
Average	53%	27.6%	51.9%

*Pool of Fractions II and III and IV

Figure 6

Affinity chromatography of canine AT-III on heparin-Sepharose. Column: 5 x 9 cm; flow rate: 1.1 ml/min; fractions 10 ml; monitoring: A 280 nm (———); linear gradient: Buffer II - equilibrating, Buffer VII - limiting, with LKB 11300 Ultrograd gradient mixer, at a 16-hour scanning (x axis) rate. The molarity of the sodium chloride creating the gradient is indexed (▲———▲). Fractions were assayed (12) for biologic activity (o———o), expressed as units/ml, according to the method described in METHODS AND MATERIALS, and for specific activity (□———□), expressed as units/mg of protein. SDS-PAGE profiles (74) for pooled Fractions II, III, and IV appear above respective peaks.


A (280 nm)
NaCl (Molarity)
AT-III (units/mg)
AT-III (units/ml)
0.6
0.4
0.2
1.8
1.2
0.6
5
3
1
4.0
3.0
2.0
1.0
0
I
II
III
IV
0
200
400
600
800
1000
1200
Effluent Volume (ml)

Protein concentration by refractometry = 8.29 mg/ml

O.D. value at 280 nm (1:10 dilution) = 0.640.

$$\frac{8.29 \text{ mg/ml}}{0.640 \times 10} = \frac{10 \text{ mg/ml}}{X}$$

$$X = 7.72$$

This value served to calculate protein concentrations of all fractions of interest throughout these studies, by recording absorbancy values at 280 nm.

2. Polyacrylamide Gel Electrophoresis (PAGE) in the Presence of Sodium Dodecyl Sulfate (SDS)

a. **Assessment of Purity and Molecular Size**

Assessment of purity and estimation of molecular size of various products were done by SDS-PAGE (74). Figure 7 illustrates a comparative profile of the following:

Lane 1	. . .	Canine Fraction II
Lane 2	. . .	Canine Fraction III
Lane 3	. . .	Canine Fraction IV
Lane 4	. . .	Canine AT-III*
Lane 5	. . .	Human AT-III**
Lane 6	. . .	Rabbit AT-III***

It is evident that no obvious differences (neither in purity nor in molecular size) are noted between the various subfractions of the canine product. Also, the human and rabbit product exhibit an apparently identical profile.

---

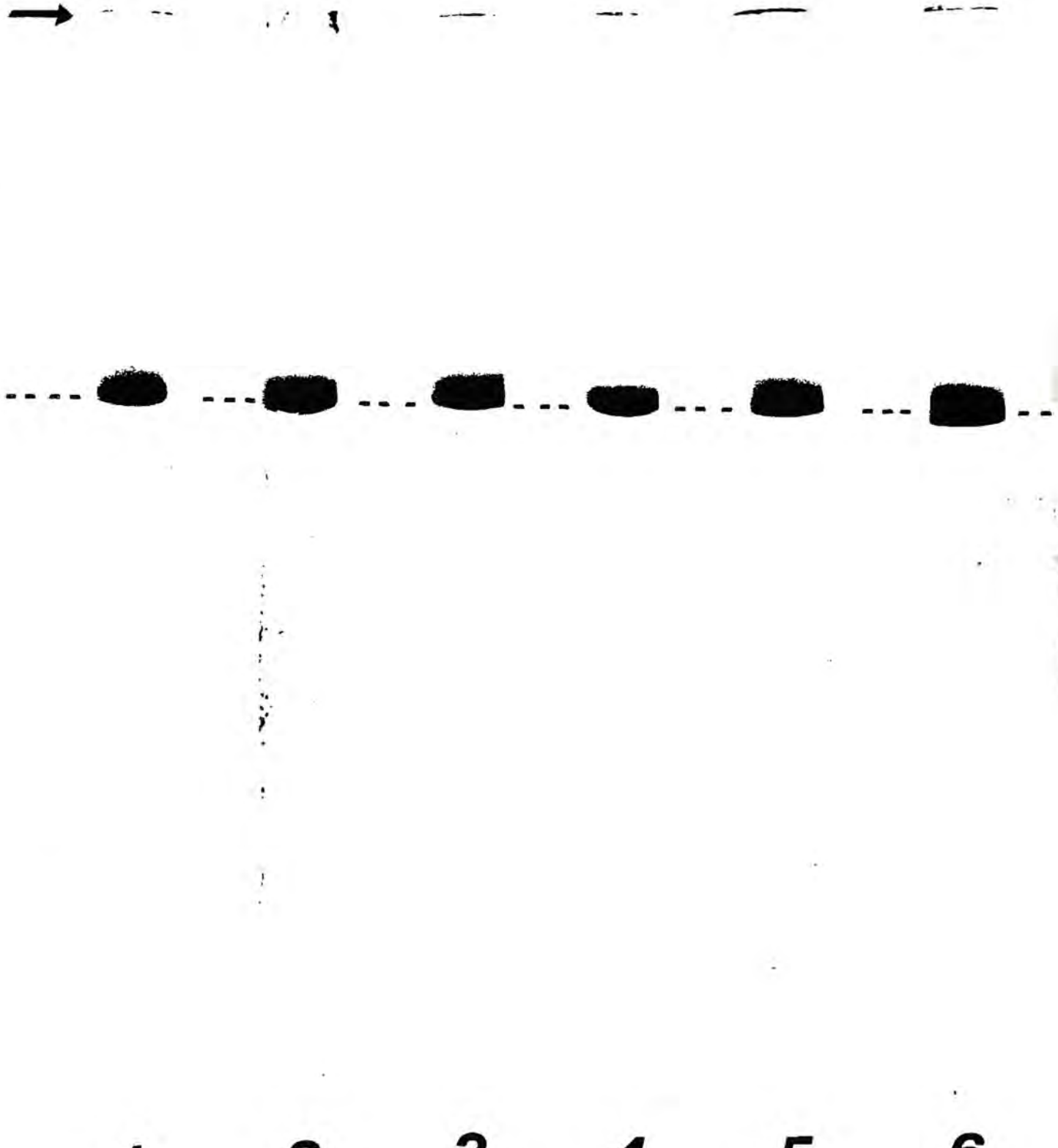
* Prepared from a single donor dog and eluted as one (pooled) fraction.

** Prepared according to Murano (28).

*** Prepared by the method described for the canine product.

Figure 7

Polyacrylamide Gel Electrophoresis in the presence of SDS-PAGE (74). 7.5% gel matrix; pH 7.4. Origin is on top (arrow). Lanes 1, 2, and 3 = Canine AT-III (Fractions II, III, and IV), respectively; Lane 4 = Canine Fractions II and III and IV (pooled) prepared from single donor dog; Lane 5 = Human AT-III (28); Lane 6 = Rabbit AT-III prepared by the method described for the canine product. The estimated molecular size of 68,000 is based on position relative to a series of standards: Ribonuclease, Chymotrypsinogen, Ovalbumin, Albumin, and Aldolase, with respective molecular weights: 14,000; 25,000; 43,000; 68,000; and 158,000. The dashed line serves as a guide for alignment.


1
2
3
4
5
6

b. **Demonstration of AT-III-Thrombin Complex Formation**

SDS-PAGE (74) was also utilized to demonstrate the formation of antithrombin-thrombin complexes. Figure 8 illustrates the following:

Lane 1	. . .	Canine AT-III (Fraction II)
Lane 2	. . .	Human thrombin*
Lane 3	. . .	AT-III and thrombin (equimolar) incubated at 22°C for 2 hours
Lane 4	. . .	AT-III and thrombin (equimolar) incubated at 22°C for 30 seconds in the presence of heparin (3 units/ml)

It is apparent from this profile that purified canine AT-III, when tested for its ability to complex with human thrombin, displays very poor "progressive" inhibitory activity (as evidenced by the lack of complex formation). It displays a powerful "heparin cofactor" activity (similarly to its human analogue) as evidenced by the virtually instantaneous appearance of a high molecular weight (approximately 95,000) complex.

3. High Resolution Liquid Chromatography (HPLC)

a. **Assessment of Purity and Molecular Size**

Assessment of purity and estimation of the molecular size of canine AT-III by HPLC was performed in comparison to purified human and rabbit AT-III. Figure 9 illustrates the elution profiles of the three molecular species. It is evident that, similarly to the patterns observed on SDS-PAGE (Figure 7), the three molecular species elute with a similar estimated molecular size of 68,000 (based on purified human albumin as a standard) and with a high degree of purity.

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* Human thrombin: Specific activity 3,700 u/mg was a gift of Dr. John Fenton. New York State Department of Health, Albany, New York.

Figure 8

Polyacrylamide Gel Electrophoresis in the presence of SDS-PAGE. 7.5% gel matrix; pH 7.4. Origin is on top (arrow) 60 ug samples. Lane 1 = Canine AT-III (AT); Lane 2 = Human thrombin (Thr); Lane 3 = equimolar mixture of canine AT-III and human thrombin incubated at 22°C for 2 hours prior to electrophoresis; Lane 4 = equimolar mixture of canine AT-III and human thrombin, incubated at 22°C for 30 seconds, in the presence of heparin - 3 units/ml - prior to electrophoresis. Estimated molecular sizes of individual protein species and AT-III-thrombin complex (CX) are indexed (- - - - -) based on standards (see Figure 7): CX = 95,000, AT = 68,000, Thr = 37,000.

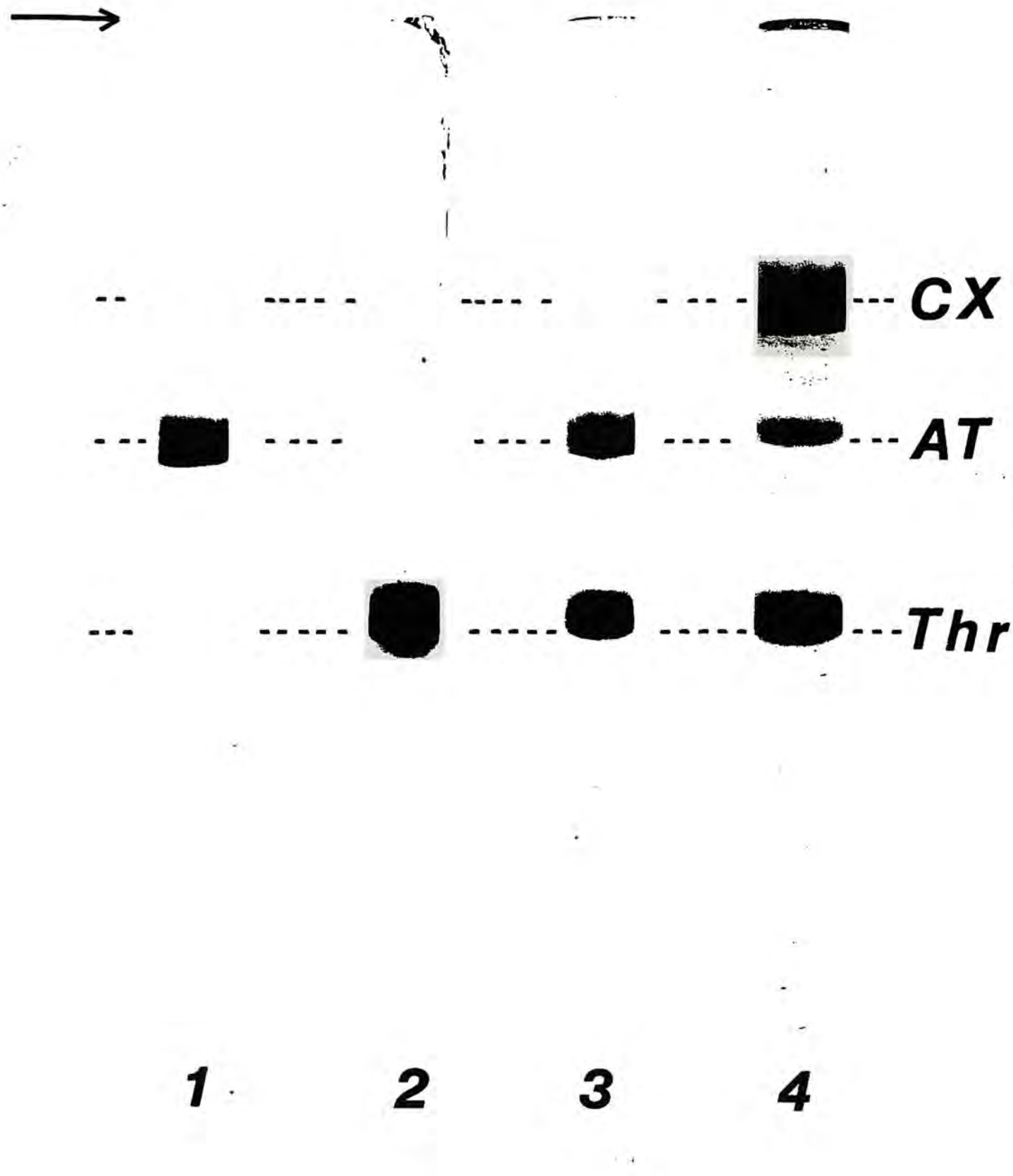
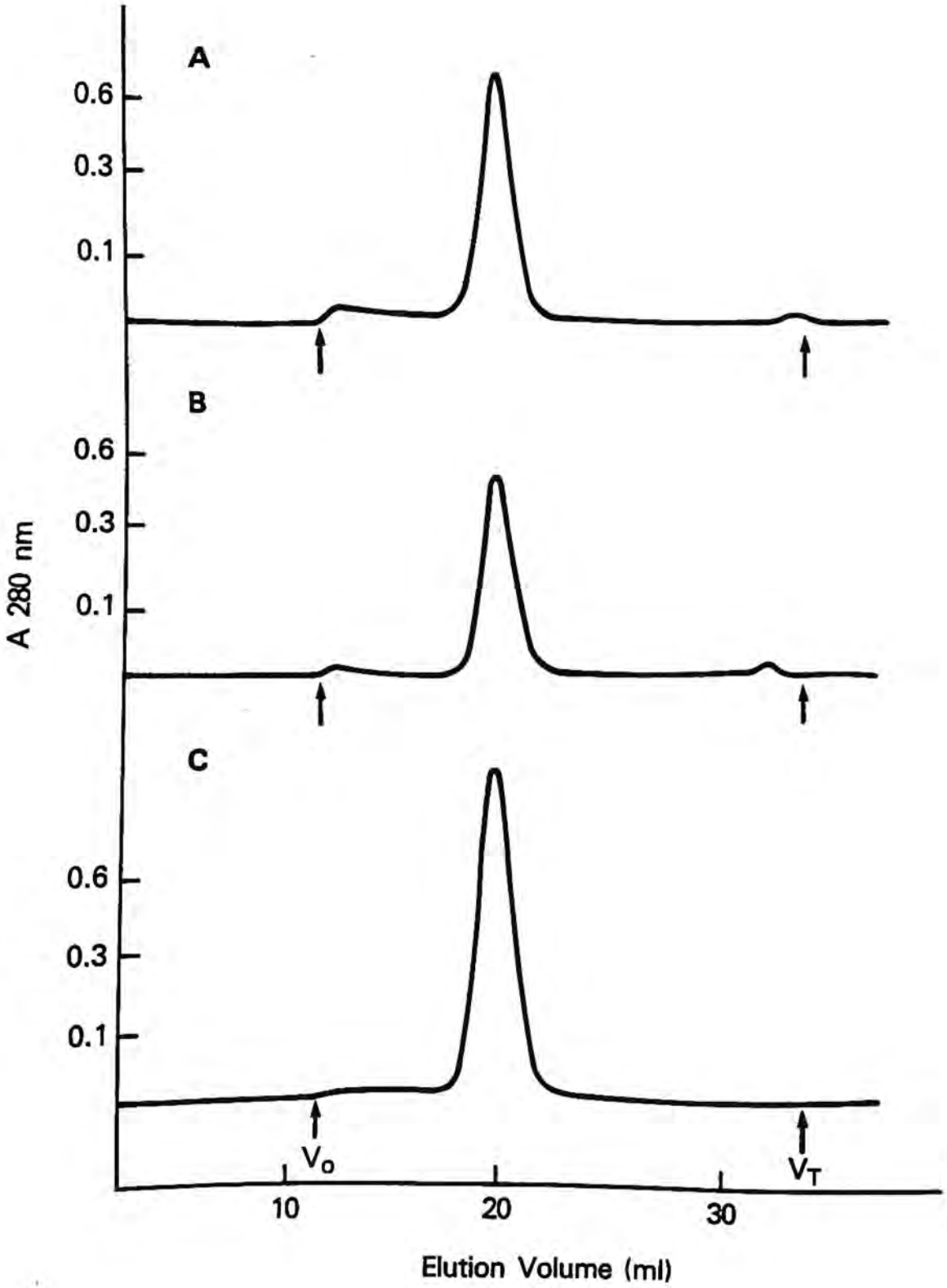

CX
AT
Thr
1
2
3
4

Figure 9

High resolution liquid chromatography (HPLC) of three purified AT-III products: Canine AT-III (A), Human AT-III (B), and Rabbit AT-III (C). Column: TSK-3000; sample volume = 100 ul; Buffer: Tris-chloride 0.1 M, sodium citrate 0.01 m, sodium chloride 0.15 M, pH 7.4; flow rate: 0.5 ml/min; monitoring: 280 nm (Beckman 450 data system controller). V_o = Void Volume; V_T = Total Volume.


A
B
C
0.6
0.3
0.1
A 280 nm
V_o
V_T
10
20
30
Elution Volume (ml)

b. **Demonstration of AT-III-Thrombin Complex Formation**

Canine AT-III-thrombin complex formation by HPLC is illustrated in the composite Figure 10. Purified human thrombin elutes in a position with apparent molecular size of about 37,000. Canine AT-III elutes in a position with an apparent molecular size of 68,000. In the presence of heparin, the complex (formed by incubating equimolar quantities of each) elutes in a position immediately adjacent to the free AT-III with an apparent molecular size of about 95,000. These observations are in accord with the profiles documented by SDS-PAGE (Figure 8). The largest peak, at V_o, most likely represents polymers of the complex (80).

4. Specificity of Antigen (AT-III) Antibody Binding

The respective antigens described: Canine AT-III, Human AT-III, and Rabbit AT-III were used to elicit antibody production in goats (see METHODS AND MATERIALS). These antisera served as a valuable source in testing potential cross (specie) reactivities - an anticipated finding in view of the apparent antigen similarities. Results obtained with Ouchterlony double diffusion are summarized in Table VII. It is evident that, as the sensitivity of this method is not optimal, reactivity appears to be primarily specie-specific. To verify or disprove this observation, the more sensitive immuno-blotting technique was used. A test matrix similar to the one described in Table VII was executed. Figure 11 illustrates results. With this technique, it appears that while reactivity is for the most part specie-specific, there exists some cross reactivity as evidenced by the light (but clearly visible) reactions between anti-canine AT-III serum and human/rabbit AT-III.

Figure 10

High resolution liquid chromatography (HPLC) of purified canine AT-III (A), purified human thrombin (B); complex (C); prepared by incubating equimolar quantities of thrombin and AT-III for four hours at 22°C. Complex (D) prepared by incubating equimolar quantities of thrombin and AT-III (the same amounts as in composite C) for 3 minutes, in the presence of heparin (3 units/ml), at 22°C. Column: TSK-3000; sample volume = 100 ul; Buffer: Tris-chloride 0.1 M; sodium citrate 0.01 M, sodium chloride 0.15 M, pH 7.4; flow rate = 0.5 ml/min; monitoring: 280 nm (Beckman 450 data system controller). V_o = void volume; V_T = total volume.

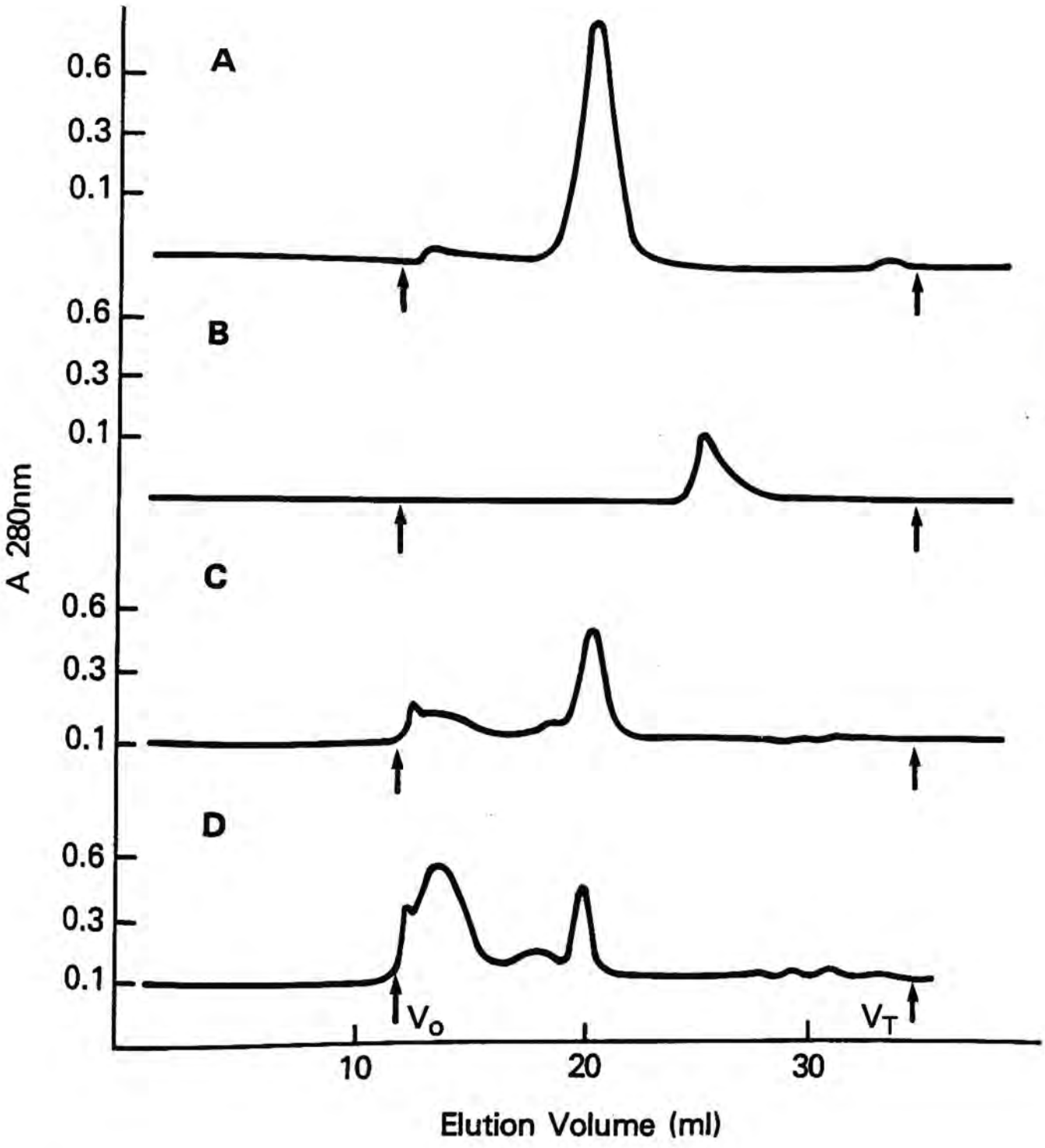

A
B
C
D
0.6
0.3
0.1
A 280nm
V_o
V_T
10
20
30
Elution Volume (ml)

TABLE VII

ANTIGEN-ANTIBODY SPECIFICITIES OF CANINE, HUMAN AND RABBIT AT-III WITH RESPECTIVE ANTISERA*

Antigen	Antibody to	Results
Canine AT-III	Human AT-III	No Rx
Rabbit AT-III	Human AT-III	No Rx
Human AT-III	Human AT-III	Single Precipitin line
Canine AT-III	Rabbit AT-III	No Rx
Rabbit AT-III	Rabbit AT-III	Single Precipitin line
Human AT-III	Rabbit AT-III	No Rx
Canine AT-III	Canine AT-III	Single Precipitin line
Rabbit AT-III	Canine AT-III	No Rx
Human AT-III	Canine AT-III	No Rx

*Developed in goats (see METHODS AND MATERIALS).

Figure 11

Immuno-blots of purified canine, (C), rabbit (R) and human (H) AT-III using respective and heterologous antisera developed in goats (METHODS AND MATERIALS). - - - - Sample (antigen) application a-C: anti-canine AT-III; a-H: anti-human AT-III; a-R: anti-rabbit AT-III. Electrophoresis: two hours. 1% Agarose in sodium barbital buffer, 0.05 M, pH 8.6, 6 volts/cm. Blotted onto nitrocellulose sheets, reacted with respective primary antibodies, and developed with secondary antibody (rabbit antigoat IgG conjugated with HRP).

a-C
a-H
a-R
+
-
H R C H R C H R C

5. Crossed Immunoelectrophoresis (C.I.E.)

Crossed immunoelectrophoresis (C.I.E.) of purified canine AT-III (Fraction II) revealed one single (diffuse peak) indicative of a single molecular species. As no effort was made to study the product under varying conditions of ionic strength, electrophoretic time, with or without heparin, it is difficult to assess the degree of purity by these results alone.

6. Comparative Amino Acid Analysis of Canine, Rabbit and Human AT-III

Table VIII summarizes the amino acid residue profiles for the three molecular species. Cysteine and methionine were not determined. Also, aspartate and glutamate are expressed as the cumulative content of aspartic acid/asparagine and glutamic acid/glutamine, respectively. It is apparent from these profiles that the amino acid composition of the three molecular species demonstrates unremarkable differences. Indeed, a striking (expected) similarity is noted, implying a similar primary structure.

7. Comparative Partial N-Terminal Primary Structure of Canine, Rabbit and Human AT-III

Figure 12 illustrates the partial N-Terminal amino acid sequence of human, canine and rabbit AT-III. For comparative purposes, the published (81) human data appear on the top line and serves as a reference for alignment. Although a number of residues (X) were unidentifiable, it is clear that there exist major similarities and some dissimilarities between the three molecular species. Clearly, the N-Terminal amino acid of the canine molecule is proline, which is different from that of the human and rabbit molecule, which is histidine. Interestingly, with the exception of a short five residue N-Terminal sequence

TABLE VIII

COMPARATIVE AMINO ACID COMPOSITION
PROFILES OF CANINE, HUMAN AND RABBIT AT-III

	CANINE AT-III n moles	HUMAN AT-III n moles	RABBIT AT-III n moles
ASP	39	43	46
THR	19	21	22
SER	23	27	26
GLU	41	49	43
GLY	19	19	18
ALA	28	30	27
VAL	30	29	30
ILE	18	20	16
LEU	33	37	40
TYR	9	8	9
PHE	16	18	16
HIS	--	--	16
LYS	26	34	34
ARG	21	21	20

- - unidentifiable residues. The following amino acids were not studied: cysteine, methionine, tryptophan.

Figure 12

Partial N-Terminal amino acid sequence analysis of purified canine, rabbit, and human AT-III, by the automated Edman degradation procedure (78,79) described in METHODS AND MATERIALS. The human sequence indexed (*) is that reported by Peterson (81). N-Terminal residue for human and rabbit AT-III is histidine (HIS); for canine AT-III it is proline (PRO). Note alignment beginning with position 5. (X) indicates unidentifiable residues.

	1				5							10				15	
Human*		HIS	GLY	SER	PRO	VAL	ASP	ILE	CYS	THR	ALA	LYS	PRO	ARG	ASP	ILE	PRO
Human		HIS	GLY	SER	PRO	VAL	ASP	ILE	X	THR	ALA	LYS	PRO	ARG	ASP	ILE	PRO
Canine	PRO	TRP	SER	PRO	GLY	X	ASP	ILE	X	THR	ALA	LYS	PRO	ARG	ASP	ILE	PRO
Rabbit		HIS	X	SER	PRO	VAL	ASP										

	17			20					25					30				
Human*	MET	ASN	PRO	MET	CYS	ILE	TYR	ARG	SER	PRO	GLU	LYS	LYS	ALA	THR	GLU	ASP	GLU
Human	MET	ASN	PRO	MET	X	ILE	TYR	ARG	SER	PRO	X	LYS	X	ALA	X	X	X	GLU
Canine	X	ASN	X	MET	X	PHE	TYR	ARG	X	X	X	X	X	PHE				

which is different in the dog, beginning with position 6, there appears to be an almost perfect homology to the human product.

D. IMMUNOLOGIC QUANTITATION OF CANINE AT-III

The radial immunodiffusion of Mancini, et al. (71) was used to assess the immunologic concentration of AT-III in normal canine plasma. Figure 13 illustrates the relationship between the square of the precipitin diameter $(d)^2$ and the concentration of purified canine AT-III. This standard curve served to interpolate $(d)^2$ values for normal dog plasma pools (4 animals) and for 2 individual (independent) animals. Values ranged between 205 and 230 ug/ml, with a mean of 214$\pm$ 12 ug/ml.

E. STABILITY OF PURIFIED CANINE AT-III

The biologic activity of purified canine AT-III, stored at four temperatures: 22°C, 4°C, -20°C, and -70°C, was monitored for a period of three months. Aliquots of appropriately stored samples (with no precautions to assure sterility) were assayed periodically. Figure 14 profiles residual biologic activity (12) for the four products. It is evident that, stored in liquid form at ambient temperature, loss of activity is precipitous in comparison to essentially complete stability when stored frozen.

Figure 13

Radial immunodiffusion (71) quantitative relationship between the square of the precipitin diameter and the concentration of purified canine AT-III. Antiserum preparation in goats and diffusion matrix preparations are described in METHODS AND MATERIALS. Standard curve served to interpolate unknown values from normal (individual or pooled) canine plasma. Insert denotes $(d)^2$ and respective interpolated concentration values from two separate dog plasma pools and two separate dog (individual) plasmas.

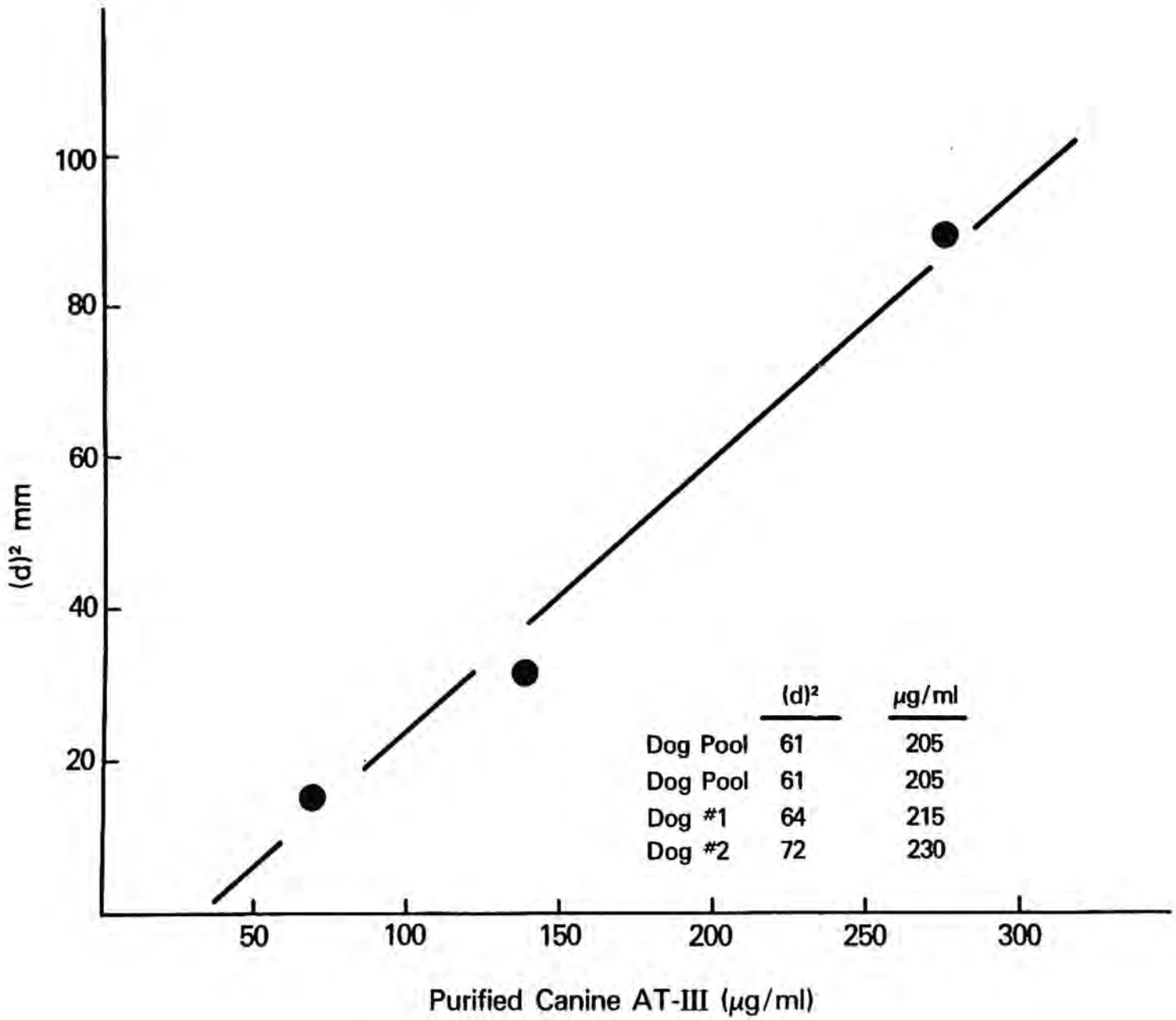
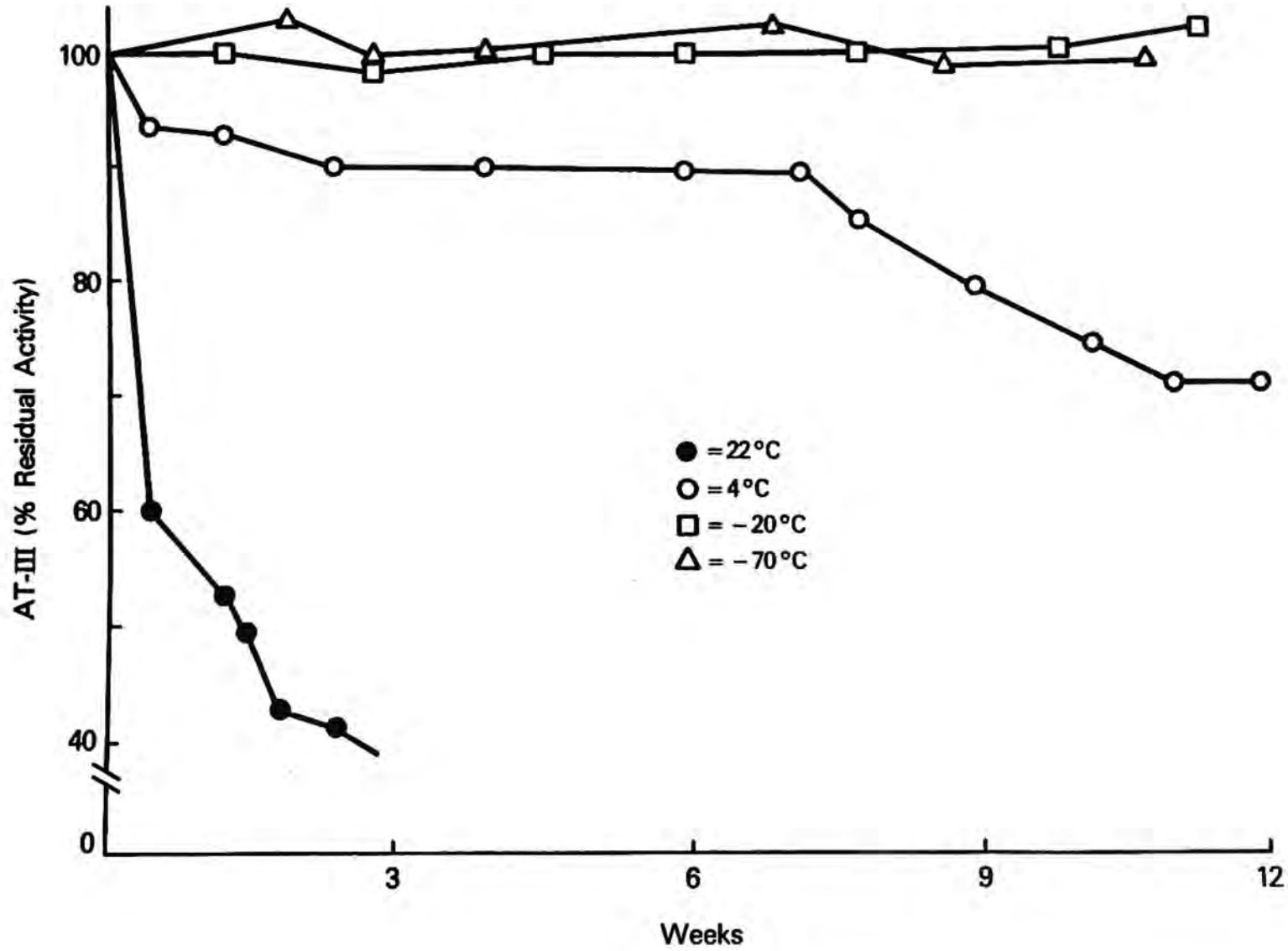

100
80
60
40
20
(d)² mm
50
100
150
200
250
300
Purified Canine AT-III (µg/ml)
(d)²
µg/ml
Dog Pool 61 205
Dog Pool 61 205
Dog #1 64 215
Dog #2 72 230

Figure 14

Biologic activity profile of purified canine AT-III stored at 22°C, 4°C, -20°C and -70°C. At designated time intervals, aliquots of samples stored at the indexed temperature were removed and assayed (as described in METHODS AND MATERIALS) at a 1:30 dilution. The residual biologic activity (%) is in comparison to the starting value which was designated 100%.


100
80
60
40
0
AT-III (% Residual Activity)
3
6
9
12
Weeks
● = 22°C
○ = 4°C
□ = −20°C
△ = −70°C

V. DISCUSSION AND CONCLUSIONS

A. PREFACE AND RATIONALE

Antithrombin-III (AT-III), in the human, has been identified as the primary modulator (inhibitor) of blood coagulation. Indeed, although to varying degrees, most serine proteases generated during clotting are inhibited (controlled) by AT-III (17-23,26) and this process is accelerated dramatically in the presence of heparin or analogues. The complete primary structure (35,81) and mechanism of action of the human molecule have been elucidated (28,32).

Appreciation for the complex functional interrelationships between various "triggers" and multiple anatomic/biochemical compartments in syndromes of disseminated intravascular coagulation (DIC) has served as impetus for the development of therapeutic modalities in an effort to blunt/arrest the often catastrophic consequences of such insults to human physiology. Purified human AT-III has been infused in a number of patients with a multitude of pathologic conditions - and with symptoms indicative of DIC (11,47,50-60,82,83). Unfortunately, to date, evaluation of therapeutic efficacy has been impossible, as none of these studies have been controlled with respect to underlying pathology, concomitant therapy, alternative therapy, long-term follow-up, et cetera. Nevertheless, the "generally favorable" response noted by most investigators has generated substantial enthusiasm for the development of appropriate animal study models. To date, the rabbit and the dog have been explored as potentially useful resources and, indeed, depending on the qualitative and quantitative form of injury (trigger), the dog seems to lend itself as a useful model (33,64-67). In those instances where the "trigger" does not appear to elicit an appropriate (expected) consumption of AT-III, it is likely that the sensitivity of the response correlates

best with the depletion of other components, for example, heparin cofactor-II. The extent to which results obtained in the dog are deemed interpretable (with respect to evaluating the therapeutic efficacy of AT-III), is predicated on the availability of large quantities of canine AT-III and on the elucidation of the structure-function relationship of the molecule.

B. PREPARATION OF CANINE AT-III

The preparative procedure described in this study, while in principle similar to others described (affinity chromatography on immobilized heparin) (30-33), is economical, involves a minimum of maneuvers, and yields a product of reproducible high quality/purity/stability. The total recovery (yield) of biologically active material is respectably equal to or better than any described for the human product (24,32). It can be concentrated up to almost 30 times with no apparent loss of activity, and can be kept stored (frozen) for extended periods. (No loss of activity was noted for a period of three months at -20°C or colder). All these properties favor the procurement of such a product (canine AT-III) in practical quantities to satisfy needs for therapeutic studies in a canine model of DIC.

C. ADSORPTION CHARACTERISTICS OF CANINE AT-III TO HEPARIN-SEPHAROSE

In the course of preparing several batches of canine AT-III, it was noted that a portion of (but persistent quantity) AT-III activity (about 20%) could not be adsorbed onto the immobilized heparin matrix. This phenomenon has been observed by others (32,34,80) working with the human analogue and has been suspected to be related to specific avidities of high or low molecular weight heparin for AT-III. Indeed, it is documented that there exist several distinct

forms of heparin, differing in their ability to bind and "activate" AT-III (32,34,84-86). Clearly, this does not explain the nonadsorbing properties noted, since repeated attempts to adsorb this residual activity onto fresh -virgin - matrix proved futile.

It is interesting to speculate that the molecular species which does not adsorb onto immobilized heparin indeed cannot adsorb for one of two reasons: (a) it is denatured, thereby it lacks appropriate configuration/binding sites; (b) it represents a molecule which coincidentally exhibits heparin cofactor activity in an in vitro assay system, but in reality is not AT-III. The nature of this phenomenon remains elusive.

D. BIOCHEMICAL PROPERTIES OF PURIFIED CANINE AT-III

1. Elution Characteristics from Affinity Matrix

Similar to observations made with the human (32,80) and rabbit (31) product, it was noted that, with the gradient described, canine AT-III elutes from immobilized heparin affinity matrix as a polydisperse protein peak - a profile which could be modified to produce a single sharp peak by using a steeper gradient. The three major activity peaks noted in Figure 6 were anticipated to display different biochemical properties. Disappointingly, all three fractions displayed similar specific activities and, further supporting this observation, identical electrophoretic profiles in the presence of sodium dodecyl sulfate (SDS) as indicated in the inserts in Figure 6. One likely explanation of the polydisperse nature of the elution may be related to the differential binding properties of multiple molecular species of immobilized heparin (32,34,84-86) as discussed earlier. Another possibility may be that there indeed exist multiple molecular species of AT-III, as in the human (28,32) and that these molecules differ so subtly

in chemical composition as to escape detection. Perhaps heterogeneity of carbohydrate structure may play a role.

2. Absorbance Coefficient

The absorbance coefficient* for canine AT-III reported in the literature ranges between 6.2 and 8.6 (30,33). The value for the human molecule is 7.2 (28). The value for canine AT-III obtained in the present study is 7.72. This is very similar to that of the human and rabbit molecule and indeed, not surprising in view of the virtually identical amino acid composition, especially with respect to the tyrosine content.

Interestingly, the exact same value was obtained whether canine AT-III, Fraction II alone or the combined pool of Fractions II, III and IV were analyzed, lending further support to the thesis that the heterogeneity in elution observed in the gradient chromatography is due to the heterogeneity of heparin and not of AT-III.

3. Purity and Molecular Size

Utilizing polyacrylamide gel electrophoresis (PAGE) in the presence of SDS and high resolution liquid chromatography (HPLC) as analytical tools, it was established that the canine AT-III product described in this study appears pure in that a single band or a single peak (primarily) was resolved by staining or by optical density measurement, respectively. It is conceivable that the product could be still contaminated with a foreign protein of a similar molecular size. However, this is unlikely, as the specific activity of the purified product (approximately 5 units/mg) is precisely in line with expectations based on its concentration in plasma. It was established that the canine molecule, by this

*Defined as the optical density reading of a 1 g/dl solution, at 280 nm.

technology, is virtually indistinguishable from the human and rabbit molecule with respect to molecular size which was calculated (estimated ±5%) at 68,000. This value, although not identical, is similar to that reported by Carlson and Atencio (31) for the rabbit AT-III (63,000-65,000); somewhat smaller than that reported by Damus and Wallace (30) for the canine AT-III (77,000); similar to that reported by Tanaka, et al. (33) for the canine AT-III (70,000); and similar to that reported by Petersen (35,81) for the human AT-III (67,000). Furthermore, by this same technology, canine AT-III Fractions II, III and IV were indistinguishable, again, lending further support to the argument that these fractions, indeed, represent one and the same molecular species.

4. Amino Acid Composition and Partial N-Terminal Sequence

Based on data obtained by others with respect to molecular size (30,31,33,35,81), and the preliminary PAGE and HPLC results obtained in this study, it was predicted that the amino acid composition and (perhaps) primary structure of the canine molecule would prove remarkably similar to the human and rabbit product. Indeed, the similarities with respect to amino acid composition are striking and to the extent that minor differences noted might be interpreted as experimental error.

The partial N-Terminal primary structure proved interesting in that, for the most part, there exists a striking homology to the human analogue. However, the leading N-Terminal sequence of the first six residues differs from the human and rabbit analogue. Indeed, as noted in Figure 12, the Pro - Trp - Ser - Pro - Gly - sequence is unique to the canine molecule. Mechanistically, one might speculate that the AT-III molecule is synthesized as a precursor and modified to its functional form by proteolysis. In the human and in the rabbit, the result of

this process yields a single chain polypeptide with histidine as its N-Terminal residue. In the dog, the process differs enough to yield a molecule larger by (at least) one N-Terminal residue: proline. The significance of this observation is unclear.

5. Functional Characteristics

The ability of human AT-III to complex with various serine proteases involved, in blood clotting, has been abundantly documented (17-23,26) and reviewed in the introduction. In order to develop confidence in the future use of the dog as an appropriate study model for DIC, it became imperative that not only the structure of the canine molecule be characterized, but its function as well. In this study, salient features of the functional properties of canine AT-III were addressed with experiments designed to illustrate that the molecule is involved in the neutralization of the key enzyme thrombin by a process similar to (or different from) that of the human analogue - the formation of a stable complex.

Utilizing PAGE in the presence of SDS and HPLC, it was demonstrated that, similarly to the human analogue, purified canine AT-III, when incubated with purified human thrombin, exhibits an extremely sluggish (progressive) inhibitory activity. This was demonstrated by the dramatically slow rate for complex formation, for example, the appearance of a high molecular weight band in SDS-PAGE (Figure 8) and high molecular weight peak by HPLC (Figure 10). In the presence of heparin, the appearance of the complex was virtually instantaneous - as in the human (24), implying a similar if not identical mechanism of neutralization.

It is of interest to note from the HPLC profiles, especially in the presence of heparin, that not only is the thrombin-AT-III complex formed rapidly, but, it in turn polymerizes into a very large macromolecular structure eluting at

the void volume of the column. This polymerization has been noted with human AT-III-thrombin complexes as well (80).

It is of further interest to note that the progressive inhibitory activity of the canine AT-III appears to be dramatically sluggish in comparison to the human counterpart (hours versus minutes) (24). This observation, however, should not be interpreted without considerations for the following. With human product (24), the progressive activity was quantitated by incubating human AT-III with human thrombin. In the present work, canine AT-III was incubated with human thrombin. Perhaps the enormous difference in complexing rate is a consequence of this "cross specie" configuration.

While it may be argued that these results do not reflect a true quantitative mechanistic perspective, as the reactions studied involved two species (dog and human), it is clear that, as in the human, a complex does form and it appears most rapidly in the presence of heparin. This establishes the "heparin cofactor" properties of canine AT-III.

E. SPECIFICITY OF POLYCLONAL ANTIBODIES TO CANINE AT-III

In the course of exploring potential animal models for study in therapy, it is imperative that if immunologic quantitation of a component is being considered, the antibodies used to quantitate must be characterized especially with respect to interspecie cross reactivities. This is of particular importance when a therapeutic component is derived from another species - as has been the case in studies involving the infusion of human AT-III in rabbits (61,62) or dogs (64) - but has similarities in biochemical structure. As noted earlier, it is clear that the chemical composition and structure (excluding carbohydrate) of canine, human, and rabbit AT-III are similar. It was anticipated, therefore, that some

cross species reactivities would be noted. Indeed, as summarized in Figure 11, there exists a small but significant amount of cross reactivity between these three species. While difficult to argue in the absence of comparative data, it is interesting to speculate that primary difference in immunologic profiles for the three species are predicated on the carbohydrate composition of each.

F. IMMUNOLOGIC CONCENTRATION OF CANINE AT-III IN NORMAL CANINE PLASMA

The concentration of AT-III in normal human plasma is 150-200 ug/ml (28). In the dog, the concentration was calculated at 200-230 ug/ml. This value (mean of 214 ug/ml) was obtained either with a normal plasma pool or with individual animals. While no studies were performed to assess the daily variation of this concentration in the dog, data involving measurements of biologic activity over a ten-day period (12), indicates that fluctuation is less than 10%.

Based on the values obtained in this study (214 ug/ml) and based on the definition of an AT-III unit (as that amount contained in one ml of normal plasma), the calculated maximal specific activity possible is 4.7 units/mg of protein. Within the limits of experimental error, similar values were obtained for purified Fractions II, III, and IV (Figure 6). It is interesting to note that the specific activity of human AT-III products used experimentally in therapy is about 4 units/mg of protein (80).

G. BIOLOGIC STABILITY OF PURIFIED CANINE AT-III

Of the multitude of needs in preparation for performing therapeutic trials in an animal model, a most essential is the availability of an abundant quantity of a product with acceptable biologic stability. The canine AT-III

product described in these studies displays excellent stability when stored for periods up to three months, at neutral pH and at temperatures of -20°C or lower. This stability profile is identical to that described for the human product. Interestingly, the AT-III molecule, regardless of the species of origin, is a remarkably stable protein, even at 4°C, providing precautions are exercised with respect to sterile preparation (80).

VI. SUMMARY

Canine AT-III has been isolated from normal dog plasma utilizing immobilized heparin as an affinity matrix. The two-step procedure is rapid, economical and reproducible. The product can be obtained with a final yield of approximately 30%. It is stable for periods of at least three months when stored at neutral pH at -22°C or lower. Biochemically, the product displays many similarities to AT-III isolated from human and from rabbit plasma. Its molecular weight is 68,000, it is a single polypeptide chain, which interacts with thrombin in a manner similar to that described for the human analogue. Its concentration in normal dog plasma is 214 ug/ml, indicating a specific activity of about 5 units/mg of protein. Its amino acid composition is virtually identical to the rabbit and human analogue. For the most part, its N-Terminal sequence (primary structure) is also dramatically homologous to the human counterpart.

It is concluded that this product may serve as a valuable resource in animal studies (canine) involving the development of an appropriate model to assess the therapeutic efficacy of this important protease inhibitor in blunting the often catastrophic sequelae of disseminated intravascular coagulation.

VII. PERSPECTIVE

In order to lend validity and substance to the effort initiated in this work, it is important to continue these studies along the following lines:

1. The animal model eventually chosen for studying the potential therapeutic efficacy of AT-III should reflect elements of (a) precision and reproducibility with respect to response to specific trigger(s); (b) accuracy of analytic measurements with maximal attention for quality control procedures; (c) quantitative characterization of compensatory metabolic processes in response to specific trigger(s); (d) ethical/humane treatment whether the model is acute or chronic.

2. The molecule itself might be further characterized (comparatively to the human analogue) in terms of (a) carbohydrate composition (qualitatively and quantitatively); (b) complete primary structure; (c) two-dimensional electrophoresis of intact, reduced, and digested molecule ("fingerprinted"); (d) kinetics of inhibition with canine thrombin and canine Factor Xa; (e) heparin cofactor activity with high and low molecular weight heparin and heparin analogues.

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AUTOBIOGRAPHY

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I [redacted]. I obtained my early education from the local private and public school systems, graduating from Freedom High School in June, 1969.

I attended the University of Pennsylvania (Penn), in Philadelphia, Pennsylvania, and obtained a Bachelor of Arts Degree in Sociology in June, 1973, graduating cum laude. While attending Penn, I attended the three-year diploma school of nursing at the Hospital of the University of Pennsylvania where I obtained the registered nurse degree in 1972.

In June 1976, I was awarded a Masters of Science degree in Nursing, magna cum laude, from the Catholic University of America, Washington, D.C. Thesis title: Weaning Ventilator Patients: Intermittent Mandatory Ventilation versus Standard Technique. In 1976 I passed my examination in critical care nursing and received certification as a critical care registered nurse. In that same year I was inducted into the National Honor Society for nurses, Sigma Theta Tau-Kappa Chapter.

I was employed as a clinical nurse specialist in critical care at Johns Hopkins Hospital, Baltimore, Maryland, from 1976-1978. For the next five years I was the Nursing Director and Chief Flight Nurse at the Medical Shock-Trauma Acute Resuscitation Unit (MedSTAR), Washington Hospital Center, Washington, D.C.

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