THE ASSAY OF ANTITHROMBIN III AND HEPARIN
USING SYNTHETIC CHROMOGENIC SUBSTRATES

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INTRODUCTION

A. STATEMENT OF THE PROBLEM

An exciting new laboratory tool in clinical investiga-
tion and diagnosis is the use of synthetic rather than
natural substrates for the assay of proteolytic enzymes,
allowing specific and sensitive enzymatic analysis.

Substrates for the measurement of fibrinolytic and
coagulation enzymes are becoming increasingly available.
Their use allows analysis of enzyme activity using spectro-
photometric initial rate or end point analysis, rather than
the traditional and often more difficult detection of
fibrin clot formation. These assays can be modified to
measure inhibitors of the coagulation system as well as the
effects of the enzymes themselves.

Antithrombin (heparin cofactor) (AT III) and heparin are
very important inhibitors of the coagulation system. AT III,
a natural inhibitor of thrombin (as well as of factors IXa,
Xa, XIa, and XIIa) is reduced to levels 40-50% of normal
in hereditary AT III deficiency (28,47). This entity results
in a significant increase in morbidity and mortality due to
thromboembolism in affected individuals (47). Acquired
AT III deficiencies are reported in liver cirrhosis (4,21,
50,81), disseminated intravascular coagulation (4,11,15,21,
81), oral contraceptive therapy (31,32,59,75,77,93,113,119,
120), and with continuous heparin therapy (13,70). The
identification of low AT III levels is important due to the

availability of treatment through the use of warfarin (69, 112), fresh frozen plasma (29), and eventually AT III concentrates (88,96).

Heparin, a most important therapeutic agent, is administered in the treatment and prophylaxis of thromboembolism to accelerate the inhibitory action of AT III. However, if too much heparin is administered, bleeding occurs (9,107). Conversely, if too little heparin is given in the treatment of thromboembolism, recurrence of the thrombosis may result (9,107). The administration of heparin therapy must be carefully monitored to avoid these complications.

Established methods for measuring AT III and heparin are time consuming, nonspecific, and many are inaccurate. AT III may be measured by clotting assays, requiring defibrination and timing the formation of a fibrin clot (3,10). Such methods are tedious and may be influenced by other plasma factors. Immunologic assays, such as passive radial immunodiffusion (68) and immunoelectrophoresis (61) require a minimum of several hours for completion. A further disadvantage is that the true functional activity of the AT III molecule may not be reflected, and a normal concentration may be measured when inhibitory activity is actually low (6,94).

The best way to monitor heparin therapy remains controversial. Heparin concentration may be measured directly or its in vivo anticoagulant effect can be assessed. Heparin

measurement by polybrene titration (43) requires many dilutions for accuracy (103) and clotting methods based on the potentiation of factor Xa (25,118) have been found to be inaccurate in pathologic samples (103). Tests used to measure the anticoagulant activity of heparin, such as the widely accepted activated partial thromboplastin time (52, 97), the whole blood clotting time (97), activated clotting time (48), thrombin time (108), and recalcification time (45) are not totally effective and may not reflect the true anticoagulated state of the patient (42,52,54,105).

It was therefore decided to apply the concept of the potential use of synthetic substrates in measuring inhibitors of blood clotting and their specificity to the measurement of AT III and heparin. AT III was measured using S-2238 in normal individuals, hereditary AT III deficiency, liver cirrhosis, and disseminated intravascular coagulation. The results were compared to a biologic clotting assay for AT III based on the inactivation of factor Xa (39) as well as to Laurell immunoelectrophoresis (61). Heparin was measured with S-2222 and an attempt made to correlate heparin concentration with activated partial thromboplastin times.

Further study was made of the relationship between AT III and heparin by measuring both levels in patients receiving continuous infusion heparin therapy. Such treatment has been reported to reduce AT III levels (13,70).

This study will show the synthetic chromogenic substrates are accurate, sensitive and rapid analytic tools. They have

vast potential in the measurement of coagulation enzymes and inhibitors.

B.   BACKGROUND

1.   Development of Synthetic Substrates

The synthetic substrates used for proteolytic enzyme assays have been developed by patterning the amino acid sequence of the substrate after the natural substrate of the enzyme. The chromophore, p-nitroaniline (pNA), is attached by amide linkage to the substrate and enzymatic hydrolysis of this bond releases pNA, which is subsequently measured spectrophotometrically at a wavelength of 405 nm (85).

Substrates are now available for the measurement of plasminogen, plasmin inhibitors, prekallikrein, and kallikrein inhibitors, making it possible to use these substrates in the study of the fibrinolytic and kinin systems. The development of synthetic substrates susceptible to thrombin and factor Xa proteolysis has uncovered new methods of studying the coagulation system and its inhibitors.

Clotting methods for coagulation testing are based on timing the endpoint of a series of enzymatic reactions involving a number of clotting factors and also depend on a source of fibrinogen to produce a fibrin clot. The use of synthetic substrates modifies this concept considerably by making it possible to measure a specific enzyme's activity directly, as well as an inhibitor's activity directed toward that enzyme. The dependence of the assay on subsequent

coagulation factors leading to the formation of the fibrin clot is thereby eliminated.

S-2160 (Benzoyl-L-Phe-Val-Arg-p-Nitroanilide HCl) was the first synthetic substrate with specificity for thrombin released by Kabi, Stockholm, Sweden. Assays for AT III using this substrate showed excellent correlation of AT III levels with clotting and immunologic assays (1,4,80,81). Moreover, work with S-2160 led to the discovery that the addition of EDTA and heparin to a buffer of pH 8.6-9.1 and ionic strength 0.2-0.33 retarded fibrin formation and the previously required defibrination step could thus be eliminated (4,80,81).

S-2238 (H-D-Phenylalanyl-L-Pipecolyl-L-Arginine-para-Nitroanilide dihydrochloride) was subsequently developed. This substrate was reported to be more soluble and have a greater specificity for thrombin, measuring as little as 0.1 NIH U/ml human thrombin and was more sensitive to alpha thrombin than S-2160 (73). Shorter reaction times were possible and higher dilutions of plasma used, helping to eliminate the interference effects of hyperbilirubinemia and hyperlipidemia (5).

S-2238 is modeled after the N-terminal end of fibrinogen's A alpha chain, which is the natural substrate of thrombin (73). Thrombin has a specificity for arginine-glycine bonds, with the arginine component being particularly important (92, 100). The phenylalanine in S-2238 is thought to aid in blocking the alpha amino group of arginine (100), which is

important in the rate of hydrolysis (63). Pipecolic acid is an analog of proline, having a six carbon rather than a five carbon ring (73). Proline also accelerates the hydrolytic action of thrombin (63).

A third substrate, S-2222 (N-Benzoyl-L-Isoleucyl-L-Gluta-myl-Glycyl-L-Arginine-para-Nitroanilide hydrochloride and its methyl ester) has a specificity for factor Xa and may be used for the assay of factor Xa, AT III (as antifactor Xa activity), and heparin. Teien developed a rapid heparin assay using S-2222 and found the method had a greater accuracy in patho-logical as well as normal samples than previous methods (104, 106). Mattler and Bang found a S-2222 heparin assay to be more sensitive and reproducible than a clotting assay (73). This substrate is patterned after the sequence found at the site in prothrombin where factor Xa cleaves the thrombin A chain from fragment two and the site at which factor Xa cleaves the thrombin A and B chains (73). S-2222 is quite sensitive to factor Xa, the chromophore being split from the substrate by factor Xa 14 times faster than by thrombin (84). Bovine thrombin splits S-2238 100 times faster than factor Xa (84) but both S-2238 and S-2222 were found to be very susceptible to trypsin and fairly sensitive to plasmin (73).

A possible limitation of the use of S-2238 was its re-ported inability to be used in standardizing thrombin pre-parations. Thrombin activity of stored preparations was not shown to decrease with time, although clotting assays did detect a decreased activity (36). The possible significance

of this finding with respect to the specificity of S-2238 for thrombin for use in clinical studies can be evaluated by using hirudin as a specific thrombin inhibitor (71,84).

Another potential drawback of the synthetic substrates is their high cost. However, increased demand and avail- ability should lower costs and increase accessibility for routine use.

The importance of AT III, its relationship with heparin, and the need for accurate clinical methods of assay have been briefly discussed in the introduction. A more detailed ac- count of the discovery of AT III and heparin and the present knowledge regarding AT III and the AT III-heparin interaction is needed as a basis for evaluating the accuracy, sensitivity, and value of chromogenic substrates in measuring AT III in various inherited and acquired conditions.

2. Discovery of Heparin and AT III

In 1916, Jay McLean, while attempting to demonstrate the thromboplastic action of a group of phosphatids, isolated "courin" from heart and "heparphosphatid" from liver. He noted these two substances, later identified as heparin, had the ability to inhibit the coagulation of blood (64). Howell and Holt in 1918 reported heparin had the ability to slow or prevent coagulation of blood by preventing conver- sion of prothrombin to thrombin. It was also suggested that heparin caused the production of antithrombin from pro- antithrombin (56).

In 1939, Brinkhouse, et al, reported the discovery of a

plasma factor involved in the inhibition of thrombin forma-
tion. They noted that neither this plasma factor nor
heparin alone exhibited such a property, but the two in
combination were exceptionally effective in preventing the
formation of thrombin. The unknown factor was found in
both plasma and serum and was non-dialyzable. They suggested
that this substance not only prevented thrombin formation
but also destroyed any remaining thrombin present in the
solution (16). Since their initial discoveries, it has been
further shown that both heparin-cofactor activity and anti-
thrombin activity as well as other clotting factor inhibitory
activity are shared by the same molecule (74,90).

3. Chemical Composition of AT III

AT III is a glycoprotein, migrating electrophoretically
as an alpha-2-globulin, with a molecular weight of about
62,000 daltons and a concentration in plasma of approximately
29 mg/dl. The molecule is composed of a single polypeptide
chain (47) consisting of some 538 residues, including 44
lysine residues and 25 arginine residues (74). UV studies
have indicated phenylalanine, tryptophane, and tyrosine side
chains (76). The AT III molecule appears to have a more asym-
metric and extended shape than other globular proteins (74,
76). Release of sialic acid from the AT III molecule using
neuraminidase showed that sialic acid is not necessary for the
function of the AT III molecule and thus may delay or in-
hibit clearance of AT III from the bloodstream, as has been
shown for other glycoproteins (22).

4. Metabolism of AT III

Little is known of the metabolism of AT III. Synthesis is thought to occur in the liver since plasma levels are reduced in hepatic failure due to cirrhosis. In a patient with acute phosphorous poisoning in whom all liver function abruptly ceased, the half-life of AT III was estimated to be 48 hours (51). A second study employed radioactive labeled exogenous AT III which was injected intravenously into control subjects and patients. They found a normal AT III concentration of 19.6 ± 2.3 mg/dl; the intravascular fraction to be 0.45 ± 0.05; the fractional catabolic rate 0.55 ± 0.02 of the plasma pool per day; and a half-life of 2.83 ± 0.26 days (67 hours). The urinary excretion of radioactivity was a constant fraction of the plasma radioactivity, with the exception of a smaller amount the first day. This may represent transfer of AT III to the extravascular compartment. One patient with hemophilia A had a normal AT III half-life and this suggested to the investigators that continuous in vivo coagulation is not the normal route for AT III catabolism (20).

5. Interaction of AT III with Thrombin and Heparin

Thrombin is a serine protease which cleaves arginine-glycine bonds of fibrinogen, releasing fibrinopeptides and leading to development of fibrin monomer and ultimately a fibrin clot. Inactivation of thrombin results from the formation of a 1:1 thrombin-antithrombin complex that is extremely stable (47). Experiments blocking the active

serine of thrombin using diisopropylphosphorofluroridate (DFP), showed that the site is essential for thrombin-antithrombin complex formation. The reactive site of AT III, however, was shown to involve arginine residues. It is suggested that a unique arginine residue is required for complex formation since the serine of thrombin has a known specificity for unique arginine bonds (90).

In the absence of heparin, thrombin inactivation by AT III is a slow, progressive process. However, in the presence of heparin, inactivation of thrombin is markedly accelerated and is virtually instantaneous (47). There are two main theories as to how heparin accelerates the rate of thrombin inactivation.

The most widely accepted hypothesis is that heparin binds to epsilon amino lysyl groups on AT III, causing a conformational change in the AT III molecule which leads to the reactive arginine being more accessible to the active serine site of thrombin (89). This conformational change involves exposure of two tyrosine residues and a partial burial of a tryptophane residue (30,111). One investigator found evidence for two heparin binding sites on AT III (30). However, it is not known if both sites must be filled to accelerate the rate of thrombin inactivation.

There is also evidence that heparin acts as a catalyst in the thrombin-antithrombin reaction without changing the 1:1 stoichiometry of the complex. As little as 0.01 U/ml of heparin was found to be effective in enhancing the rate of

reaction (1). The plasma half-lives of heparin (about two hours) and AT III (about 67 hours) are quite different, suggesting only small amounts of heparin are bound to AT III (101). Heparin is easily dissociated from, and has less affinity for the stable thrombin-AT III complex than free AT III. This leaves the heparin free to activate another AT III molecule (12,18).

Heparin is a high molecular weight muco-polysaccharide containing highly negatively charged sulfate groups, which are thought to be involved in the anticoagulant action of the molecule (35,89). The negatively charged groups are thought to bind to the positively charged lysine residues of AT III (88). Variation in molecular weight and chemical composition are not well correlated with anticoagulant action (89), although certain sequences of substituted sugar residues may be necessary (55). It now appears different fractions of heparin may have varying anticoagulant activities (55,89,107). Rosenberg found one fraction comprising two-thirds of the starting material had only 15% of the anticoagulant activity while the remaining one-third contained 85% of the anticoagulant activity and bound tightly to AT III (89).

A second hypothesis of the mechanism of heparin action is that heparin binds to thrombin rather than AT III, causing allosteric changes in the conformation of thrombin and leading to an increased reaction with AT III (65). Studies have shown a greater affinity of thrombin for heparin than for AT III-heparin complexes (49) and kinetic analyses

supported the binding of heparin to thrombin, resulting in more prompt inactivation by AT III (99).

Elucidation of the AT III-thrombin-heparin interaction is further complicated by the discovery of multiple forms of thrombin of which both alpha and beta forms are active. For example, it has been shown that the inactivation of alpha thrombin by AT III is accelerated by heparin, while the inactivation of beta thrombin is only slightly affected (66).

In summary, there are two mechanisms proposed to explain the AT III-heparin-thrombin interaction. One proposes that heparin causes a conformational change in thrombin, leading to its interaction with AT III. However, most of the evidence favors the theory that heparin, acting as a catalyst, binds to the lysyl groups of AT III causing a conformational change in AT III. This causes the arginine of AT III to be more accessible for binding with the active site serine of thrombin, and the stable AT III-thrombin complex is formed.

AT III inactivates factors Xa, IXa, XIa, and XIIa, which also are serine proteases. As with thrombin, heparin accelerates their 1:1 complex formation with AT III. Factor VII appears to react differently and is only slowly neutralized by AT III (47).

Probably the in vivo therapeutic role of heparin is to accelerate the inactivation of small amounts of factor Xa by AT III (62,79,114,117). Inhibition of one unit of factor Xa prevents 50 units of thrombin from being formed (107), and thus heparin used prophylactically accelerates the

neutralization of factor Xa by AT III, preventing thrombus formation. Smaller amounts of heparin are needed for this purpose than in the event where large amounts of thrombin are already formed, as in acute pulmonary embolism (107).

AT III also is an inhibitor of the plasmin system. The presence of heparin greatly accelerates the inhibition of plasmin by AT III. Kallikrein is slowly neutralized by AT III and its inhibition is accelerated only slightly in the presence of heparin, indicating some special specificity of the serine protease must be necessary for the accelerating action of heparin on AT III (47).

Heparin has been isolated from several organs, mast cells, and basophils. Heparan sulfate, which is chemically and functionally similar to heparin, is found on cell surfaces such as endothelial cells and platelets. It is possible heparin and heparin-like substances may play a role in coagulation or fibrinolysis on vessel surfaces. Consequently, lesions of vessel walls or platelet abnormalities might hinder normal hemostatic reactions and contribute to thrombotic complications (88,89).

6. AT III Variation with Age and Sex

AT III levels vary somewhat with age and sex, but do not generally differ sufficiently to warrant the use of specific normal age and sex percentiles (82) with the exception of young infants. Infants before one-month-of-age have AT III levels about half those normally found in adults. AT III increases with age until six months when adult levels

are reached (102).

Women of childbearing age have somewhat lower AT III levels than those found in males (31,82). AT III levels tend to decrease with age (51) although one study confirmed this finding only in men (82). Mechanisms responsible for decreased AT III with age are unclear.

7. Hereditary AT III Deficiency

Hereditary AT III deficiency was first described by Egeberg in 1965 (28). Study of a Norwegian family found that ten of seventeen members had AT III concentrations approximately 50% of normal and seven of these had a history of pathologic thromboses.

Since that initial description, a number of other families have been reported (19,23,33,41,44,46,67,69,83,91, 94,110,112). These studies each confirm an autosomal dominant pattern of inheritance. Thrombotic symptoms do not usually occur until young adulthood, although a few cases of affected children as young as 8-years-of-age have been reported (23,28,33). Most thrombotic events occurred with such predisposing events as trauma, pleurisy, surgery, pneumonia, pregnancy (28,69,112), oral contraceptive therapy (33), influenza (33) and possibly hyperlipidemia (19,46). However, some thromboses have occurred without precipitating events (110).

Most thromboses have involved the venous side of the circulation including pulmonary embolism. However, one series reported a patient with hereditary AT III deficiency

who had a severe arterial atheroma (67) and another with coronary artery thrombosis (83).

In most individuals with hereditary AT III deficiency, AT III antigen concentration and biologic activity correlate well and are approximately 50% of normal. However, abnormal forms of AT III have been detected. In one hereditary AT III deficient patient, immunologic levels of AT III were found to be normal, yet activity assays revealed a level of 5-10% in the propositus and 20-30% in relatives (94). Two dimensional immunoelectrophoresis of plasma from the propositus showed a lower than normal amount of free AT III and the presence of a higher molecular weight AT III having no heparin cofactor activity (95). Another AT III deficient family was found to have a portion of their AT III that reacted abnormally with heparin (41).

In addition to its value as an anticoagulant, warfarin has been shown to slightly raise AT III levels (69,112) and thus has potential as a prophylactic treatment in those who have suffered a thrombosis or face a situation which may precipitate a thrombosis.

Fresh frozen plasma can also be used as treatment for hereditary AT III deficiency. In one case the administration of plasma raised the level of AT III by 20% and in conjunction with heparin therapy allowed surgery to be performed without a thrombotic episode (29). AT III concentrates, when available, may be an effective means of treatment, making it possible to raise AT III levels to normal.

8. Acquired AT III Deficiencies

    a. Disseminated Intravascular Coagulation (DIC)

Decreased AT III concentration has been reported during local intravascular clotting, such as deep vein thrombosis and pulmonary embolism (2,4,57). The mechanism of such a decrease is thought to be consumption of the inhibitor during the clotting process (2,11). The same mechanism appears to lower AT III levels during DIC, with mean values as low as 38-56% of normal having been demonstrated (4,11,15,21,81). In this acquired coagulopathy, circulating procoagulants are converted to their activated form and therefore may require neutralization by clotting factor inhibitors. The lowered AT III concentration, in addition to the increased release of platelet factor 4, a heparin antagonist from platelet destruction, may render the patient with DIC relatively refractory to the action of therapeutic heparin (88). Infusion of experimental AT III concentrates activated with heparin were shown to aid in producing near-normal hemostasis in three patients with severe DIC (96).

    b. Hepatic Cirrhosis

AT III activity and antigen have been reported to be reduced to 30-60% of normal in patients with severe cirrhosis of the liver (4,21,50,81). Since the liver is thought to be the site of production of AT III, liver disease may lead to decreased production of AT III. For the same reason, however, circulating procoagulant clotting factors

are also reduced, and fibrinolysis may be accelerated. Perhaps for these reasons there appears to be no increased incidence of thrombosis in patients with liver failure (50, 69).

    c. Estrogen and Oral Contraceptive Therapy

    Studies of AT III levels in women using oral contraceptives containing estrogens have led to the conclusion that AT III is lowered by estrogens and that this may be one factor in the increased incidence of thrombosis associated with oral contraceptive use (75,93). AT III levels 9-19% lower in those women using oral contraceptives have been reported (31,32).

    Synthetic estrogens (ethinyl oestradiol and mestranol) appear to be implicated in the fall of AT III (77,119) and also tend to increase procoagulant factors (77). Of interest, the conjugated estrogens used by post-menopausal women do not appear to significantly decrease AT III (77,113).

    The estrogen induced decline in AT III may be dose related, although this allegation is controversial. Contraceptive preparations containing less than 50 µg estrogen failed to decrease AT III levels (50). In another study, a cyclic rise in AT III levels correlated with the week in which treatment was discontinued (31). However, one investigator failed to confirm that the daily dose of estrogen was related to the reduction in AT III (119). The definitive mechanisms for these variations of AT III concentrations are unknown (31,59,77,120).

9. Effect of Anticoagulants on AT III

    a. Warfarin

Warfarin has been shown to be effective in raising AT III levels in hereditary AT III deficiency. One investigator reported raising AT III levels 21-62% with warfarin treatment of 1-9 months (69). Other studies in normal individuals have confirmed an increase in AT III (72) as much as 18% in one study of 100 patients receiving warfarin (115). Moreover, in studying levels in patients who required warfarin, AT III was found to decrease significantly over 2-3 weeks after abrupt cessation of warfarin (87). The mechanisms for the increase in AT III with warfarin have not yet been elucidated.

    b. Heparin Therapy

As early as 1963, a decrease in AT III with heparin therapy was reported (13). Injections of heparin into dogs and humans caused a continuous decrease in heparin cofactor activity until heparin was withdrawn.

Recently, Marciniak and Gockerman studied AT III levels in 24 patients receiving continuous IV heparin and two receiving repeated IV injections (70). AT III was measured by a factor Xa inhibition assay, Laurell immunoelectrophoresis, and radial immunodiffusion. Within 3-5 days of institution of heparin therapy, AT III values dropped to a mean of 70% of the initial AT III level. In one patient with hereditary AT III deficiency, AT III fell to extremely low levels. No correlation of the fall in AT III levels with the dose of

heparin given was found (70). Subcutaneous heparin therapy also was found to significantly lower AT III (78).

This decrease in AT III concentration with heparin raises concern over the possibility of recurrent thromboembolism occurring with the cessation of heparin therapy. As long as heparin is still circulating, the increased activity of the AT III should compensate for the lower AT III values. However, the time period when the anticoagulant is no longer present and AT III has not yet risen may be critical for the recurrence of thromboembolic phenomenon (70).

The increased risk of thromboembolism is emphasized by a case report in which a patient with pulmonary embolism was initially treated with heparin and then switched to warfarin. Upon cessation of heparin, deep vein thrombosis occurred and low levels of AT III were demonstrated (34). Other reports confirm the possibility of thromboembolism with the cessation of heparin therapy (109).

The metabolism of AT III in patients receiving heparin was studied in a series of patients as well as normal control subjects by injecting labeled AT III and monitoring its catabolism. The patients with venous thrombosis were treated with heparin and only in those patients was the plasma half-life of AT III significantly shortened and the fractional catabolic rate increased. Thus, heparin appears to increase AT III turnover due either to consumption following in vivo coagulation or to greater turnover of

AT III-heparin complexes (20).

　　10.　Control of Heparin Therapy

Although the therapeutic and prophylactic use of heparin for the treatment of thromboembolism is well established (37), the control of heparin therapy remains a dilemma.

One approach to the management of heparin dosage has been to measure the effect of heparin on the clotting time. The whole blood clotting time was one of the earlier tests used.　Inherent in this test are many disadvantages, including the need for immediate bedside determination, it is time consuming (52,86), it has poor precision due to variations in technique (54,97), and it is influenced by oral anticoagulants (54).

The activated clotting time was developed and reported to have increased precision and sensitivity over the whole blood clotting time, as well as being less time consuming. However, it still requires performance at the bedside. Other clotting tests used for measurement of the anticoagulant effect of heparin have been the thrombin time (108) and whole blood recalcification time (45), but the test that is currently most widely accepted and used is the activated partial thromboplastin time (aPTT) (26,37).

The aPTT has the advantage of being able to be performed in the laboratory, is quick, reproducible, and reliable (42, 52).　One study found use of the aPTT for monitoring heparin therapy was helpful in the prevention of the recurrence of venous thromboembolism, although it was not a good prediction

of bleeding complications (9).

Despite its widespread use, there are still many criti-
cisims of the aPTT. One study found no advantage to using
the aPTT over other tests, including protamine titration
(86). The aPTT is also influenced by other coagulation
factors and inhibitors as well as activators of the coagula-
tion system. The aPTT measured during heparin therapy needs
to be compared to a pre-treatment time (105). The pos-
sibility remains that other factors may influence the aPTT
during treatment, making comparison to a baseline value of
limited use (54).

An alternative to the indirect tests of heparin dis-
cussed above is the measurement of actual plasma heparin
concentrations. Polybrene titration (40,43) has been found
to be accurate (103) but it is time consuming and tedious,
requiring many dilutions to be accurate. It does have the
advantage of allowing calculation of the amount of pro-
tamine sulfate needed for heparin neutralization in heparin
overdose or after bypass procedures (43).

Newer heparin assays developed were clotting methods
based on the acceleration of heparin in the inactivation of
factor Xa (25,118). These assays were found to be sensitive,
but inaccurate in pathologic samples (103).

Recently Teien described a plasma heparin assay utilizing
the chromogenic substrate S-2222 (104,106), which is
sensitive to Xa. The use of S-2222 for heparin measurement
has been found to be accurate, sensitive (73,104,106) and

less influenced by other factors in plasma than clotting methods (60).

Thus the control of heparin therapy remains controversial. It has been suggested it may be advantageous to measure both heparin activity and concentration (42), especially when a patient appears overanticoagulated by activity assays (103).

MATERIALS AND METHODS

A. PLASMA PREPARATION

1. Preparation of Pooled Normal Human Plasma

Every six weeks nine parts blood was drawn by veni-
puncture, with minimal trauma, using plastic syringes and
disposable 20 gauge needles from 20 healthy male donors and
added to a polypropylene tube containing one part Ware's
anticoagulant. (6 parts 0.1 M sodium citrate [Mallinckrodt
Chemical Works, St. Louis, Mo.] and 4 parts 0.1 M citric
acid [Mallinckrodt Chemical Works]). The contents were
gently mixed and centrifuged 10 minutes at 7,000xg and 4°C
in a Beckman refrigerated centrifuge model J21B. A pro-
thrombin time and activated partial thromboplastin time were
performed on the supernatant and plasmas, abnormal clotting
times were discarded. The remaining plasmas were pooled in
a plastic beaker, dispensed into small-capped plastic vials
and flash frozen with acetone-alcohol and dry ice. The
plasma was stored at -70°C.

2. Preparation of Test Plasmas

For AT III determinations plasmas were prepared as
described above. The plasma was kept at 4°C if analyzed
within a few hours or kept frozen at -20°C or lower until
tested.

Blood drawn for heparin assay was kept on melting ice
and centrifuged within one-half hour of venipuncture. Plasma
was prepared as described above.

B. S-2238 ACTIVITY ASSAY FOR AT III

1. Reagents

S-2238

The contents of a 25 mg vial of S-2238 (Ortho Diagnostics, Raritan, New Jersey) were dissolved in 40 ml sterile distilled water to make a 1.0 mmol/liter stock solution. The dry powder was stored in a desiccator at 4°C and used before the expiration date indicated by the manufacturer. The 1.0 mmol/liter solution was stored at 4°C, protected from the light by wrapping the container with aluminum foil and was used before the indicated expiration date.

Polybrene

A polybrene solution, (Sigma Chemical Company, St. Louis, Missouri) was prepared by dissolving 3 mg polybrene per ml of sterile distilled water. The solution was stored at -20°C in 2 ml aliquots for up to 6 months.

S-2238 Working Solution

A working substrate solution was prepared by mixing 7.5 ml of the 1.0 mmol/liter stock S-2238, 1.0 ml of the 3 mg/ml polybrene solution, and 1.5 ml sterile distilled water, resulting in a 0.75 mmol/liter concentration of S-2238. This solution was stable for 1 week stored at -4°C and protected from the light by wrapping the container with aluminum foil.

Thrombin-Roche

Bovine thrombin, kindly supplied by Hoffman-LaRoche, Basel, Switzerland (courtesy of Dr. W. E. Scott, Hoffman-La-

Roche, Nutley, New Jersey) was obtained with an activity of 51 NIH U/mg. To prepare a 10 NIH U/ml solution, 2 mg thrombin Roche was dissolved in 10.2 ml sterile normal saline (McGaw Laboratories, Irvine, Calif.) containing 5 g/l polyethylene glycol of 6000 molecular weight (Sigma Chemical Company, St. Louis, Mo.). For some experiments a solution of 20 NIH U/ml was used. The thrombin was kept on melting ice during the assay. The solutions were stable for 1 week at 4°C.

Parke-Davis Thrombin

Topical bovine thrombin (Parke-Davis, Detroit, Michigan) was obtained in vials containing 1000 NIH U/ml. The contents of the vial were dissolved in 100 ml sterile normal saline containing 5 g/l polyethylene glycol to give a final concentration of 10 NIH U/ml. For some experiments 20 NIH U/ml were used.

Human Thrombin

Highly purified human thrombin, kindly supplied by Dr. Art Thompson, U.S. Public Health Service Hospital, Seattle, Washington, was reported to have an activity of 2500 NIH U/ml and to contain 5-10% beta and/or gamma thrombin. The material was diluted to a concentration of 10 NIH U/ml in sterile normal saline containing 5 g/l polyethylene glycol. For some experiments 20 NIH U/ml was used.

Buffer with Heparin

The buffer was prepared by dissolving 6.1 g Trizma base (Sigma Chemical Company, St. Louis, Mo.), 2.8 g

$Na_2 \cdot EDTA \cdot 2H_2O$ (Mallinckrodt Chemical Works, St. Louis, Mo.) and 10.2 g NaCl (Mallinckrodt Chemical Works) in 800 ml sterile distilled water. The pH of the buffer was adjusted to 8.4 by adding 1 N HCl. Heparin (3000 U) (Panheprin ®, Abbott Laboratories, N. Chicago, Illinois) was added and the total volume adjusted to 1000 ml with sterile distilled water. The buffer was stable 2 months at 4°C.

Acetic Acid

A 50% acetic acid solution was prepared by adding an equal volume of concentrated acetic acid (Scientific Products, McGaw Park, Illinois) to distilled water.

Hirudin

Hirudin extracted from leeches was obtained from Sigma Chemical Co., St Louis, Mo. Activity was 100 antithrombin units per vial and was reconstituted with 1 ml sterile normal saline.

2. Equipment

Spectrophotometer

Absorbance was measured with a Beckman DU spectrophotometer (Model 2400, Beckman Instruments, Fullerton, Ca.) with a Gilford attachment (Model 222, Gilford Instrument, Oberlin, Ohio) using disposable semi-microcuvettes, 1 cm x 0.4 cm with a 10 mm light path supplied by Walter Sarstedt, Inc., Princeton, N.J.

Oxford Pipettors

Automatic pipettors in 50, 100, 200, 300 and 400 µl sizes were obtained from Scientific Products, McGaw Park, Il.

Heating Block

Incubations were performed at 37°C in a Constantemp heating block (Roeco Mfg. Service, Monterey Park, Calif.).

3. ### Method

Rationale

AT III is assayed by diluting test plasma with a buffer containing heparin to maximally increase the activity of AT III, and then incubating the diluted plasma with excess thrombin. The amount of thrombin that is inactivated is in proportion to the amount of AT III present in the incubation mixture. A substrate (S-2238)-polybrene mixture is added and any excess heparin present is quickly neutralized by the polybrene. The remaining thrombin splits the terminal peptide bond in the substrate, releasing the chromophore pNA. The reaction is stopped instantly by the addition of acetic acid. The released yellow-colored pNA is measured spectrophotometrically. A linear relationship is observed between absorbance and the concentration of AT III present in test plasma.

Procedure

a. Reference Curve

To set up the reference curve, 300 μl normal human plasma was diluted with 3.25 ml buffer and then diluted in 12 x 75 mm plastic disposable tubes as follows:

Diluted Normal Plasma	Buffer	Equivalent AT III Concentration
µl	µl	units/ml
50	1150	0.25
100	1100	0.50
150	1050	0.75
200	1000	1.00
250	950	1.25

b.   Test Plasma

Test plasma was prepared by diluting 50 µl test plasma with 3.50 ml buffer. If the resulting absorbance was outside the range of the curve, 100 µl test plasma was added to 3.45 ml buffer.

c.   Analysis

Plasma was tested by preincubating 400 µl of appropriately diluted plasma 2-6 minutes at 37°C. Thrombin, 100 µl of a 10 NIH U/ml solution, was added with mixing and the incubation continued. Exactly 30 seconds later, 300 µl of S-2238 working solution, prewarmed to 37°C was added with vigorous mixing and returned to the heating block. Exactly 30 seconds after addition of the substrate, 300 µl 50% acetic acid was added with thorough mixing to stop the reaction. The reaction mixture was shielded from light until absorbance at 405 nm was measured. A diluted normal plasma blank containing 400 µl of the 1.00 AT III units/ml solution, 300 µl 50% acetic acid, and 400 µl distilled water was used. If test plasma was found to be icteric or lipemic, a test plasma blank (rather than normal plasma)

was used containing 400 µl test plasma dilution, 300 µl acetic acid, and 400 µl distilled water. All samples were studied in duplicate.

A reference curve was drawn on arithmetic graph paper by plotting absorbance vs. AT III U/ml contained in the standard plasma dilutions. AT III concentration in test plasma could then be obtained from the reference curve.

In experiments using hirudin to inhibit thrombin activity, concentrations of 5.0, 2.5, 1.25, and 0.625 antithrombin units (AT-U) of hirudin per 1 unit thrombin were used. Dilutions of the hirudin were made in normal saline. Thrombin (20 U/ml) was incubated with an equal volume of diluted hirudin to yield a final concentration of 10 NIH U/ml thrombin in the test system. Human thrombin was tested at a final concentration of 5 NIH U/ml. Buffer with heparin was used in place of the test plasma in the AT III assay. Each hirudin-thrombin dilution was incubated 5 minutes at 37°C. 100 µl of the hirudin-thrombin mixture was added to prewarmed buffer and the procedure outlined above for AT III assay followed.

Blanks were analyzed as described above, substituting saline for thrombin with each hirudin concentration. Absorbance was measured against a blank solution containing 400 µl buffer, 300 µl acetic acid and 400 µl distilled water.

4. Obtaining the Normal Population

Specimens were collected at the American Red Cross Blood Center with the kind permission of Dr. Frans Peetoom, Director, for the determination of AT III concentration in a

normal population. Donors were excluded if they had used aspirin within 24 hours or were on oral contraceptive therapy. Following the standard donation of 450 ml blood, 4.5 ml blood was mixed with 0.5 ml Ware's anticoagulant. Specimens were centrifuged within 30 minutes of collection and the supernatant plasma kept on ice until tested. Additional normal volunteers were obtained at the University of Oregon Health Sciences Center. At least five men and five women in each of the age groups 20-29, 30-39, 40-49, and 50-59 years were tested for a total of 43 individuals obtained at both places.

C. LAURELL IMMUNOELECTROPHORESIS FOR AT III ANTIGEN DETERMINATION

1. Reagents

AT III Antiserum

Rabbit antiserum to human AT III was obtained from Behring Diagnostics, American Hoeschst Corp., Somerville, N.J.

Tank Buffer

Tank buffer at pH 8.6 was prepared by adding 50.28 g sodium barbital C-IV (Fisher Scientific Co.), 7.8 g barbital C-IV (Fisher Scientific Co.), and 1.57 g calcium lactate (Matheson Coleman and Bell Manufacturing Chemists, Norwood, Ohio) to 4 liters distilled water at approximately 60°C. The solution was heated and stirred on a magnetic stir plate until the solutes were dissolved.

Gel Buffer

Gel buffer, pH 8.6, was prepared by adding 4.74 g sodium barbital C-IV, 0.68 g barbital, and 0.196 g calcium lactate to 1 liter distilled water at approximately 60°C. The buffer was heated and stirred on a magnetic stir plate until solutes were dissolved.

Agarose Gel

A 1% agarose solution was prepared by adding 0.5 g agarose (Kallestad, Chaska, Mn.) to 50 ml gel buffer. This was heated with continuous stirring to boiling until the agarose was completely dissolved.

Phosphomolybdic Acid

Phosphomolybdic acid (1%) was prepared by dissolving 5 g phosphomolybdic acid, 48-hydrate (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 500 ml distilled water.

2. Equipment

Power Supply

A Beckman Duostat, set for constant voltage, supplied power for the electrophoresis. Electrodes were placed in 1 liter buffer tanks on either side of the cooling plate.

Cooling Plate

A cooling plate was constructed of plexiglass and connected to a continuous supply of cold flowing water.

Mold for Agarose Gel

Two glass plates 160 mm x 150 mm separated by a plastic frame 1.5 mm thick and 14 mm wide were held together by clamps. Using this form, a flat homogeneous gel measuring

134 mm x 80 mm x 1.5 mm could be reproducibly obtained.

Gilson Pipetman

A digital pushbutton microliter pipette #P20 with a reproducibility of <0.06 μl and error of <1% at 10 μl was supplied by Rainin Instrument Co., Inc., Brighton, Mass.

3. Method (7,61)

a. Rationale

Plasma containing AT III antigen is placed in a well punched in a flat agarose gel containing AT III antisera. Current is applied, causing the AT III antigen to move towards the anode. AT III antigen complexes with antibody in the gel forming a precipitin "rocket" pointed toward the anode. The length of the rocket is directly proportional to the concentration of plasma AT III antigen placed in the well.

b. Preparation of Antibody Containing Agarose Gels

Agarose, 20 ml at 100°C, was added to a 40 ml polypropylene tube. When the temperature had declined to 56°C, 0.35-0.5 cc Behring AT III antisera was added, mixed and the solution poured into the mold using a 10 ml plastic pipette. The agarose was allowed to set at room temperature for about 30 minutes or was covered with parafilm and stored at 4°C overnight. One glass plate and the plastic spacer were gently removed and a row of 16 wells 2 mm in diameter and 1 cm apart were punched in the gel approximately 2 cm from the bottom.

c. Electrophoresis

The gel with wells containing 7 μl of test material

was placed on the cooling plate. Buffer tanks were filled with 1 liter tank buffer. Cloth wicks were cut to match the width of the gel and voltage was set at 5 volts per cm gel using a voltmeter. The gel was covered and electrophoresis carried out for 5 hours.

d. Preparation of a Standard Curve

Immunoelectrophoresis of dilutions of normal pooled plasma in gel buffer (undiluted, 1:2, 1:4, and 1:8) was performed on each plate in addition to dilutions of test plasma (undiluted and/or 1:2).

e. Staining

Following electrophoresis the precipitin lines were developed by submerging the gels in 1% phosphomolybdic acid for 10-60 minutes. The height of the precipitin rocket was measured in mm using a light box and a mm ruler with magnification.

f. Calculations

A standard curve was drawn on log-log paper by plotting the height of the rocket obtained from each dilution of normal plasma against AT III concentration in units per ml. Test plasma values were plotted and the AT III concentration calculated from the standard curve.

D. FACTOR Xa INHIBITION ACTIVITY ASSAY FOR AT III

1. Reagents

Activated Factor X

Bovine factor Xa (Sigma Chemical Company, St. Louis, Mo.) was reconstituted to approximately 3 units/ml with Trizma

buffer and kept on melting ice during the assay. The solution was stable at least 5 hours.

Anticoagulant Free Bovine Plasma (AFBP)

Plasma harvested from bovine blood collected in 0.1 M Na oxalate (9 parts blood to one part oxalate) was dialyzed at room temperature for 4-6 hours against 1000 times its volume of 0.154 M NaCl. The dialysate was centrifuged at 2500xg for 15 minutes and the supernatant frozen in 2 ml aliquots in plastic tubes at -70°C and used within 6 months (118).

Cephalin

Cephalin was prepared from human brain according to the method of Hjort, Rapaport, and Owen (53). One cerebral hemisphere was stripped of its membranes, washed, and 200 g brain tissue ground with 150 ml acetone using a mortar and pestle. The acetone was decanted and the washing repeated five times. Ethyl ether, 700 ml, was added to the residue and left overnight at 4°C. The ether portion was decanted and saved and the residue discarded. The ether was evaporated using a vacuum aspirator and the orange phospholipid containing material washed with 200 ml acetone and evaporated to dryness. Washing was repeated and the residue again dissolved in 100 ml ethyl ether. After evaporation to dryness the weight of the residue was recorded and was dissolved in veronal buffer to a concentration of 0.09 g%. The solution was homogenized with a Potter tissue homogenizer, ultrasonicated, and frozen in small aliquots at -20°C.

Cephalin-AFBP

Cephalin and AFBP were mixed in equal parts. The mixture was stable for 8 hours at room temperature and for 3 hours at 37°C.

Plasma-CEF Reagent

As an alternative to the cephalin AFBP described above, plasma-CEF reagent supplied by Sigma Chemical Co., St Louis, Mo. was used. This reagent contains rabbit brain cephalin in AFBP, and was stored at 4°C. After reconstitution with 37°C distilled water, the contents were swirled and kept at room temperature. The solution was stable 8 hours at room temperature and 3 hours at 37°C.

Heparin

Panheprin ® (Abbott Laboratories, N. Chicago, Ill.) was diluted to 0.5 U/ml with sterile normal saline.

Tris-Maleate Buffer

Trizma maleate (Sigma Chemical Co., St. Louis, Mo.) was dissolved in distilled water to a concentration of 0.02 M. The pH was adjusted to 7.5 and the solution stored at 4°C.

Tris-Maleate Buffer with Bovine Serum Albumin

Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in tris-maleate buffer using 1 g per 100 ml buffer to prepare a 1% solution.

$CaCl_2$

$CaCl_2$ (Mallinckrodt Chemical Works, St. Louis, Mo.) was diluted to a concentration of 0.025 M with distilled water.

2. <u>Equipment</u>

<u>Automated Coagulation Testing Device</u>

A Coag-a-Mate/Single Channel, (manufactured by Warner Lambert Co., Morris Plains, N.J.) was used to determine and record clotting times of test plasmas.

<u>Heating Block</u>

A Constantemp Heating Block, manufactured by Roeco Mfg. Service, Monterey Park, Calif., was set to 37°C for incubations.

<u>Oxford Automatic Pipettors</u>

Automatic pipettors, in 100 μl, 200 μl, and 300 μl sizes (Scientific Products, McGaw Park, Illinois) were used for sampling.

3. <u>Method</u>

a. Rationale

AT III will neutralize factor Xa at a rapid rate in the presence of heparin. Test plasma is diluted and incubated with heparin to maximally activate the AT III present in the mixture. A standard solution of factor Xa is added, of which a variable amount will be inactivated in proportion to the AT III present in the test plasma. $CaCl_2$ and AFBP-cephalin are added to the incubation mixture and the clotting time determined. There is a linear relationship between clotting time and the concentration of remaining factor Xa. Since AT III binds stoichiometrically (1:1) with the factor Xa, the concentration of AT III can be calculated from the quantity of factor Xa inhibited.

b. Reference Curve

Serial dilutions of factor Xa in tris-maleate buffer with bovine serum albumin were prepared using the undiluted reconstituted factor Xa as 100% and diluting to 50, 25, and 12.5%. The dilutions were kept on melting ice.

Each dilution of Xa (0.1 ml) was added to 0.3 ml prewarmed (37°C) tris-maleate buffer and 0.1 ml 0.5 U/ml heparin and incubated 5 minutes. Prewarmed $CaCl_2$ (37°C), 0.1 ml, was added to a 0.1 ml aliquot of the primary incubation mixture and then AFBP-cephalin was added by the Coag-a-mate. Clotting time was determined using an optical endpoint on the Coag-a-mate. Tests were run in duplicate and results averaged and plotted vs. the Xa activity of each dilution on log-log graph paper.

c. Analysis of Test Plasma

Tris-maleate buffer, 0.2 ml, and 0.1 ml 0.5 U/ml heparin were prewarmed in a 12 x 75 mm glass tube and 0.1 ml diluted test plasma added. This mixture was incubated exactly 30 seconds and 0.1 ml undiluted factor Xa added. This was incubated 5-20 minutes and the clotting times determined in duplicate as described for the reference curve.

d. Standardization of Reagents

The test system was standardized and the optimal dilution for test plasmas determined by assaying AT III in dilutions of normal pooled plasma. The pooled plasma was diluted 1:20 and higher dilutions prepared from it (1:180-1:250 for the reagents used in this study). The optimal

dilution was that dilution which inhibited 40-70% of the factor Xa. Test plasmas were first analyzed using the optimal dilution and then, if necessary, appropriately diluted to inhibit 40-70% of the factor Xa.

e. Calculations

Following analysis of test plasma, the percentage of factor Xa remaining was determined from the reference curve. AT III units per ml plasma were then determined using the following calculation (one AT III unit is the concentration of AT III per 1 ml of normal pooled plasma):

1. 100 - %Xa remaining = %Xa inhibited

2. $\dfrac{\text{%Xa inhibited}}{100}$ = activity units

3. Units x dilution of plasma = units/0.1 ml plasma

4. Units/0.1 ml x 10 = units/1.0 ml

5. $\dfrac{\text{Units/ml test plasma}}{\text{units/ml normal pooled plasma}}$ = % of normal pooled plasma

6. % of normal pooled plasma/100 = U/ml AT III

4. Obtaining the Normal Population

Twenty-two healthy male volunteers were tested and plasma prepared as described above. S-2238 and Xa inhibition activity assays as well as Laurell immunoelectrophoresis were performed.

E. S-2222 PLASMA HEPARIN ASSAY

1. Reagents

S-2222

The chromogenic substrate S-2222 was supplied by Ortho Diagnostics, Raritan, N.J., in 25 mg vials. A 1 mmol/l solution was prepared by adding 25 mg of the substrate to 34 ml sterile distilled water warmed to 50°C and stirring for 1 hour. The solution was stored at 4°C protected from light by covering the container with aluminum foil. The substrate was used prior to the expiration date indicated on the vial.

Factor Xa

Bovine factor Xa (Sigma Chemical Co., St. Louis, Mo.) was reconstituted to approximately 8 U/ml with sterile distilled water. The reconstituted Xa was kept at room temperature and was stable 10 hours.

Tris Buffer

Buffer was prepared by dissolving 6.1 g Trizma base (Sigma Chemical Co., St. Louis, Mo.), 2.8 g $Na_2EDTA \cdot 2H_2O$ (Mallinckrodt Chemical Works, St. Louis, Mo.), and 10.2 g NaCl (Mallinckrodt Chemical Works, St. Louis, Mo.) in 800 ml sterile distilled water. pH was adjusted to 8.4 with 1 mol/l HCl and the volume adjusted to 1000 ml with sterile distilled water. The buffer was stable 2 months when stored at 4°C.

Heparin

Panheprin ® (Abbott Laboratories, N. Chicago, Illinois) 5000 U/ml was used in these studies.

Acetic Acid

A 50% acetic acid solution was prepared by adding an equal volume of concentrated acetic acid (Scientific Products,

McGaw Park, Ill.) to distilled water.

2. Equipment

Spectrophotometer

Absorbance was measured with a Beckman DU Spectrophotometer Model 2400 (Beckman Instruments, Fullerton, Ca.) with a Gilford attachment Model 222 in disposable semi-microcuvettes 1 cm x 0.4 cm with a 10 mm light path (Walter Sarstedt, Inc., Princeton, N.J.).

Heating Block

Incubations were performed at 37°C in a Constantemp heating block manufactured by Roeco Mfg. Service, Monterey Park, Calif.

Automatic Pipettors

Oxford pipettors (Scientific Products, McGaw Park, Ill.) in 50 μl, 100 μl, and 300 μl sizes were used for sampling.

3. Method

a. Rationale

Heparin in test plasma is measured as a heparin-AT III complex, based on the ability of this complex to inactivate factor Xa in proportion to the concentration of the heparin-AT III. Normal pooled plasma is added to the reaction mixture as a standard source of AT III. The remaining factor Xa cleaves a peptide bond in the substrate, releasing pNA. The reaction is allowed to proceed and instantly stopped by the addition of acetic acid. The absorbance is measured at 405 nm. The absorbance decreases in direct proportion to heparin concentration.

b. Standard Curves

Standards were prepared by diluting 300 µl heparin (the same brand of heparin used for treatment of the patient) with 100 ml saline to make a 15 U/ml solution and 100 µl was added to 1400 µl normal plasma giving a concentration of 1 U/ml. Further dilutions were made to encompass a 0.0-0.8 U/ml range and 0.0-0.20 U/ml range of values. All dilutions were made in plastic 12 x 75 mm tubes and kept at 4°C until used.

High Concentration Range: 0.0-0.8 U heparin per ml plasma.

1 U/ml Heparin in Normal Plasma µl	Normal Plasma µl	Equivalent Heparin Concentration U/ml
0	500	0.00
100	400	0.20
200	300	0.40
300	200	0.60
400	100	0.80

Low Concentration Range: 0.0-0.20 U heparin per ml plasma.

1 U/ml heparin in Normal Plasma µl	Normal Plasma µl	Equivalent Heparin Concentration U/ml
0	1000	0.00
50	950	0.05
100	900	0.10
150	850	0.15
200	800	0.20

c. Procedure

In the first tube, 100 µl standard or test plasma, 100 µl normal plasma, and 800 µl buffer were mixed and kept on ice.

A 200 ml aliquot of the contents of the first tube was

added to a second tube and incubated at 37°C. Three to four minutes later 100 μl of factor Xa was added and the contents of the tube mixed and incubated at 37°C for 30 seconds for the high concentration range and 180 seconds for the low concentration range. S-2222, 200 μl, prewarmed to 37°C was added to the second tube, mixed vigorously and incubated at 37°C for an additional 180 seconds. The reaction was stopped by the addition of 300 μl of 50% acetic acid with thorough mixing.

The reaction mixtures were protected from light until absorbance was measured at 405 nm against a distilled water blank. If the test plasma was lipemic or icteric, the absorbance of a test plasma blank (200 μl diluted plasma, 300 μl 50% acetic acid, and 300 μl distilled water) was measured against a normal plasma blank (200 μl of the 0.00 U heparin/ml standard, 300 μl 50% acetic acid, and 300 μl distilled water). The absorbance of the test plasma blank was subtracted from the absorbance of the test plasma and this value used in the calculation of heparin concentration.

The reference curve was drawn by plotting the absorbance obtained for each standard sample against the known heparin concentration. Heparin concentration in the test plasma was calculated by referring to the standard curve.

F. COAGULATION SCREENING TESTS

The following assays were kindly performed by the Hematology Division of the Clinical Pathology Laboratory

according to Hematology Division procedures:

Fibrin Degradation Products (FDP)

FDP were measured using a staphylococcal clumping test.
(Procedure No. 534.)

Fibrinogen

Fibrinogen was measured with the Dade Data-Fi Fibrin-
ogen Kit supplied by Dade Division – American Hospital
Supply Corp., Miami, Florida.   (Procedure No. 532.)

Platelet Counts

Platelet counts were performed on a Coulter Thrombo-
counter (Coulter Electronics, Inc., Florida) (Procedure
No. 565) or by phase contrast microscope (American Optical
Co.) and hemocytometer (American Optical Co.) (Procedure
No. 504).

Activated Partial Thromboplastin Times and Prothrombin Times

aPTT (Procedure No. 528a) and PT (Procedure No. 530a)
were measured by optical density detection of clotting on a
Lancer Coagulyzer (Sherwood Medical Industries, St. Louis,
Mo.).

Plasma Protamine Paracoagulation Test (3P)

3P was measured using 1% protamine sulfate, supplied by
Lilly Pharmaceutical, to detect the presence of fibrin mono-
mer (Procedure No. 526).

G. STATISTICS

Statistical methods, such as mean, standard deviation,

student t test, paired t test, correlation (r), and linear
regression were performed using a Hewlett-Packard programmable
calculator and standard statistical methods (27,116).

H.   STUDY OF PATIENTS RECEIVING HEPARIN THERAPY

   1.   Selection and Informed Consent

   Patients with deep vein thrombosis and/or pulmonary
emboli were sought for study. Patients were accepted only
within twenty-four hours of starting continuous infusion
heparin therapy. Written informed consent (see Appendix A)
was obtained and patients advised that they were free to
withdraw from the study at any time, as dictated by the
human experimentation committee of the University of Oregon
Health Sciences Center.

   2.   Obtaining Blood Samples

   Daily samples of 18.5 ml blood were drawn by atraumatic
venipuncture. Nine ml was mixed with 1 ml Ware's anticoag-
ulant in a polypropylene tube for AT III and heparin determin-
ations. This tube was immediately placed on ice and plasma
prepared as described. Samples were frozen until assay. An
aliquot of 4.5 ml blood was added to a Venoject vacuum blood
collection tube (Kimble-Terumo, Inc., Elkton, Maryland) con-
taining 3.8% sodium citrate for determination of PT, aPTT,
fibrinogen, and 3P. Platelet counts and hematocrits were
performed with 2 ml blood added to 3 mg $Na_2 \cdot EDTA$ in a Venoject
vacuum blood collection tube. An additional 2 ml blood was
added to a Vacutainer tube (Becton-Dickinson, and Co.,

Rutherford, New Jersey) containing soybean trypsin inhibitor and thrombin for FDP determination.

3. Assays Performed

AT III measurements by the S-2238 and Xa inhibition activity assays and Laurell immunoelectrophoresis for antigen determination were performed on each daily sample. Heparin levels were measured with S-2222. PT, aPTT, fibrinogen, FDP, 3P, and platelet count (corrected for hematocrit) were performed by the Clinical Pathology Laboratory. Record was made of heparin and Coumadin therapy as well as other medications taken by the patients.

I.   STUDY OF AT III DEFICIENT STATES

Four patients with hereditary AT III deficiency from four different families were studied with AT III activity and antigen assays. Two patients with DIC as well as two with severe liver disease were tested.

J.   CASE HISTORIES

1. Hereditary AT III Deficiency

Representatives of four families having hereditary AT III deficiency were studied.

a.   The S. family was first diagnosed as having AT III deficiency in France when the sister of C.S. was tested after suffering mesenteric vein thrombosis and found to have an AT III level of approximately 50%. The father, a nephew, and possibly a half-brother of C.S. were found to be affected.

C.S. had a deep vein thrombosis at age 32 and another one the next year which led to a bilateral iliac vein ligation. Following childbirth she suffered a mesenteric vein thrombosis, but has been asymptomatic since that time except for occasional transient episodes of tenderness and swelling of her legs.

R.S., the 21-year-old son of C.S., has not suffered from any thromboembolic events.

b. M.F., a 30-year-old female, has had multiple pulmonary emboli and thrombophlebitis since age 17. Her mother was diagnosed as having hereditary AT III deficiency and is currently being treated with anticoagulants. A 33-year-old sister had a pulmonary embolus with pregnancy. The grandmother died at age 36 and an aunt at age 42 of pulmonary emboli.

c. C.V. was diagnosed at age 41 as having hereditary AT III deficiency. Since age 18 or 19 he had had at least 10 episodes of deep vein thrombosis and on two occasions pulmonary emboli.

d. O.H., a white male in his sixties, has suffered multiple episodes of thrombophlebitis since approximately age 14. R.H., his 35-year-old son, is mentally retarded as a result of cerebral hemorrhage followed by thrombotic events. He has experienced multiple thrombotic episodes since that time. Another son with the same history died at age 25. A third son also has a history of thrombosis, although is not debilitated.

2. <u>Disseminated</u> <u>Intravascular</u> <u>Coagulation</u>

    a.   L.S. was admitted with a gunshot wound to the head and a DIC work-up was positive: prolonged prothrombin time and activated partial prothrombin time, fibrinogen of 23 mg/dl, and positive plasma protamine paracoagulation test.

    b.   F.M. was admitted to intensive care suffering congestive heart failure, chronic obstructive pulmonary disease, digitalis toxicity, and shock. A DIC work-up was positive: prolonged prothrombin time and activated partial thromboplastin time, fibrinogen of 90 mg/dl, positive plasma protamine paracoagulation test, and a platelet count of 28,000/cumm.

3. <u>Liver</u> <u>Disease</u>

    a.   L.S., was a white male with severe liver failure as well as primary fibrinogenolysis.

    b.   M.C., a young girl approximately 6-years-of-age, had severe liver disease. A DIC workup was negative.

4. <u>Heparin</u> <u>Therapy</u>

    a.   Patient one was a black male in his late twenties with a deep vein thrombosis, possibly a result of drug abuse. He was treated with continuous infusion heparin therapy and Coumadin (warfarin).

    b.   Patient two was a white male in his sixties with a recurrent right leg thrombophlebitis. Oral anticoagulation had been insufficient following the first thrombotic incident and led to his rehospitalization. He also had a history of heart disease and was treated with many medications including

Slow-K, Colace, quinidine, Norpace, and Lasix as well as continuous infusion heparin and Coumadin.

      c.   Patient three was a white male in his early twenties admitted with the diagnosis of pulmonary embolism. He also had a heart abnormality and was treated with digoxin and KCl as well as continuous infusion heparin therapy and Coumadin.

RESULTS

A. BASIC STUDIES: S-2238 ACTIVITY ASSAY

Initial studies using the synthetic chromogenic substrate S-2238 for the measurement of AT III were directed toward verifying that the concentration of substrate was sufficient for the assay and to document optimal incubation times. For these studies, 0.75 mmol/l substrate was incubated with 10 U/ml thrombin and 0.25 U/ml AT III contained in a dilution of normal pooled plasma obtained from twenty donors. Incubation times ranging from 5-50 seconds were chosen. At the end of each thrombin-substrate incubation, the reaction was immediately stopped with acetic acid and the absorbance measured at 405 nm. Under these conditions, incubation times from 10-45 seconds gave a linear response with absorbance (Figure 1). As a result of these studies an incubation time of 30 seconds was used for all subsequent assays.

A second experiment was performed to determine the optimal concentration of thrombin for use in the AT III assay. Concentrations of thrombin Roche from 0-10 U/ml were incubated with 0.75 mmol/l S-2238 for 30 seconds at 37°C. The absorbance was measured after the addition of acetic acid to stop the reaction. As shown in Figure 2, a linear relationship existed between absorbance and decreasing thrombin concentration. Since the addition of increasing amounts of AT III would result in a decrease of absorbance, a concentration of 10 U/ml of thrombin was selected for future assays.

Two bovine thrombin preparations were tested; that obtained from Hoffman-LaRoche (Switzerland) and that from Parke-Davis (USA). A comparison of these two preparations was carried out using a 10 U/ml concentration of each. This concentration was produced by diluting appropriate amounts of lyophilized thrombin as indicated on the label. These concentrations were not compared to an NIH thrombin standard. The AT III assay was performed using 10 U/ml thrombin, varying amounts of normal human plasma pool as a source of AT III, and the absorbance of pNA determined after a 30 second incubation. Both thrombin preparations gave a linear relationship between AT III concentration and absorbance (Figure 3). However, repetitive experiments indicated there was considerable variation of activity with different lots of Parke-Davis thrombin. For this reason, thrombin Roche was used for future experiments.

One question that has not been answered to date is whether thrombin alone is cleaving pNA from the chromogenic substrate or whether other serum proteases in the bovine preparation may be active. Since hirudin (a protein derived from leeches) is known to react exclusively with thrombin and to be nonreactive in the presence of factor Xa, plasmin, trypsin, and other serine proteases, a series of experiments was carried out to determine whether hirudin would diminish hydrolysis of pNA from S-2238. Increasing amounts of hirudin from 0 to 10 antithrombin units were incubated with equal amounts of 20 U/ml thrombin Roche or 10 U/ml human thrombin

(to yield final concentrations of 0-5 AT-U hirudin and 10 U/ml or 5 U/ml thrombin) for 5 minutes at 37°C. An aliquot of this incubation mixture was removed and added to 400 μl buffer, after which 300 μl S-2238 was added. After 30 seconds of incubation, the reaction was stopped with acetic acid and the absorbance measured. As is indicated in Figure 4, both the bovine and human thrombin were completely inhibited by the hirudin. At a concentration of 2.5 AT-U hirudin, no measurable pNA was found. The curve was linear until a concentration of 1.25 AT-U hirudin was added. These experiments indicated that other non-thrombin serine proteases were not present in significant quantities in the bovine thrombin preparations used for the assay. Furthermore, the bovine thrombin used in these experiments gave results that were almost identical to a highly purified preparation of human thrombin.

Based on these initial experiments, a standard set of conditions were determined for future AT III assays using the chromogenic substrate S-2238. A working substrate concentration of 0.75 mmol/l, a thrombin concentration of 10 U/ml utilizing thrombin Roche, and a 30 second thrombin-substrate incubation period were used. When serial dilutions of normal pooled plasma were assayed, a straight line was obtained (Figure 5). To determine the variation in the reference curve, nine consecutive reference curves obtained over a two month time period were studied. The mean value for each data point, plus or minus one standard deviation

was plotted (Figure 6). Again, remarkably little variation in the curve was seen.

In order for the assay using the chromogenic substrate for AT III to be clinically useful, the stability of test plasma upon storage under various conditions needed to be determined. As shown in Table 1, storage of 3 normal plasmas at temperatures ranging from -70°C to 25°C varied little over time periods ranging from 1 hour to 4 weeks. Even plasma that was left on red cells for 4 hours at room temperature gave normal results. As indicated, all values except one were within 0.05 U/ml of the AT III concentration measured immediately on fresh plasma. These studies indicated that AT III contained in plasma samples was very stable upon storage.

For this assay to be effective in patients who are receiving bolus or continuous infusion heparin, studies were required to exclude the effect of exogenous heparin on the assay. In these experiments, 0.1 ml of diluted heparin or normal saline was added to 0.9 ml of normal pooled plasma and the S-2238 assay used to determine AT III concentration. Concentrations of heparin used in this experiment varied from 0-50 U/ml. (The highest heparin level observed in this study was 0.332 U/ml.) As indicated in Table 2, heparin concentrations of up to 50 U/ml had virtually no effect on AT III concentration. Therefore, this assay may be used in patients receiving even large amounts of intravenous therapeutic heparin.

The reproducibility of the assay was determined by measuring AT III activity 32 times on the same plasma sample over a 4 week time period. The mean AT III concentration was 0.88 U/ml with a standard deviation of 0.021 and a coefficient of variation of 2.4%

B.   BASIC STUDIES: LAURELL IMMUNOELECTROPHORESIS

Optimal conditions for rocket immunoelectrophoresis were selected by varying the concentration of antisera in the agarose as well as duration and intensity of electrophoresis. Reproducible results were obtained using 0.35-0.5 ml AT III antisera and electrophoresing 5 hours at 5 volts per centimeter. A reference curve was plotted using dilutions of pooled normal plasma from 0.125-1.0 U/ml AT III. Rocket heights ranged from an average of 6.8 mm to 28 mm. There was a linear relationship between U/ml AT-111 and rocket height when plotted on log-log paper (Figure 7). Nine sequential reference curves were compiled over a 2-month time period and the mean for each point on the curve plus or minus 1 SD was plotted (Figure 8). The day-to-day variation was quite small. The same plasma sample was run 16 times over 2 days to determine the reproducibility. The mean AT III concentration was 1.03 U/ml, the standard deviation 0.05, and the coefficient of variation 4.9%.

C.   BASIC STUDIES: FACTOR Xa INHIBITION ACTIVITY ASSAY

Optimal conditions for the Xa inhibition assay were

first established. A factor Xa concentration of approximately 3 U/ml was found to give the steepest curve. The cephalin and factor Xa concentrations were titered to give a starting clotting time of 11-13 seconds. A concentration of 0.5 U/ml heparin was found to be sufficient in accelerating the AT III present in test plasma. In the absence of heparin, there was no inhibition of the factor Xa.

A standard curve was drawn on log-log paper by plotting the concentrations of dilutions of a standard solution of Xa (12.5-100%) against the clotting time of each dilution (Figure 9). Eight consecutive standard curves were compiled over a 2-month time period, again showing little variation. Figure 10 shows the mean plus or minus 1 SD for each point on the curve.

The accuracy of the assay was checked by diluting normal pooled plasma with buffer to give AT III concentrations of 0.50 U/ml and 0.25 U/ml. Analysis of the 0.50 U/ml dilution yielded 0.50 U/ml and the 0.25 U/ml dilution measured 0.18 U/ml.

The reproducibility of the assay was determined by running the same plasma sample consecutively nineteen times. The mean AT III concentration was 0.96 U/ml with a standard deviation of 0.054 and the coefficient of variation 5.7%.

D. <u>BASIC</u> <u>STUDIES</u>: <u>S-2222</u> <u>PLASMA</u> <u>HEPARIN</u> <u>ASSAY</u>

As previously described, this assay for plasma heparin is based on the ability of heparin contained in test plasma

to inhibit the activity of added factor Xa in the presence of a standard amount of AT III. It was first necessary to determine the optimal concentration of factor Xa and three concentrations were tested (4, 8, and 10 U/ml). When a standard curve encompassing 0-0.8 U/ml heparin was plotted against absorbance, the resulting curve was not linear on arithmetic paper (Figure 11). A linear relationship was obtained when the curve was replotted on log-log paper (Figure 11A). The 4 U/ml factor Xa provided a flat curve with little change in absorbance with increasing heparin concentration. The 8 and 10 U/ml factor Xa concentrations gave parallel curves with a more sharply inclined slope. Since the results appeared virtually identical with these two concentrations, the lower one (8 U/ml) was chosen because of its lesser cost.

Two concentration ranges of heparin were necessary to encompass those levels found in human plasmas receiving therapeutic anticoagulation (0-0.2 U/ml and 0-0.8 U/ml). A single representative curve in both concentration ranges is plotted in Figure 12. A composite of seven consecutive curves over a 2-month time period for the high concentration range and five curves for the low concentration range are shown in Figure 13. The mean plus or minus 1 SD is plotted for each point on the curve.

The same plasma sample was assayed ten times in the same day. The coefficient of variation was 14%.

E. NORMAL POPULATION STUDIES

AT III concentration was determined in groups of normal individuals using all three assays: S-2238 synthetic chromogenic substrate assay, Factor Xa Inhibition activity assay, and AT III antigen assay by electrophoresis. The results are plotted in Figure 14 and shown in tabular form in Table 3. Of note, the mean concentrations for all three assays were remarkably similar (0.985 U/ml - S-2238; 0.994 u/ml - Xa Inhibition; 0.980 U/ml - immunoelectrophoresis). There were no significant differences between the mean values obtained by all three assays ($p > 0.05$).

Since previous reports have indicated minimal differences in AT III activity according to age and sex, these parameters were studied in the normal population. As shown in Table 4, AT III concentration determined by S-2238 and immunoelectrophoresis were quite similar between sexes and ages. However, statistical analysis indicates that the mean AT III concentration using S-2238 for females aged 30-39 years was significantly less than for females aged 40-49 and males aged 20-29 ($p < 0.05$).

F. CLINICAL SURVEY

1. Hereditary AT III Deficiency

AT III concentration was determined in family members from the previously described four families (see patient description in the Materials and Methods section). AT III activity was reduced to an average of 0.45 U/ml (range

0.40-0.63 U/ml), with S-2238, in all affected individuals, as shown in Figure 15 and Figures 16-18. With the exception of the sole representative of one family studied, the level of AT III antigen was equivalent to AT III biologic activity. In this individual, AT III antigen was 0.66 U/ml, which was significantly higher than the AT III activity of 0.41 U/ml (S-2238) or 0.34 U/ml (Xa Inhibition). Crossed immuno-electrophoresis, kindly performed by Brian O'Hollaren by a modification of the method used by Gomperts, et. al. (41) showed two distinct peaks when run using agarose gel containing heparin. This implies a qualitative defect in this patient, as a single peak is seen in normal subjects and other patients with hereditary AT lll deficiency.

2. <u>Disseminated Intravascular Coagulation</u> (DIC)

Two patients with well documented DIC were studied (see patient descriptions in Materials and Methods section). The AT III activity of one patient, L.S., was just below the lower limits of the normal range (0.84 U/ml as measured with S-2238). The second patient, F.M., had an extremely low AT III concentration of 0.36 U/ml (S-2238). AT III activity and AT III antigen were equivalent in these patients (Figures 15-18).

3. <u>Hepatic Cirrhosis</u>

Two patients with far advanced cirrhotic liver disease were tested (see patient descriptions in Materials and Methods section). AT III activity was markedly reduced to 0.25 U/ml in one patient (L.S.) and 0.42 U/ml in the other

(M.C.). Again, AT III activity and antigen concentrations were equivalent (Figures 15-18).

4. Heparin Therapy

Ten patients were tested for AT III concentration 3 to 4 days after starting continuous infusion heparin therapy. AT III was measured by both activity and antigen assays. AT III was decreased to an average of 0.70 U/ml, with a range of 0.49-0.90 U/ml (S-2238). There was no significant difference in the mean AT III values for the three assays (Figure 15).

Three of these patients (see patient descriptions in Materials and Methods section) were studied prospectively from the inception of heparin therapy until the cessation of heparin and they were discharged. AT III concentrations plotted in relationship to the amount of heparin infused are shown in Figures 19 and 20. The amount of heparin infused was rather constant, with the exception of patient three. Although in each case AT III levels fell during heparin infusion, there was no obvious relationship of the decrease in AT III to the amount of heparin infused. The decline of AT III activity and antigen were similar in patient one (Figure 19). However, in patients two and three (Figure 20), AT III antigen was clearly higher than AT III biologic activity at the beginning of heparin infusion. This discrepancy grew less as therapy was continued and following cessation of heparin, AT III antigen and activity were similar. This discrepancy of higher

antigen than activity was also observed in one other patient tested for AT III after 3-4 days of continuous infusion heparin therapy.

G. CLINICAL SURVEY OF PLASMA HEPARIN

Plasma heparin concentration was assayed in ten random samples obtained from patients receiving continuous infusion heparin therapy and in the three previously described patients receiving continuous infusion therapy. As shown in Figure 21, plasma heparin concentration in these individuals ranged from 0 to 0.335 U/ml. (Values less than 0.02 U/ml were regarded as 0 U/ml.) When the AT III concentration was known, plasma heparin values were corrected according to the formula (106):

$$Hep_{conc} = Hep_{conc\ found} + 0.025\ \frac{100 - AT\ conc}{50}$$

Since the usual laboratory test used for following heparin therapy is the activated partial thromboplastin time, the heparin concentrations were plotted against the aPTT measured on samples obtained at the same time (Figure 21). The correlation coefficient was 0.53 (p <.001).

In order to determine if there was a relationship between AT III concentration and plasma heparin concentration during continuous infusion heparin therapy, values for these determinations were plotted on arithmetic paper (Figure 23). As shown, there appeared to be no direct relationship

between the concentration of heparin measured in plasma and the decline in AT III concentration.

H. CORRELATION OF AT III ACTIVITY AND AT III ANTIGEN IN THE CLINICAL MATERIAL

The relationship between AT III antigen, AT III activity by the biologic assay using factor Xa inhibition, and AT III activity using the chromogenic substrate S-2238 were remarkably similar, with the few exceptions previously mentioned. There was no significant difference between the mean AT III values obtained with the three assays for both normal subjects and clinical cases (Figure 15).

The correlation in normal subjects and clinical cases of AT III activity by S-2238 with that obtained using factor Xa inhibition was excellent (Figure 16), with $r = 0.98$ ($p < 0.001$). Similarly, both activity assays related very well to antigen determination by rocket electrophoresis. The correlation coefficient of S-2238 activity and antigen concentration (Figure 17) was 0.94 ($p < 0.001$). Finally, the correlation of Xa inhibition activity and antigen concentration (Figure 18) was $r = 0.96$ ($p < 0.001$).

DISCUSSION

In the past the measurement of proteolytic enzymes of the coagulation system and their inhibitors required the use of substrates purified from human plasmas, either normal plasma or plasmas deficient in certain clotting factors. Almost uniformly, the endpoint for these determinations was the timing of the formation of fibrin. Such assays, while immensely useful in the past, have several major drawbacks. Appropriate plasmas may be expensive or difficult to obtain; purification may be tedious and able to be performed in only a few laboratories; and stability of the plasma may be severely limited, requiring storage at -70°C with great difficulties in standardization and transport.

A recent advance has been the development of synthetic substrates with short chains of amino acids closely resembling the cleavage sites of the natural substrates. Proteolysis of the substrate results in the release of a colored compound (pNA) which can then be measured spectrophotometrically. Such synthetic substrates have immense potential for the simplified specific assay of proteolytic enzymes.

Because the potential usefulness of these substrates is so great in both research and clinical coagulation testing, it was decided to study some of these substrates in detail in order to learn about their specificity, sensitivity, and

usefulness in clinical laboratory testing. The chromogenic substrates S-2238 for AT III measurement and S-2222 for plasma heparin analysis were chosen for study.

An AT III assay was chosen since AT III is the natural clotting inhibitor of the most importance and because previous assays for the protein have been extremely tedious, difficult to perform, and of limited use in a clinical laboratory. A plasma heparin assay was selected so a second substrate could be tested and so that the biologic interactions of AT III and heparin in a clinical population could be explored in more detail. Neither substrate has been extensively studied in the past as to results in normal populations, or more importantly, in clinical pathologic states where results from the assays might be critical. Accordingly, chromogenic assays for AT III and plasma heparin were established, standardized, applied to normal and abnormal clinical states and compared in detail to previously used assays.

The biologic and clinical significance of AT III is just beginning to be appreciated. Clearly, the reduced levels in hereditary AT III deficiency correlate with the occurence of thromboembolism and possible death. Recognition of families and specific individuals with this disorder is crucial so that appropriate treatment with short or long term anticoagulants may be substituted. For example, warfarin may be given chronically for patients with a long history of repeated episodes of thromboembolism and other

agents may be used for short term protection during childbirth, trauma, and surgical procedures.

AT III deficiency may be acquired, as well as inherited, as seen in such clinical conditions as cirrhotic liver disease, DIC, and continuous heparin infusion. Recognition of these deficiencies may lead to improved methods of treatment in these conditions. As an example, drug manufacturers have begun to make AT III concentrates available and such preparations have already been tested in man (96).

Certainly the benefits of heparin therapy for the treatment of thromboembolism have been established (8,107), yet there are many complications of heparin use.

The risk of hemorrhage is high, with as many as 10-20% of patients receiving intermittant intravenous heparin demonstrating major bleeding episodes (26) and the threat of recurrent thromboembolism remains a problem with insufficient anticoagulation (9).

The monitoring of heparin therapy also remains a problem. Many factors appear to greatly influence the in vivo effect of the amount of heparin administered (37) and thus heparin requirements vary from patient to patient. The activated partial thromboplastin time is the generally accepted method of heparin regulation (9,26,37), yet it is not totally useful (105). The heparin assay using S-2222 is based on the interaction of heparin and AT III and the ability of this complex to inhibit activated factor X. This assay should theoretically reflect more accurately the in vivo actions of

heparin, particularly measuring that fraction of heparin which is active in the acceleration of AT III.

The results from the studies with S-2238 for AT III deficiencies indicate that this substrate will be very useful in the future. The assay is easy to perform, sensitive, specific for the action of AT III, and correlates well with other assays for AT III.

The assay may be performed by a single technologist, requiring approximately 3 minutes total incubation time per test plus minimal time to read absorbance. The plasma used in the assay is easily collected in readily available tubes. A small amount of plasma is needed (50 μl), thus the test can be used in infants and in situations where specimens are difficult to obtain. Storage and transport are easily accomplished. AT III was found to be stable for weeks when frozen, allowing transport of plasma on dry ice and this has indeed been accomplished successfully.

The test system appears to be quite specific for AT III. The specific inhibition of thrombin with hirudin blocked all hydrolysis of the substrate, indicating there are no other proteolytic enzymes in the thrombin preparation which would interfere with the assay. The results of this experiment also showed it is possible to use commercial bovine thrombin preparations rather than the expensive and difficult to purify human thrombin.

Conceivably other proteolytic enzymes in test plasma could cause hydrolysis of the substrate. However,

cross-over is reported to be minimal (73,84) and in clinical situations, levels high enough to effectively compete for the substrate must be very rare. The use of heparin in the S-2238 buffer further insures that AT III is the only thrombin inhibitor being measured. Alpha-2-macroglobulin and alpha-1-antitrypsin are inhibitors of thrombin as well, but their action is not accelerated by heparin and they are slower acting (80). Thus the presence of heparin and the short incubation times used lead to the specific measurement of AT III.

The reproducibility of the assay is excellent with a standard deviation of 0.021 and coefficient of variation of 2.4%. The assay appears to be extremely sensitive as well, capable of measuring normal levels as well as extremely low AT III levels.

Excellent correlation of this assay was seen with other assays for AT III in normal individuals as well as clinical conditions such as DIC, cirrhotic liver disease, continuous infusion heparin therapy, and hereditary AT III deficiency. For example, when compared with another biologic activity assay using activated factor X rather than thrombin and a fibrin clot end point, the correlation coefficient was 0.98 (p <0.001). Similarly, when the results were compared with an assay of AT III antigen using Laurell immunoelectrophoresis, the correlation was again excellent (r = 0.94, p <0.001) except in a few cases to be discussed later.

The substrate S-2222 for measurement of plasma heparin concentration also appeared to be a practical and sensitive

assay. Heparin levels from 0.02 to 0.332 U/ml were measured in patients receiving continuous heparin infusion. One sample measured less than 0.02 U/ml heparin, which was regarded as 0.00/ml, as the sensitivity of the assay is reported to be 0.02 U/ml (104). As Teien reported that an error of 0.025 U/ml heparin is introduced with varying AT III concentration (106), values were corrected when the AT III level was known.

Again, the substrate was found to be an efficient way of assaying heparin, requiring minimal time for completion, especially in the low concentration range. Small plasma samples (100 µl) were needed and thus collection of specimens easily accomplished.

Transport of specimens is somewhat limited as the plasma needs to be separated from the platelets within 30 minutes to avoid heparin neutralization by platelet factor 4. However, once separated, plasmas may be frozen without loss of heparin activity (106).

Plasma heparin levels measured with S-2222 were correlated with the aPTT's performed on samples drawn at the same time. The correlation coefficient was 0.53 (p <0.001), indicating a significant but not strong relationship. Although the S-2222 heparin assay is easily and rapidly performed, the aPTT is more readily accomplished. In view of this, and the high cost of S-2222, the heparin assay appears to offer no distinct advantage over the aPTT. However, the use of S-2222 still has potential for studying the relationships of

heparin and AT III. It may also be that heparin levels could be of use in the hard to manage heparinized patient.

In an effort to further study the fall of AT III with continuous infusion heparin therapy as reported by Marciniak (70), three patients receiving such heparin treatment were followed. AT III levels declined an average of 23% (measured with S-2238) with concentrations as low as 0.55-0.65 U/ml being measured. Low levels were also measured in random testing of patients receiving continuous heparin infusion with an average of 0.70 U/ml and a low value of 0.49 U/ml. With the cessation of heparin, AT III rose to normal levels within 2 days.

Of interest, two of the patients had initially low AT III levels. One possible explanation is the inhibitor was con- sumed during the clotting process (2,11). AT III in one of the patients returned to normal with the cessation of heparin, giving support to this possibility. The other patient was discharged the day heparin was stopped and thus the response of AT III was unable to be measured.

Unlike Marciniak's observations, two of the three patients studied had greater AT III antigen levels than activity at the beginning of the course of heparin treatment. This dis- crepancy diminished as the course of treatment continued. A higher antigen than activity level was noted in one other patient receiving heparin infusion who was tested only one time. There are several possible reasons for the higher antigen levels. The higher antigen could represent an

abnormal form of the AT III molecule, as has been previously reported (41,95). This phenomenon was also found in one of the seven patients having hereditary AT III deficiency studied in this paper and appears to be an abnormal form of AT III as shown by crossed immunoelectrophoresis of her plasma. Since the discrepancy was present initially in both patients and decreased, it is unlikely the heparin was a factor. The higher antigen concentration may have represented AT III which had been utilized in the inactivation of clotting factors during the thrombotic event but not yet cleared from the circulation. Both patients were also taking many drugs for heart problems, and it may be that certain drugs either interfere with normal AT III metabolism or inactivate AT III by unknown mechanisms.

There was no obvious relationship of plasma heparin concentration, the amount of heparin infused, and AT III levels measured in the three patients. The half-life of heparin (about 2 hours) is much shorter than that of AT III (67 hours) and it may be that both would need to be measured frequently to observe an obvious relationship between the two factors. All three patients were treated with Coumadin (warfarin) during part of their treatment with heparin. Since warfarin can raise AT III concentration (69,72,87,114) any relationship between heparin concentration and its effect on AT III may have been masked by the effect of Coumadin. It also has been reported heparin causes an increase in the fractional catabolic rate of AT III (20). The AT III-heparin interaction

may be studied with more relevance by following AT III metabolism with varying heparin doses.

Routine coagulation screening tests were performed in an effort to discern if a low grade DIC could be the cause of a fall in AT III during heparin therapy. The results were essentially negative, although the platelet count decreased in two patients and fibrinogen fell in two patients. However, no consistent pattern emerged and no pathologic values were obtained.

The full significance of the decrease of AT III with heparin therapy is unclear. It would appear that sudden withdrawl of heparin without sufficient overlapping with warfarin could render the patient susceptible to recurrence of thromboembolism. AT III levels approximating those seen in hereditary AT III deficiency were measured in some patients receiving heparin therapy. Marciniak points out that these low AT III levels measured in patients receiving heparin should not be interpreted as hereditary AT III deficiency (70). This emphasizes that caution is needed in interpreting AT III levels measured during heparin therapy. This caution should be extended to those patients receiving warfarin, as levels in this instance may be increased.

This study further proved the usefulness of the synthetic substrates for the assay of biologically important factors. The substrates were found to be accurate, sensitive, and easy to use. At the present time the substrates are expensive and some reagents difficult to obtain. However, with

the inevitable increased demand for the substrates, costs will certainly decrease. Other uses for the substrates are already under investigation. They are easily adapted to automation (98) and may even be used in routine coagulation testing, such as for prothrombin and activated partial thromboplastin times (73). Certain clotting factors such as factor Xa can be measured (85).

With increased availability and development of more synthetic substrates having specificity for proteolytic enzymes, testing of the coagulation and fibrinolytic systems could well be dramatically changed.

CONCLUSION

Two chromogenic substrates were evaluated for clinical use. S-2238 was used to measure AT III. Excellent correlation was found with antigen levels (except in a few cases) and with a biologic activity assay based on inactivation of factor Xa in normal subjects as well as hereditary and acquired AT III deficiencies. The method was reproducible, accurate, and easily performed.

Plasma heparin levels were measured with S-2222. Although no clear-cut clinical significance was found for use of the assay, there is potential for research applications and occassional hard to manage heparinized patients.

Based on the use of the chromogenic substrates used in this study, their continued use and further development is certainly warranted. They greatly expand the potential for improving testing of the coagulation and fibrinolytic systems.

APPENDIX A
CONSENT FORM

I, _____ agree to
    (last name)   (first name)   (middle initial)

participate in a study entitled "Complications of Heparin Therapy" under the direction of Scott H. Goodnight, Jr., M.D. The purpose of this study is to investigate the causes of excessive bleeding or blood clotting during the administration of an anticoagulant (heparin) for treatment of clots in the leg veins or lungs. Heparin treatment is the best treatment currently available for blood clots. This study will consist of measurements of various clotting factors in my blood at intervals during and after the infusion of heparin. Once or twice per day for up to ten days approximately 10-15 ml (2 - 3 teaspoonfuls) of blood will be obtained from a vein in the arm.

The risks of this study include a hematoma (bruise) around the blood drawing site on my arm. The benefits to me include careful administration and monitoring of the heparin and early recognition and treatment of potential complications of therapy such as bleeding or recurrence of thromboembolism.

I will not benefit from these procedures, but the results of these studies may in the future help many other people undergoing heparin therapy.

Dr. Scott H. Goodnight or other members of his staff have offered to answer any questions that I may have about these procedures. I understand that I am free to refuse to participate or withdraw from participation in this study at any time and it will in no way affect my relationship with the University of Oregon Health Sciences Center.

I have read the foregoing information, and agree to participate in this study.

_____   _____
Date and Time   Subject Signature

_____   _____
Date and Time   Witness

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      stasis, Vienna, Austria: G. Gistel and Cie., 1973.
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Table 1
Variation of AT III Concentration with Storage

Time	Storage	Temperature (°C)	AT III Concentration (U/ml)		
			Plasma Sample		
			A	B	C
1 hr	plasma	25	1.00	0.88	0.87
2 hr	plasma	25	1.00	0.88	0.83
3 hr	plasma	25	0.98	0.88	0.84
4 hr	plasma	25	0.95	0.88	0.81
2 hr	plasma	4	0.95	0.87	0.81
3 hr	plasma	4	0.98	0.88	0.83
4 hr	plasma	4	0.95	0.84	0.83
24 hr	plasma	4	0.94	0.89	0.88
4 hr	plasma + RBC*	25	0.96	0.86	--
4 hr	plasma + RBC**	25	1.00	0.92	0.86
4 hr	plasma	-20	0.91	0.87	0.79
4 hr	plasma	-70	0.94	0.84	0.83
24 hr	plasma	-20	0.99	0.87	0.84
24 hr	plasma	-70	1.04	0.89	0.86
2 wk	plasma	-20	0.99	0.85	0.84
2 wk	plasma	-70	0.95	0.88	0.89
4 wk	plasma	-20	1.02	0.89	0.88
4 wk	plasma	-70	1.04	0.89	0.86
		Mean	0.98	0.88	0.84
		SD	0.036	0.019	0.027

*   Whole blood was stored at room temperature without
    centrifugation until just prior to testing.

**   Whole blood was centrifuged and plasma left on RBC's
    at room temperature until just prior to testing.

Table 2

AT III Concentration (S-2238) Following the
Addition of Heparin to Normal Plasma*

Heparin	AT III Concentration
U/ml	U/ml
0	0.88
2.0	0.86
4.0	0.89
8.0	0.88
16.0	0.90
50.0	0.85

* 0.1 ml of diluted heparin or normal saline was added
  to 0.9 ml of normal plasma and the AT III concentra-
  tion determined on the mixture.

Table 3

AT III Concentration of Normal Populations by
Biologic Activity Assays (S-2238 and Xa Inhibition)
and Antigen Assay (Immunoelectrophoresis)

Method	Normal Population		
	Mean	2 SD	Range
		(AT III U/ml)	
S-2238 (n=43)	0.985	0.170	0.81 - 1.15
Xa Inhibition (n=22)	0.994	0.134	0.86 - 1.13
Immunoelectrophoresis (n=43)	0.980	0.240	0.75 - 1.22

Table 4

Mean AT III Activity and Antigen (U/ml)
According to Age and Sex

S-2238

(years)

	20-29	30-39	40-49	50-59
Males	1.02	1.02	0.95	1.00
Females	0.97	0.92[+]	1.02	0.97

Antigen

	20-29	30-39	40-49	50-59
Males	0.96	1.00	0.94	1.02
Females	0.98	0.90	1.03	1.03

[+] The level for females aged 30-39 is significantly less
(p < 0.05) than females aged 40-49, and males aged 20-29.

Figure 1.   The optimal thrombin-substrate incubation
time was determined by varying the incuba-
tion time from 5-50 seconds following the
addition of 300 µl 0.75 mmol/l substrate
to the thrombin solution. The 0.25 U/ml
AT III reference dilution of normal pooled
plasma was used. The reaction was stopped
with acetic acid and the absorbance read
at 405 nm.
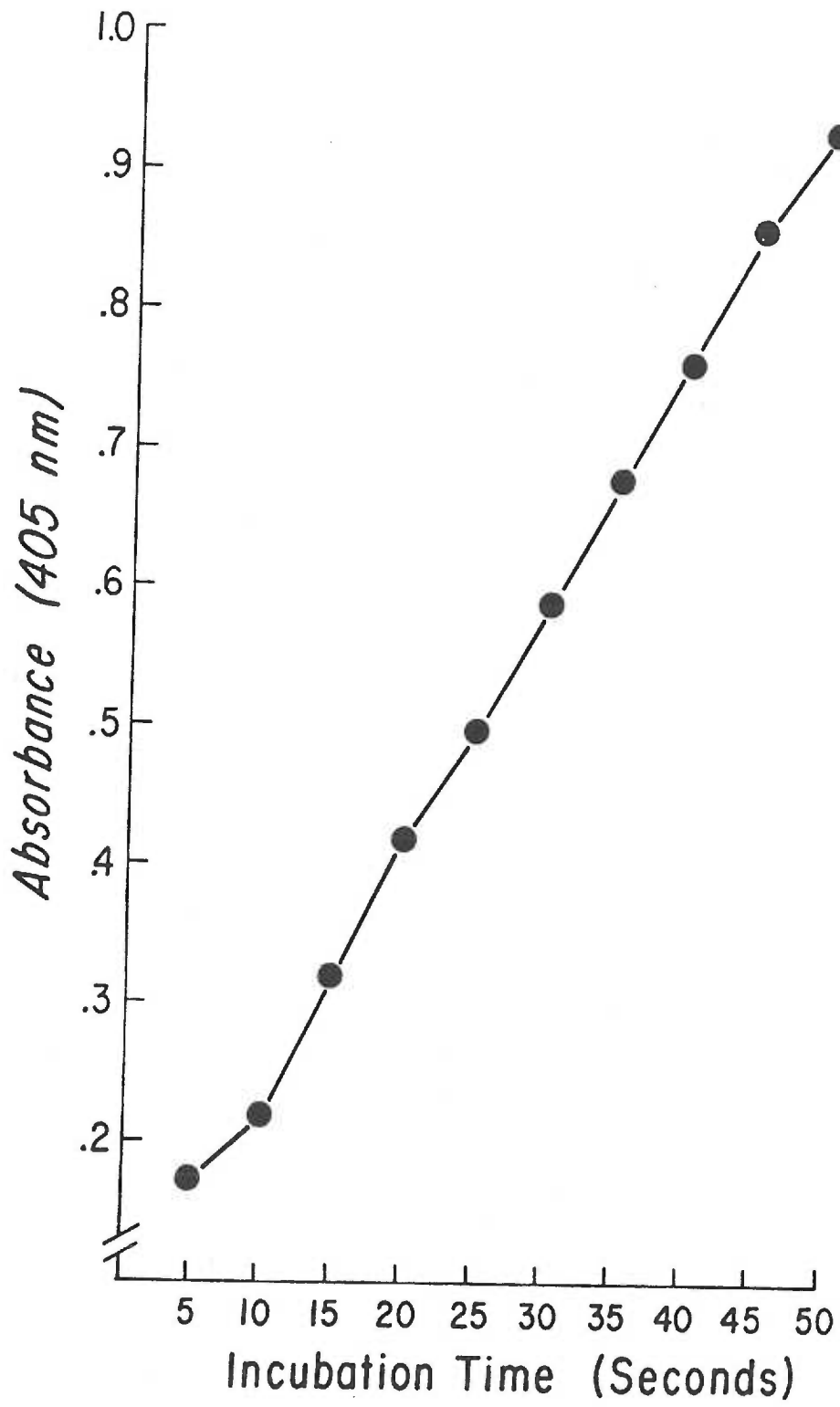
Figure 2.   To determine the optimal thrombin concen-
                 tration, 0.1 ml of 0-10 U/ml solutions
                 of thrombin Roche were incubated with
                 0.75 mmol/l S-2238. The reaction was
                 stopped after 30 seconds with acetic acid
                 and the absorbance measured at 405 nm.
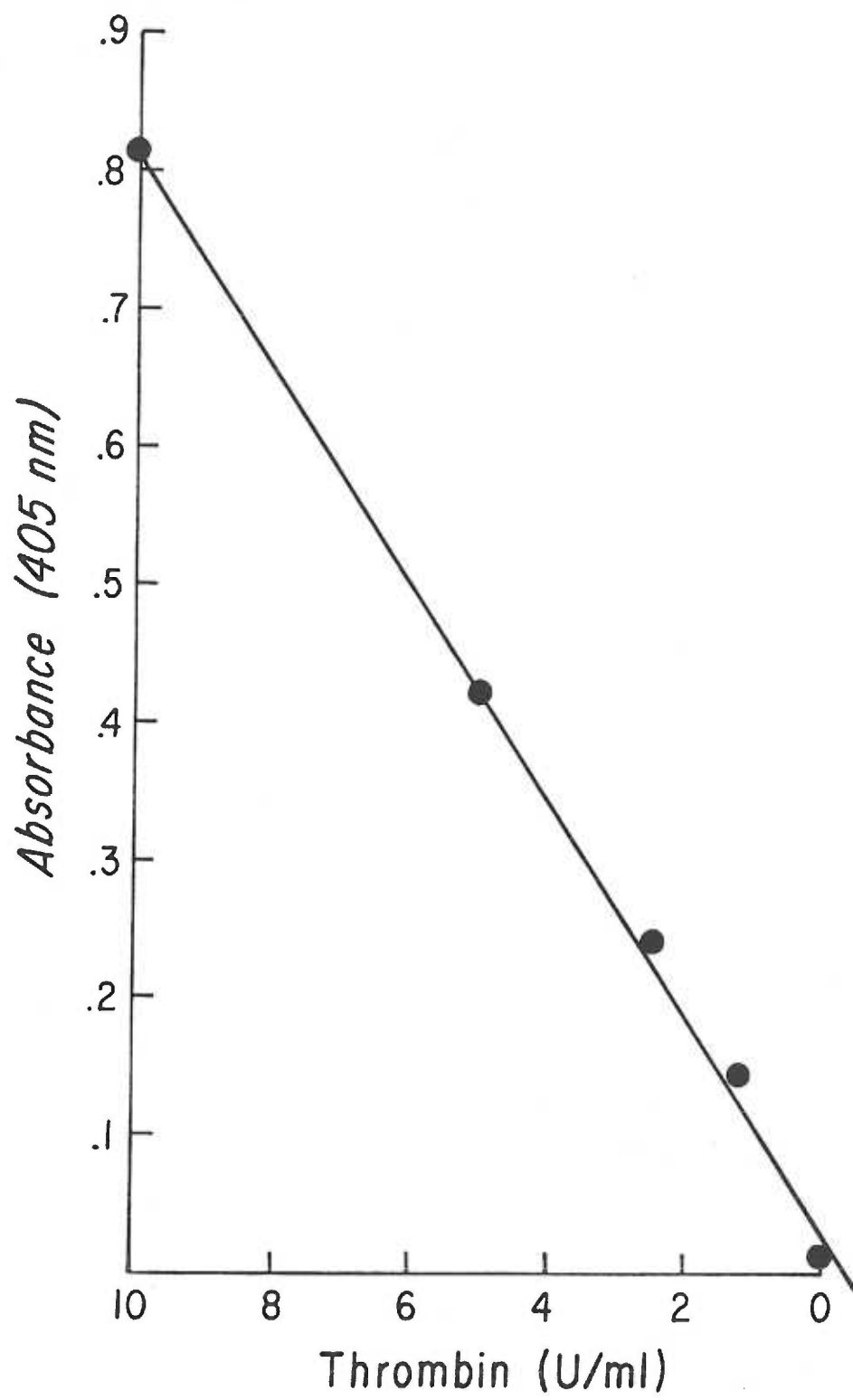
Figure 3.   Standard curves for S-2238 assay using 10
U/ml solutions of Parke-Davis thrombin
and thrombin Roche (according to manu-
facturer's information) are shown.
Represented is the average of two experi-
ments (least squares analysis).
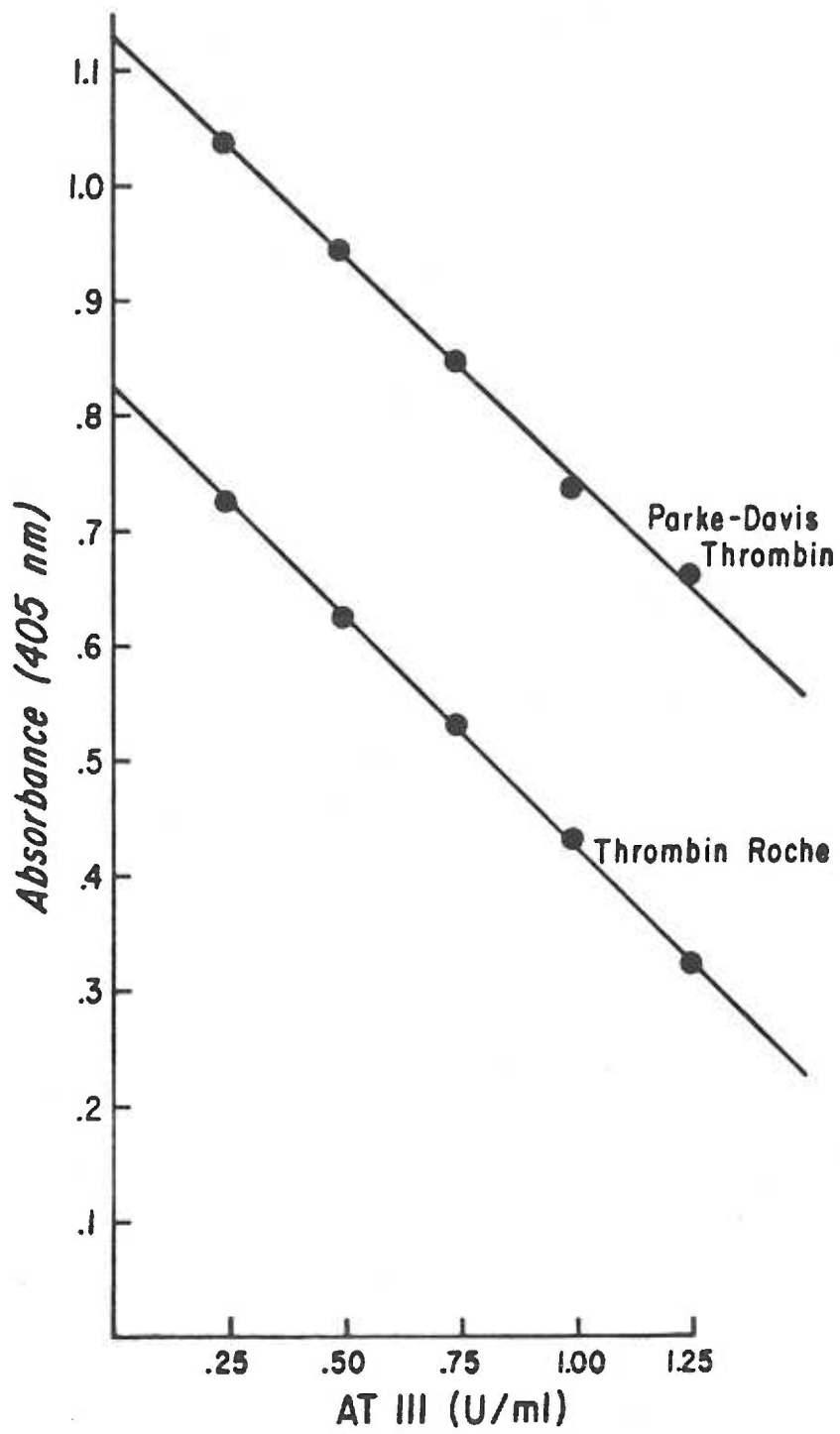
Figure 4.　　　The inhibition of thrombin by hirudin in
the S-2238 assay was studied by preincu-
bating 1 U thrombin Roche (average of two
experiments) or 0.5 U purified human
thrombin and 0 to 5 AT-U of hirudin (units
of activity taken from supplier's informa-
tion) for 5 minutes. Substrate, 300 µl of
a 0.75 mmol/l solution was added and after
30 seconds the reaction stopped with acetic
acid. Absorbance was measured at 405 nm.
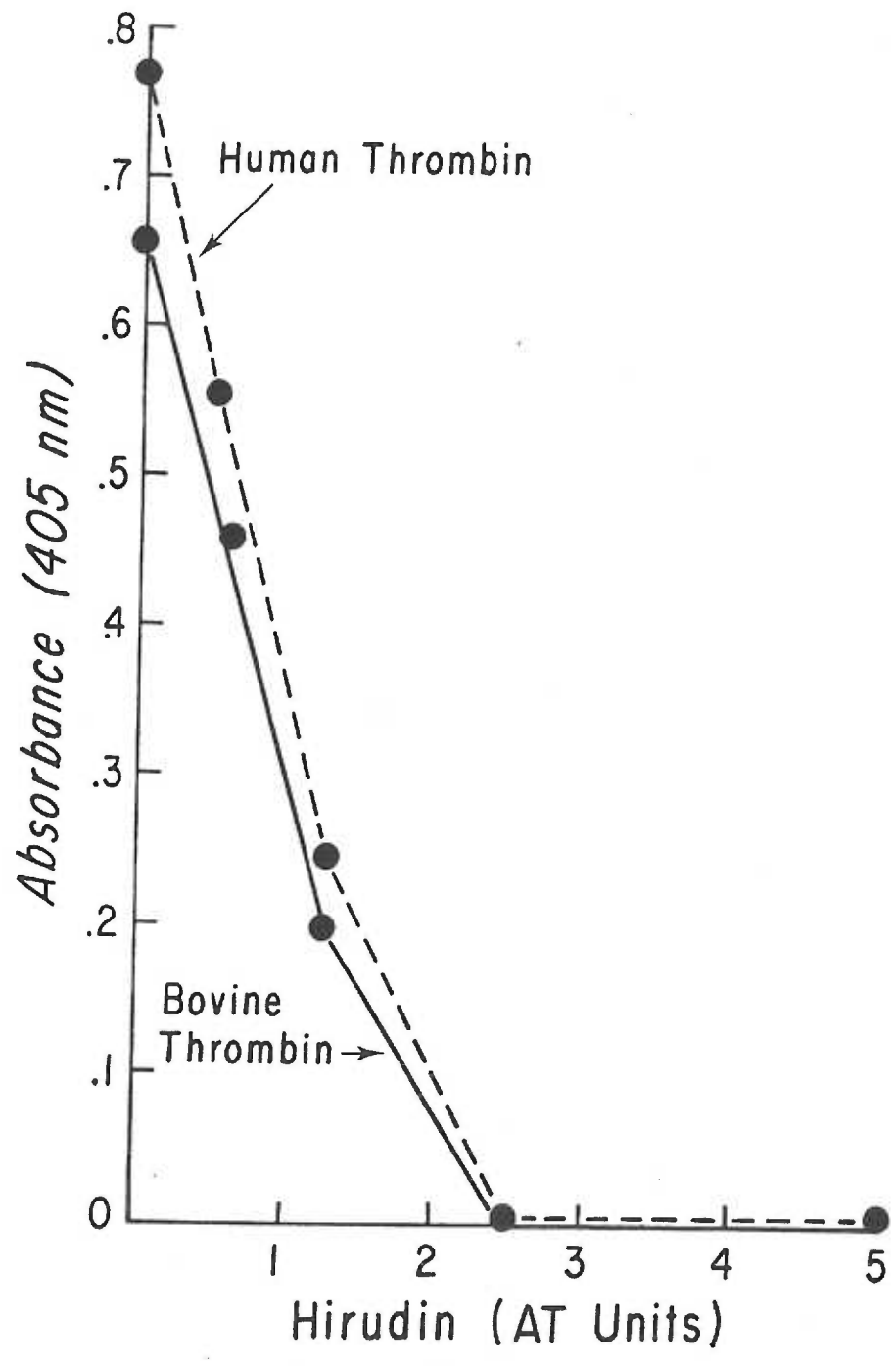
Figure 5.   A single reference curve for the S-2238
             assay of AT III. The standard conditions
             of 10 U/ml thrombin, 0.75 mmol/l S-2238
             and 30 seconds substrate-thrombin incuba-
             tion time were used. (Least squares
             analysis.)
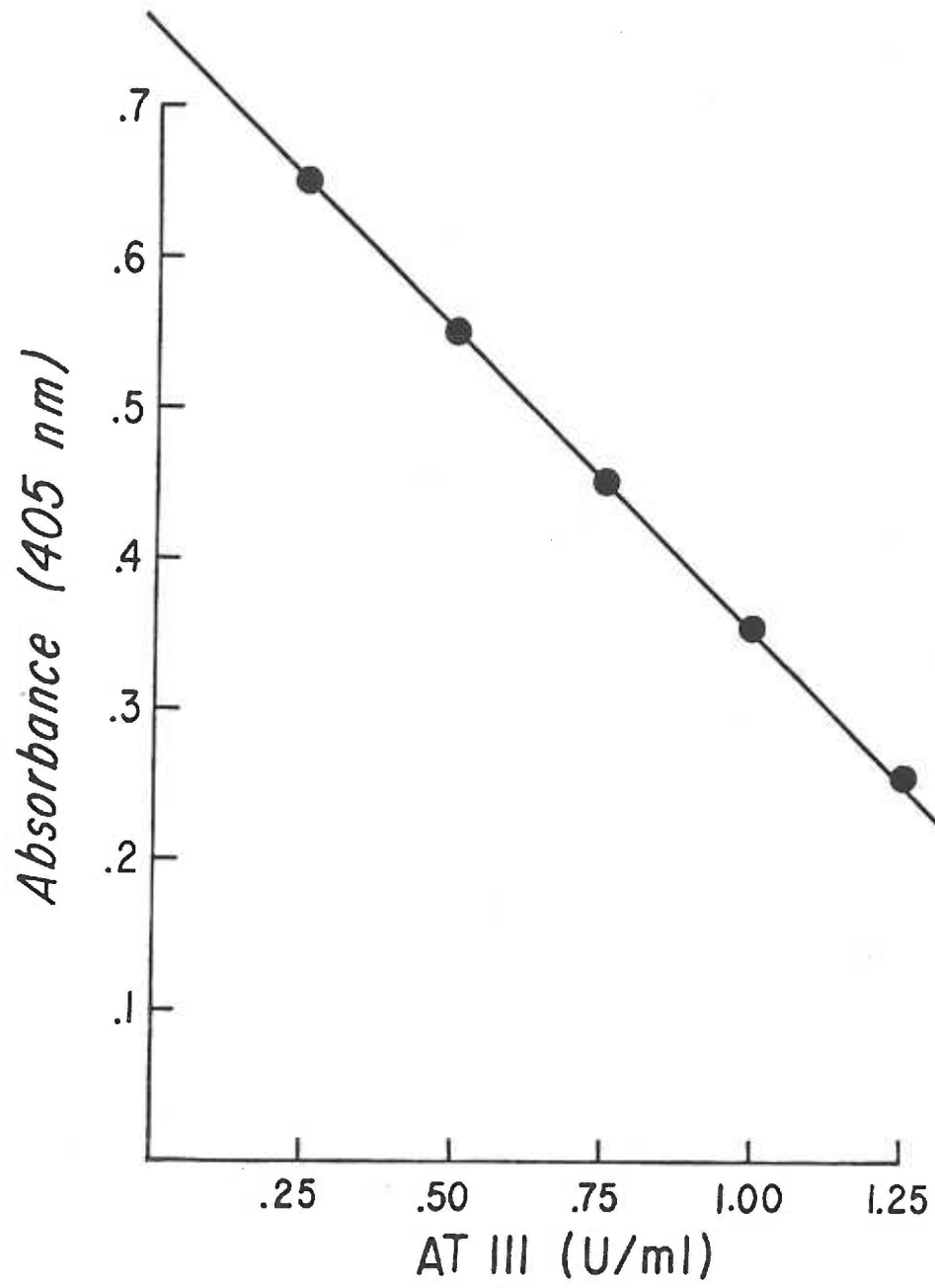
Figure 6.   A composite of nine consecutive reference
curves for the S-2238 assay of AT III over
a 2-month period of time using standard
assay conditions. Shown is the mean
$\pm$ 1 SD for each point on the curve. (Least
squares analysis.)
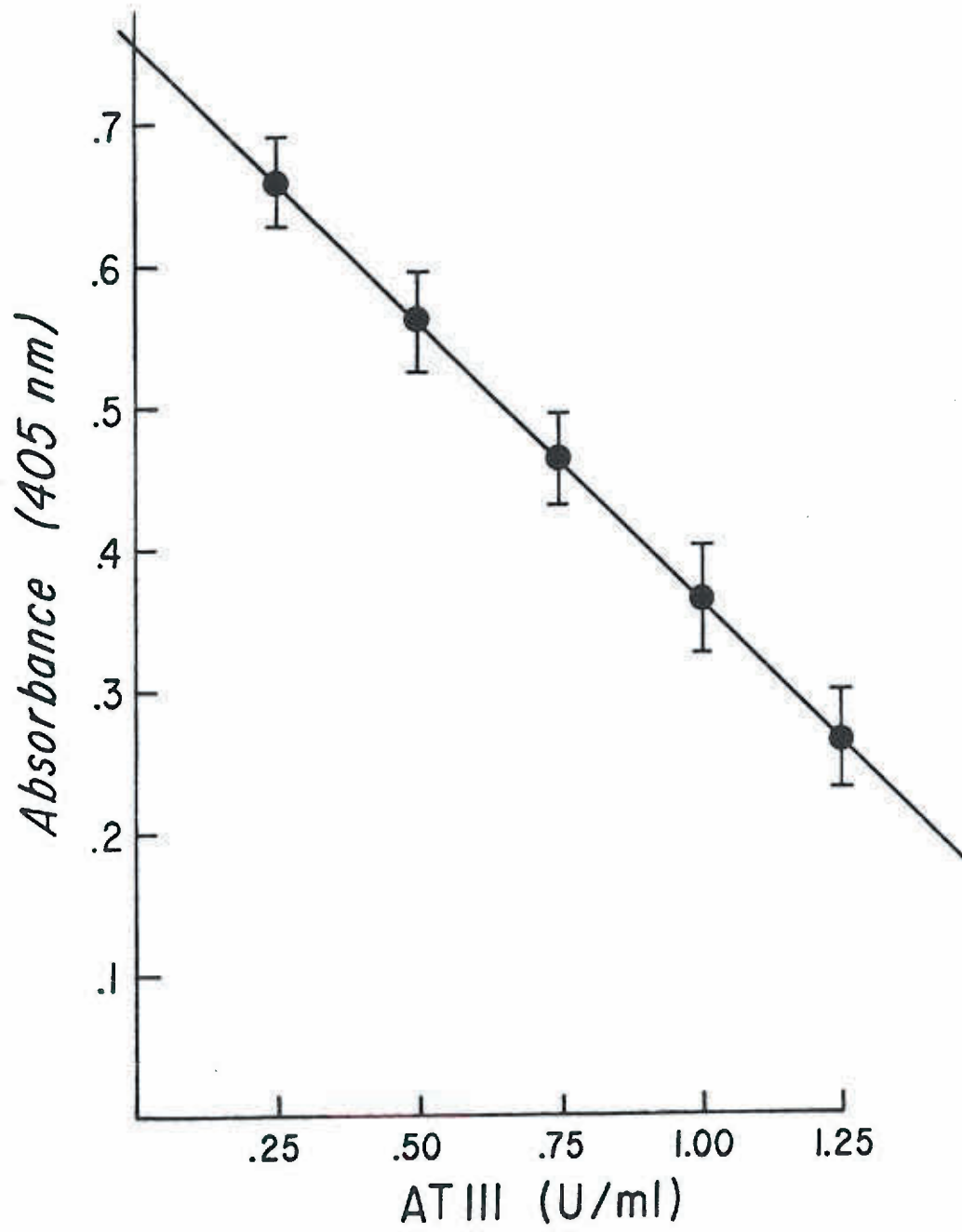
Figure 7.   A single reference curve for measurement
of AT III antigen using Laurell rocket
immunoelectrophoresis.
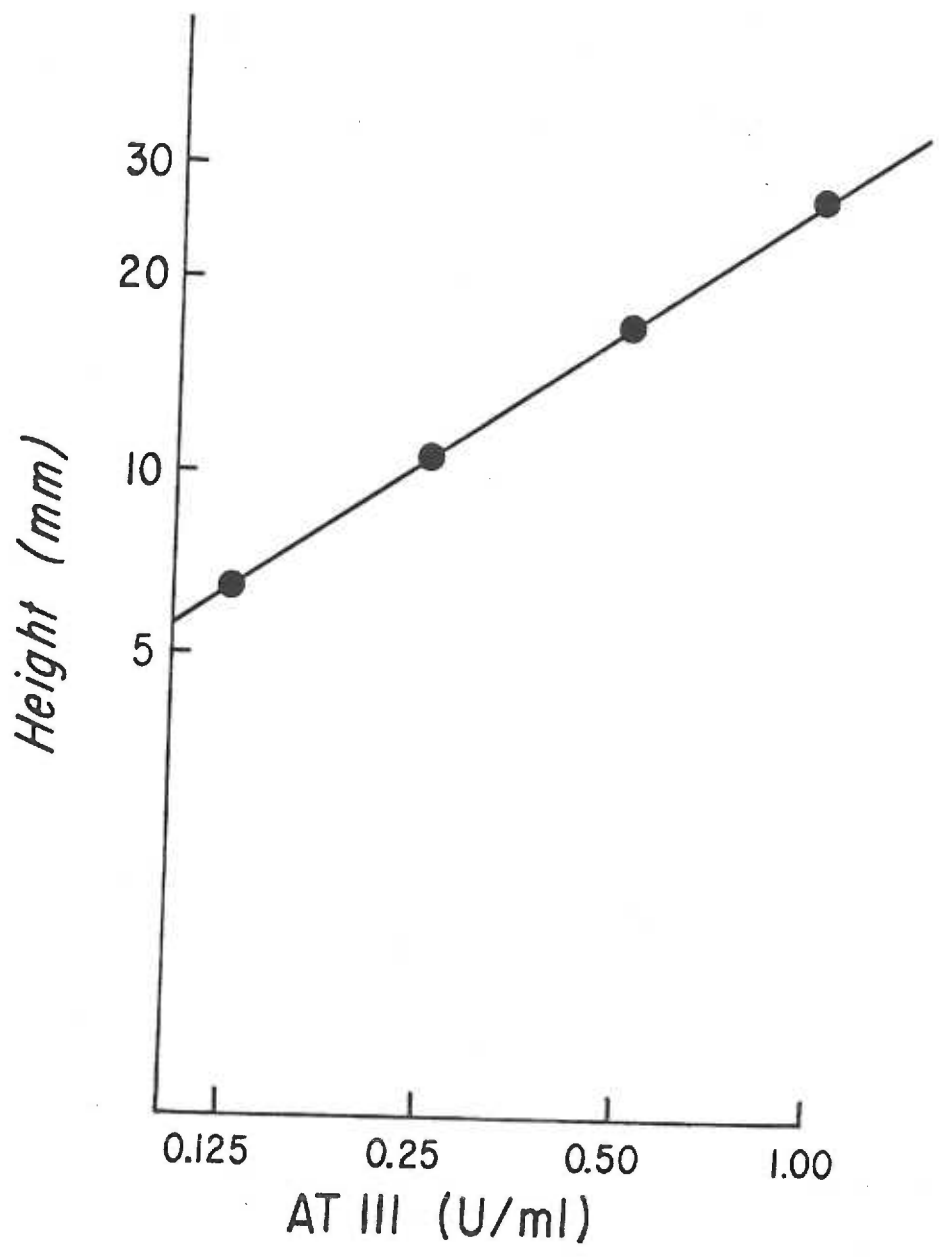
Figure 8.   A composite of nine consecutive reference
             curves over a 2-month time period for
             measurement of AT III antigen concentration
             using Laurell rocket immunoelectrophoresis.
             Shown is the mean $\pm$ 1 SD for each point
             on the curve.
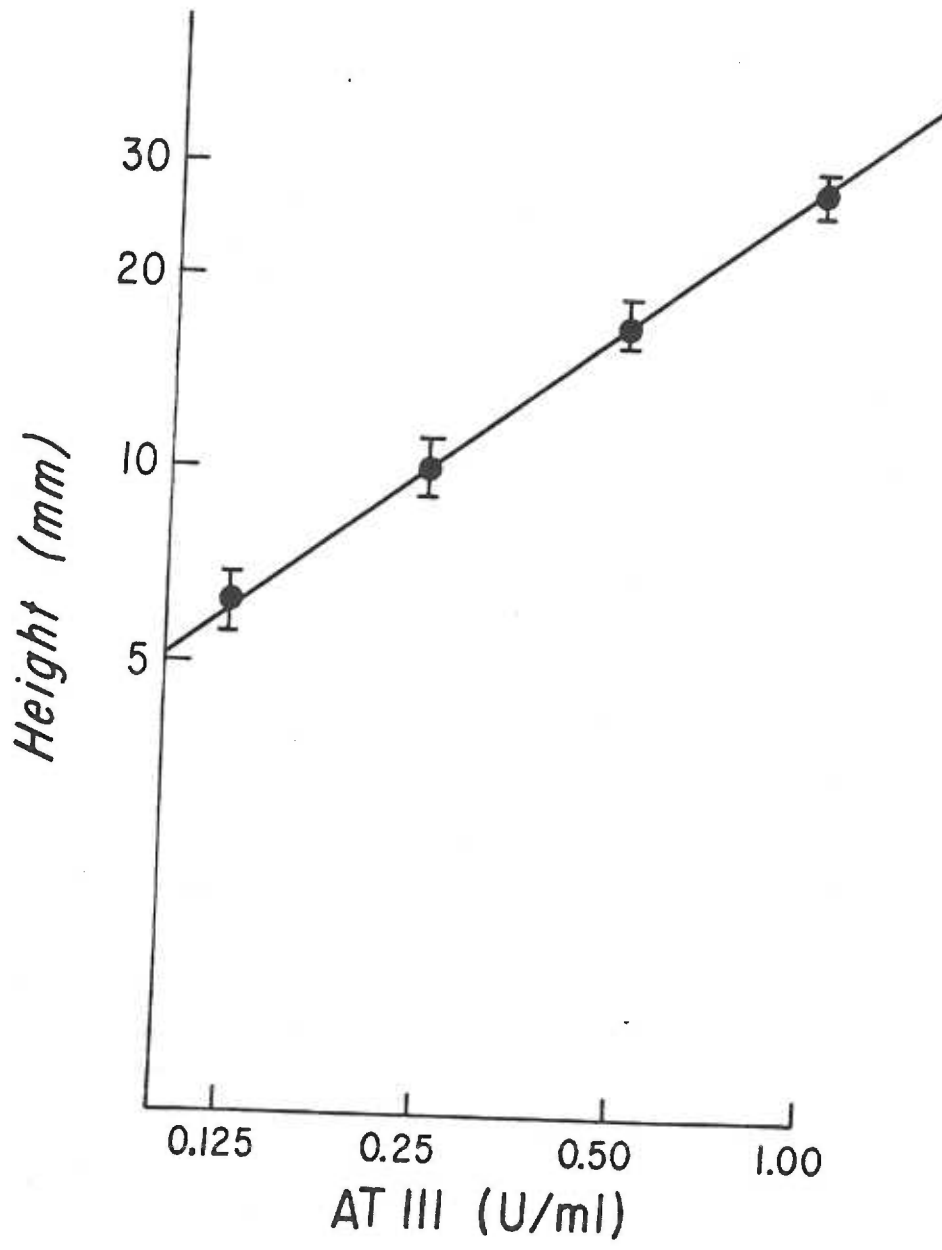
Figure 9.   A single standard curve for the factor Xa
             inhibition assay for AT III activity.
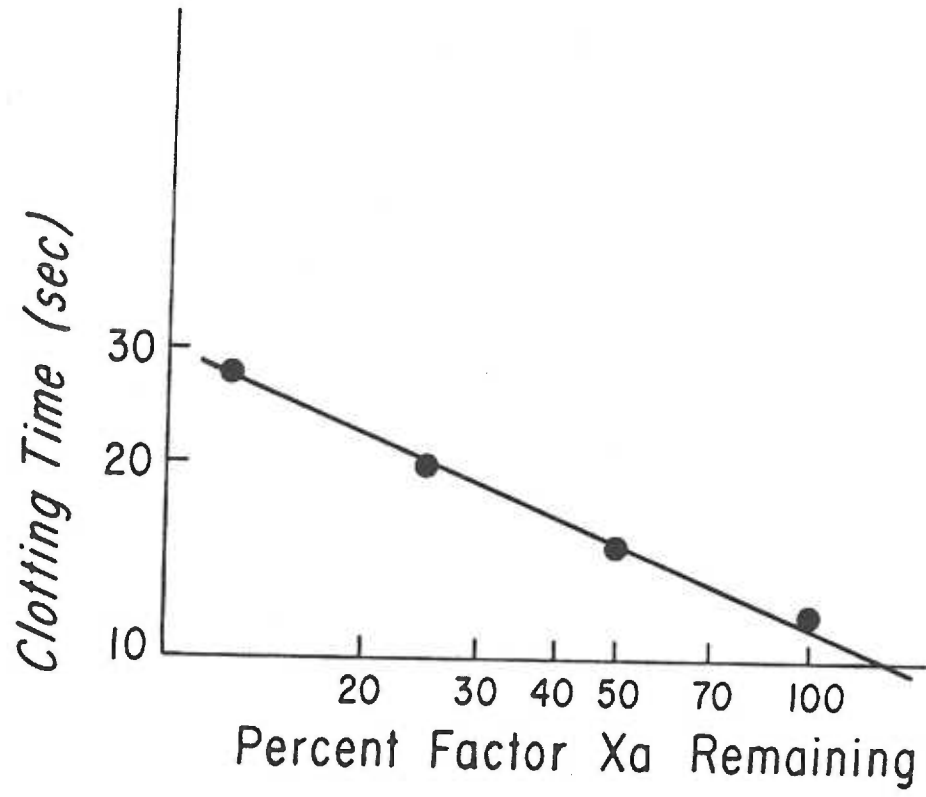
Figure 10.   A composite of eight consecutive standard
              curves compiled over a 2-month period of
              time for the factor Xa inhibition assay
              of AT III activity. Shown is the mean
              $\pm$ 1 SD for each point on the curve.
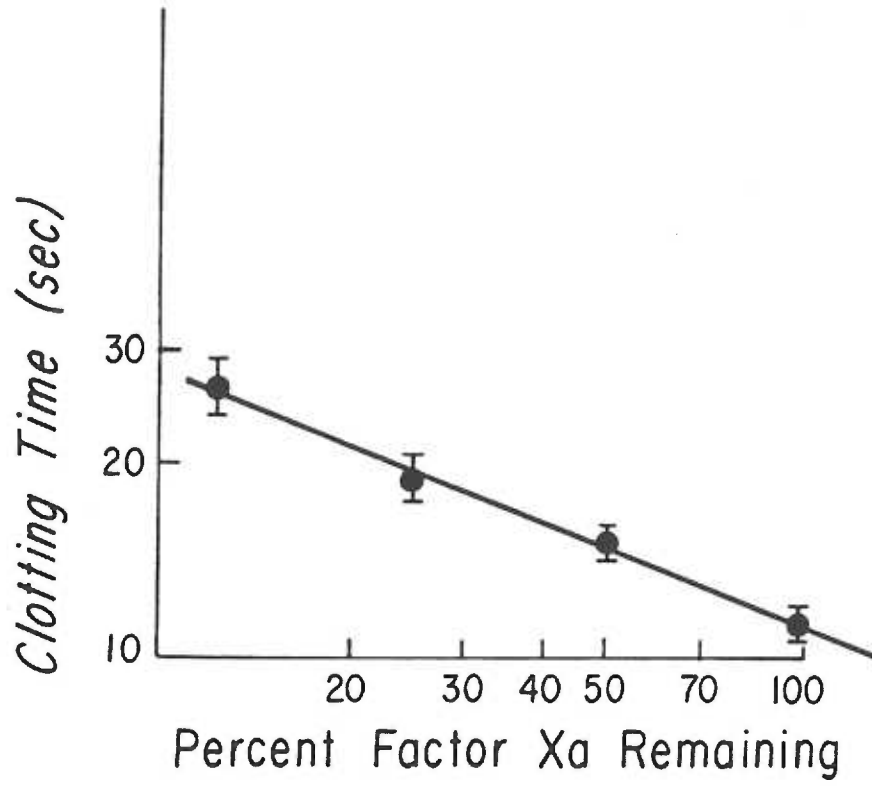
Figure 11.   Arithmetic plot of single reference curves
for the high concentration range of the
S-2222 assay for plasma heparin, using
4 U/ml, 8 U/ml, and 10 U/ml factor Xa in
the standard procedure.

Figure 11a.   Log-log plot of single reference curves
for the high concentration range of the
S-2222 assay for plasma heparin, using
4 U/ml, 8 U/ml, and 10 U/ml factor Xa
in the standard procedure.
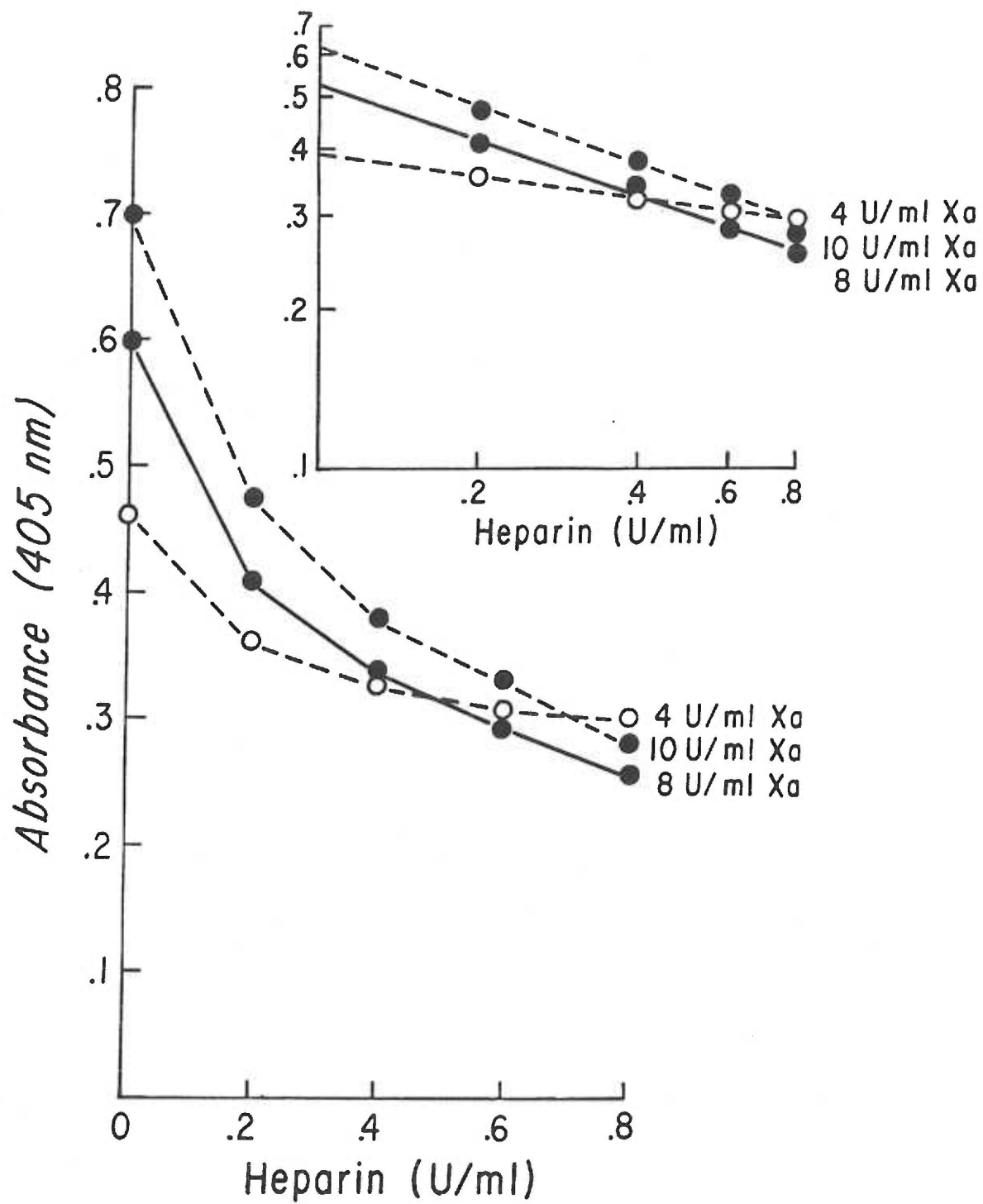
Figure 12.   Single representative reference curves
              for S-2222 plasma heparin assay, high
              concentration range and low concentra-
              tion range.

Absorbance (405 nm)

Heparin (U/ml) Low Concentration Range

Heparin (U/ml) High Concentration Range
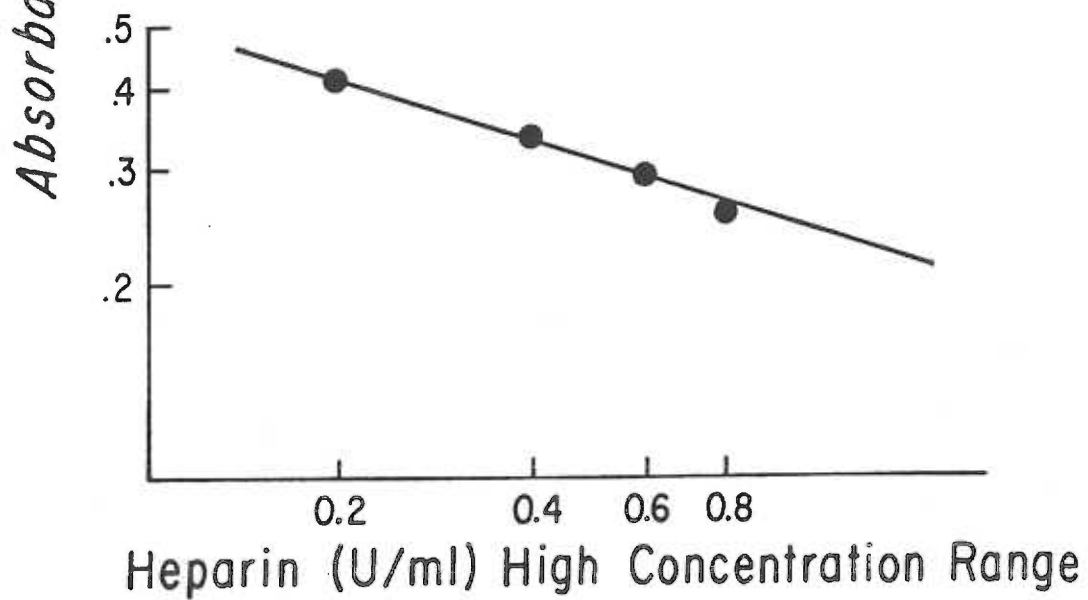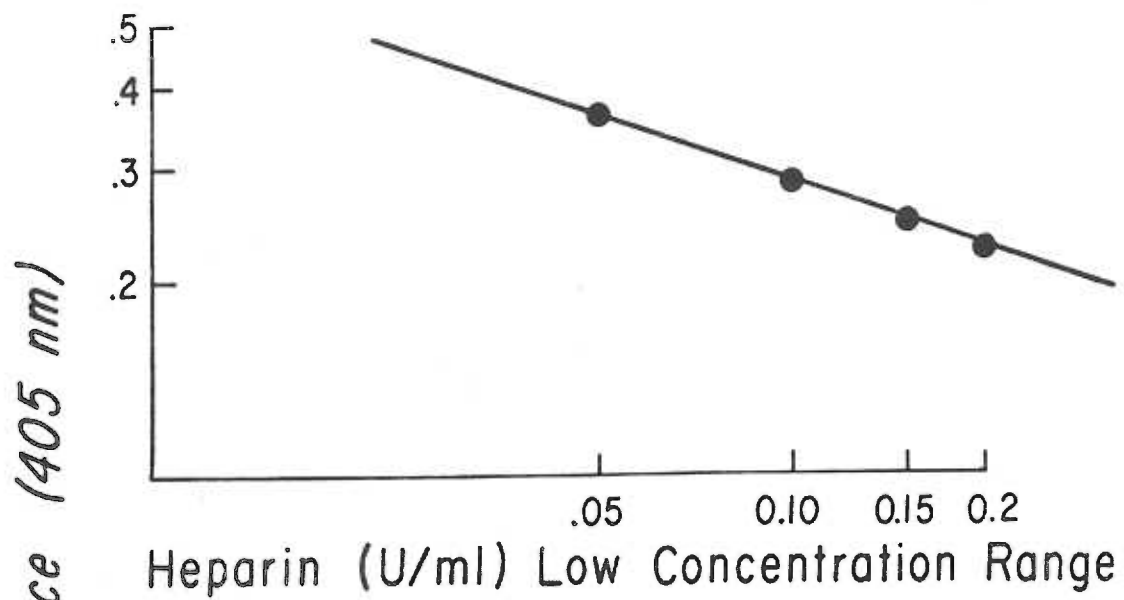
Figure 13.   A composite of seven consecutive reference
               curves for the high concentration range
               (0.0-0.8 U/ml) and five consecutive
               reference curves for the low concentration
               range (0.0-0.20) of the S-2222 plasma
               heparin assay. Shown is the mean
               absorbance $\pm$ 1 SD for each point on the
               curve.

Absorbance (405 nm)

Heparin (U/ml) Low Concentration Range

Heparin (U/ml) High Concentration Range
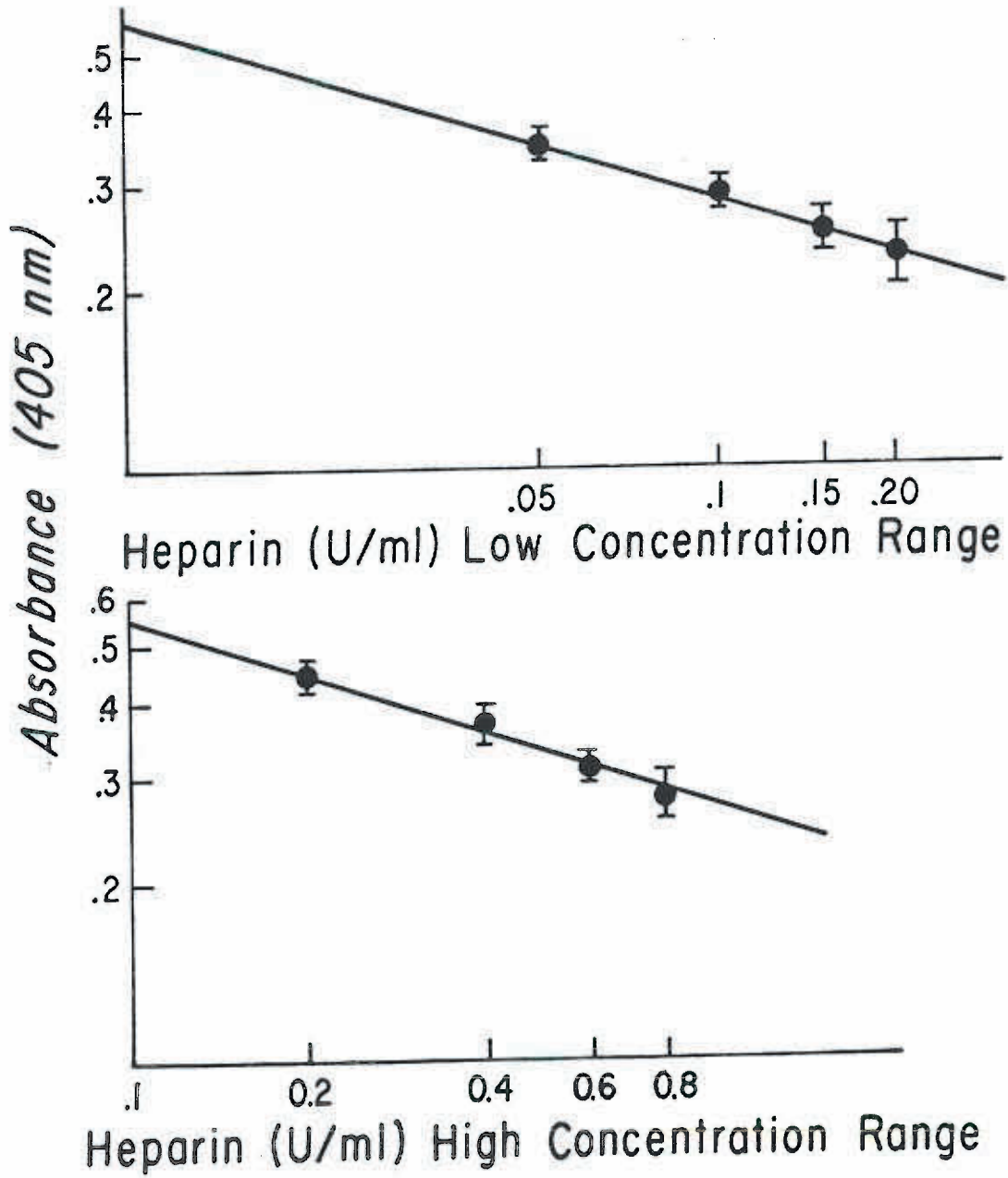
Figure 14.   Normal ranges (mean $\pm$ 2 SD) for S-2238
             activity assay, Xa Inhibition assay, and
             Laurell electrophoresis for AT III con-
             centration.
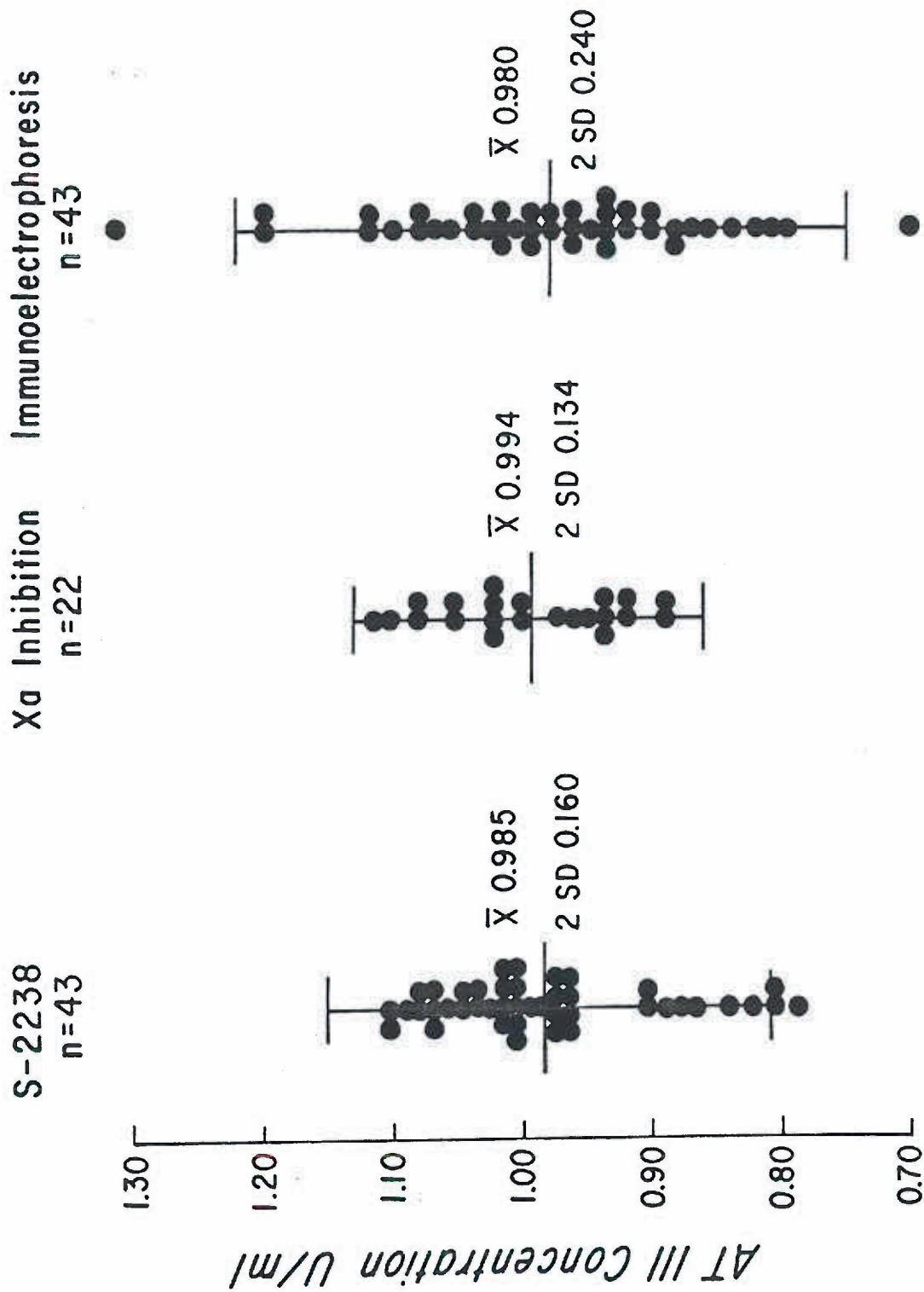
Figure 15.   The mean AT III concentrations measured
with S-2238 activity, Xa inhibition, and
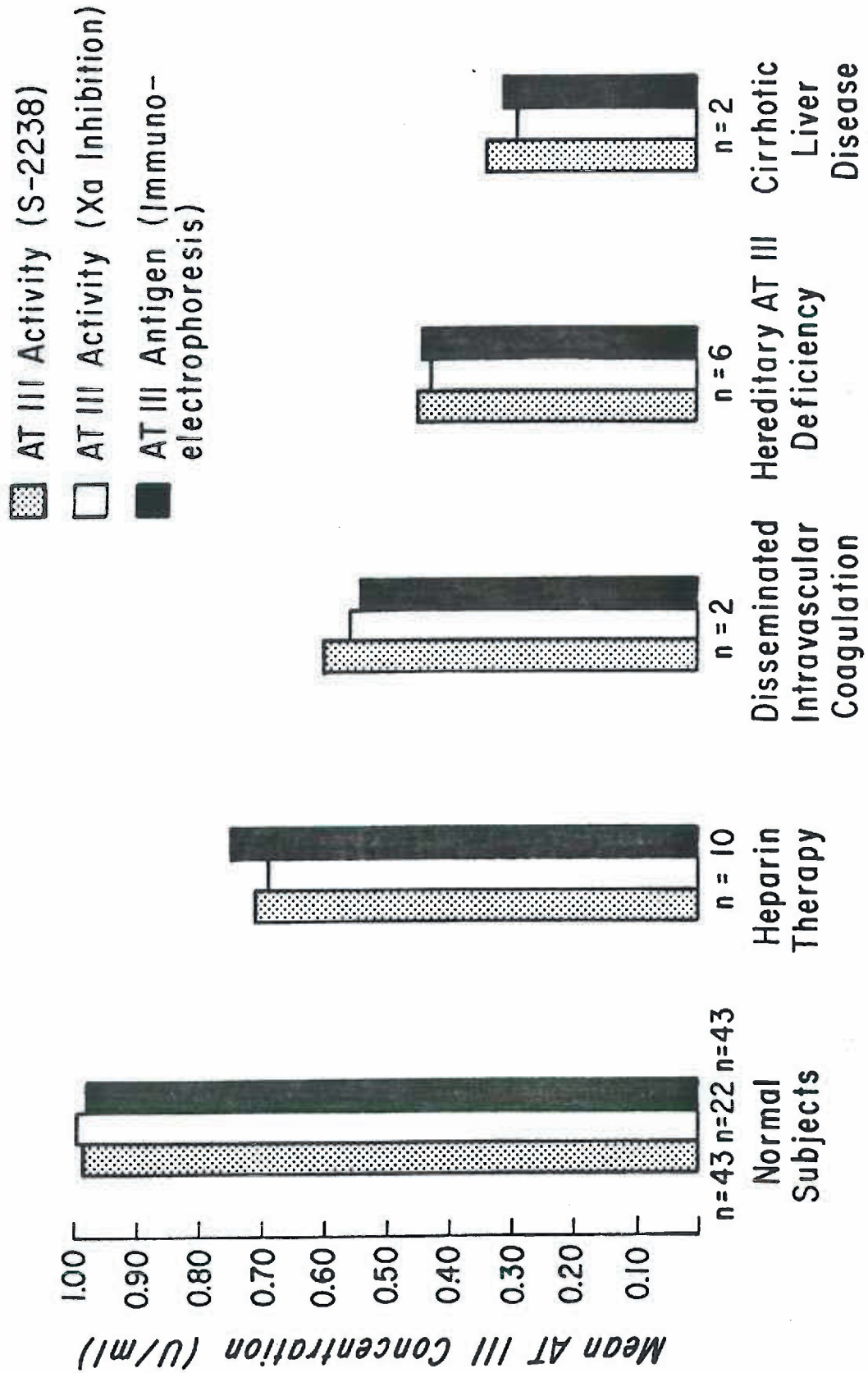Laurell immunoelectrophoresis for normal
subjects and clinical states.

Figure 16.   Correlation of S-2238 activity and Xa
inhibition activity assays for AT III in
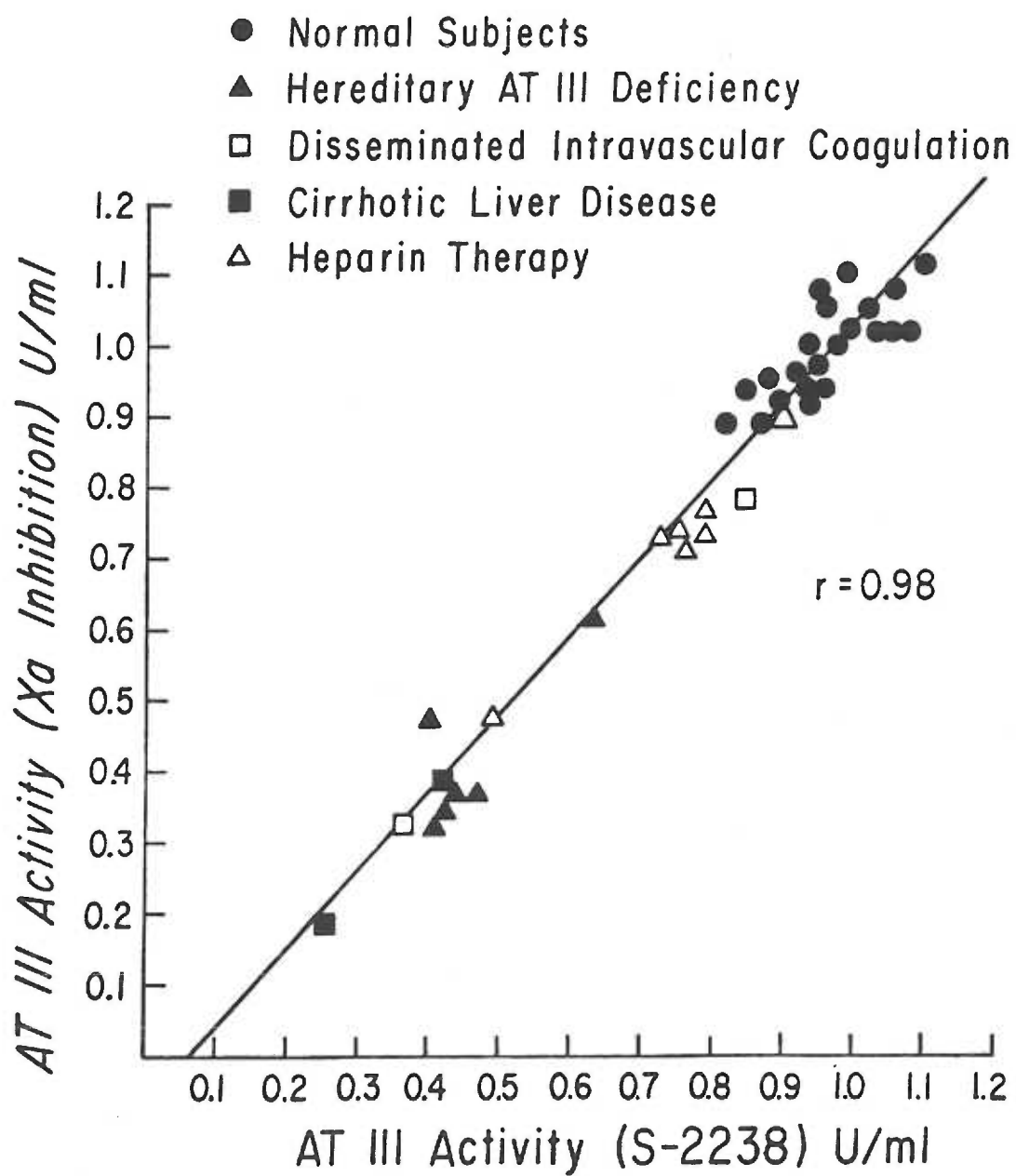normal subjects and clinical states.
(Least squares analysis.)

Figure 17.   Correlation of S-2238 activity assay and
               Laurell immunoelectrophoresis for AT III
               in normal subjects and clinical states.
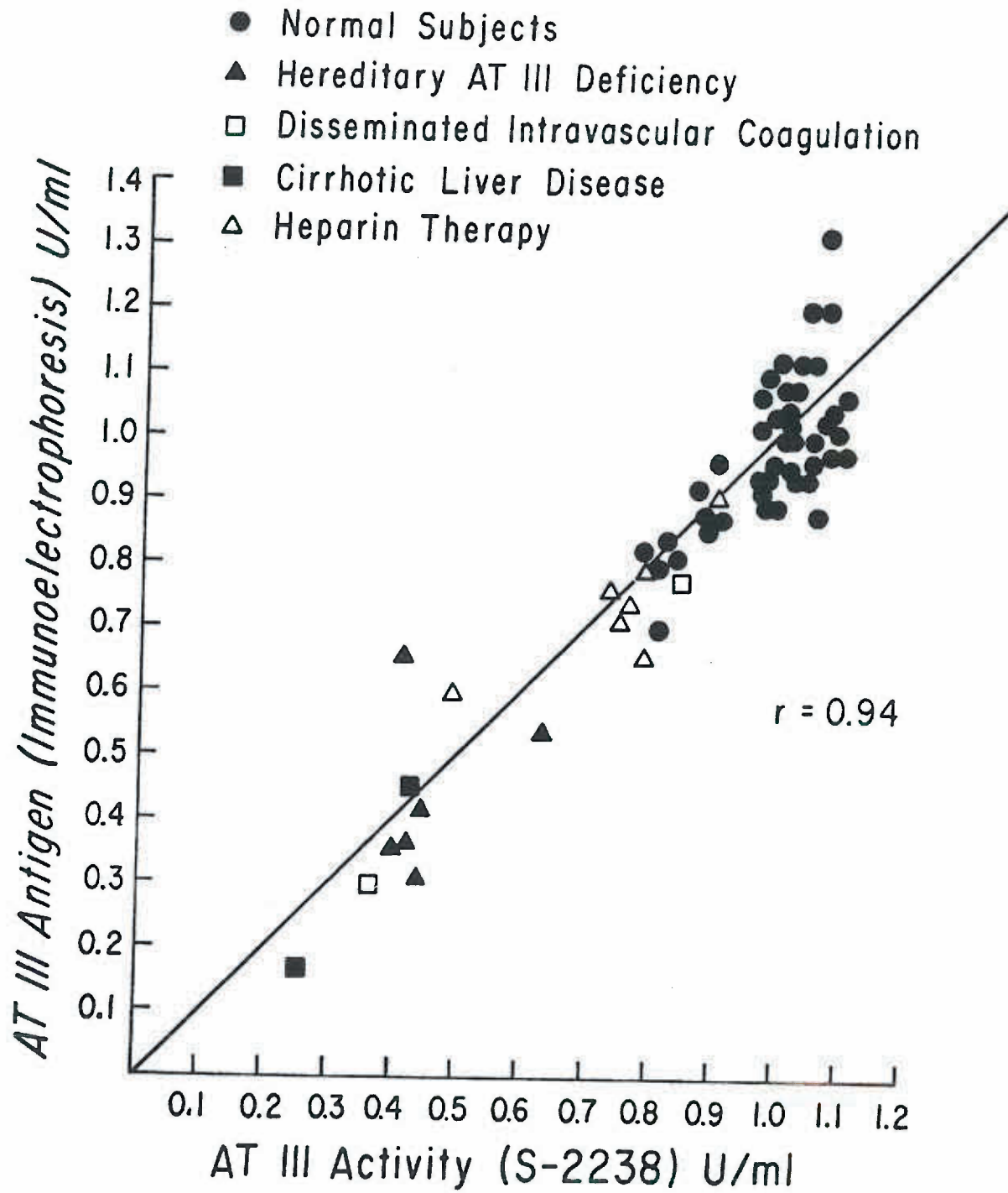   (Least squares analysis.)

Figure 18.   Correlation of Xa inhibition activity and
Laurell immunoelectrophoresis for AT III in
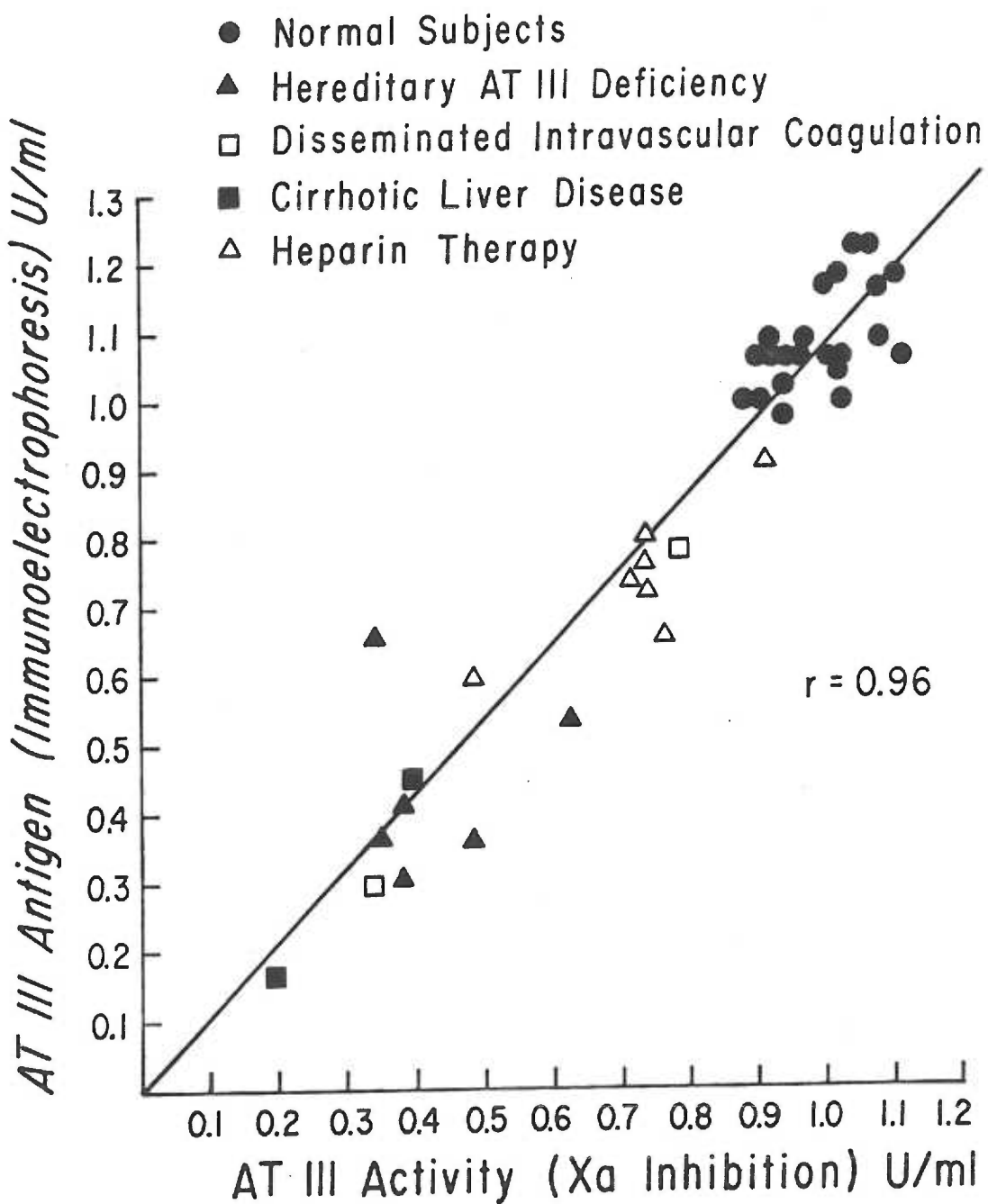normal subjects and clinical states.   (Least
squares analysis.)

Figure 19.   Patient #1: AT III levels by S-2238, Xa
              inhibition, and Laurell immunoelectro-
              phoresis during heparin infusion therapy.
              Shown also are heparin concentrations in-
              fused and Coumadin dosages.

AT III U/ml

Antigen

S-2238

Xa Inhibition

1.1
1.0
.9
.8
.7
.6
.5

40,000
30,000 Heparin (Units/24 hours)
20,000
10,000 Coumadin 10 mg/day

0 1 2 3 4 5 6 7 8 9 10 11

Days from Institution of Heparin Therapy
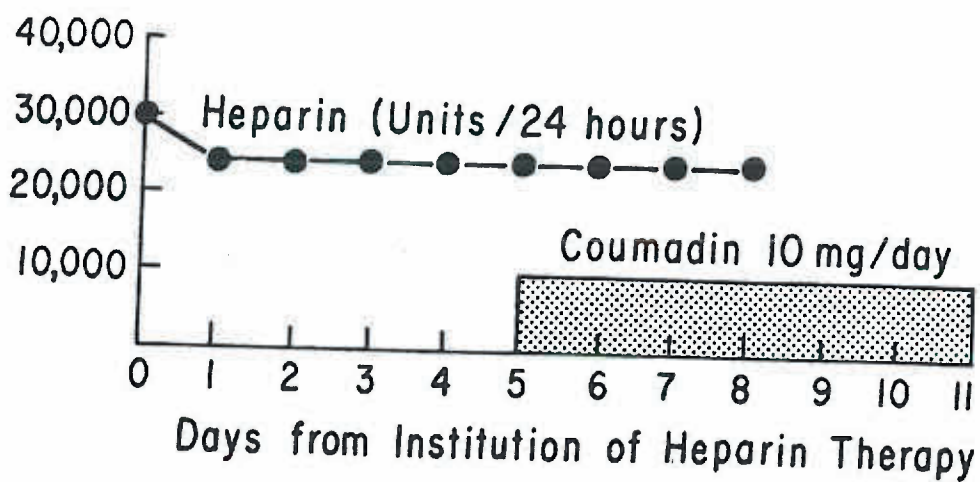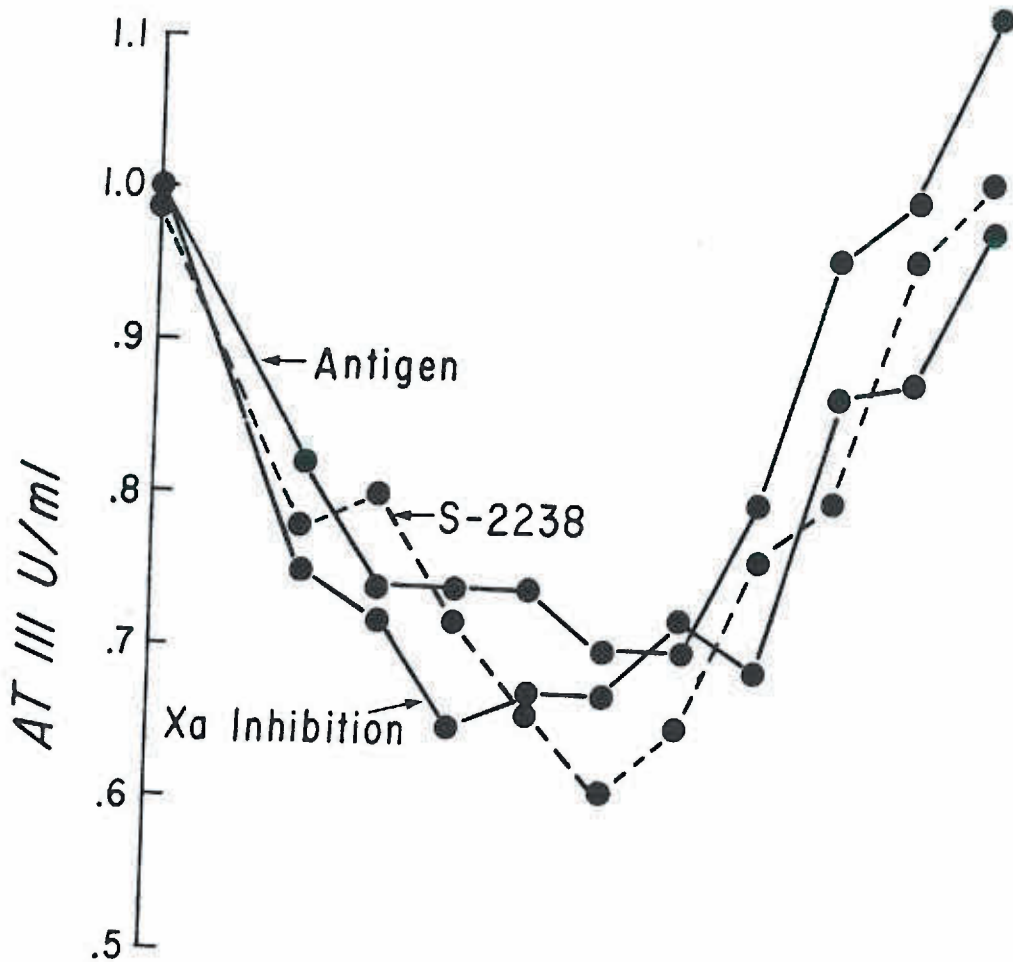
Figure 20.   Patients #2 and #3: AT III levels by S-2238,
                 Xa inhibition, and Laurell immunoelectro-
                 phoresis during and after heparin infusion
                 therapy. Shown also are heparin concentra-
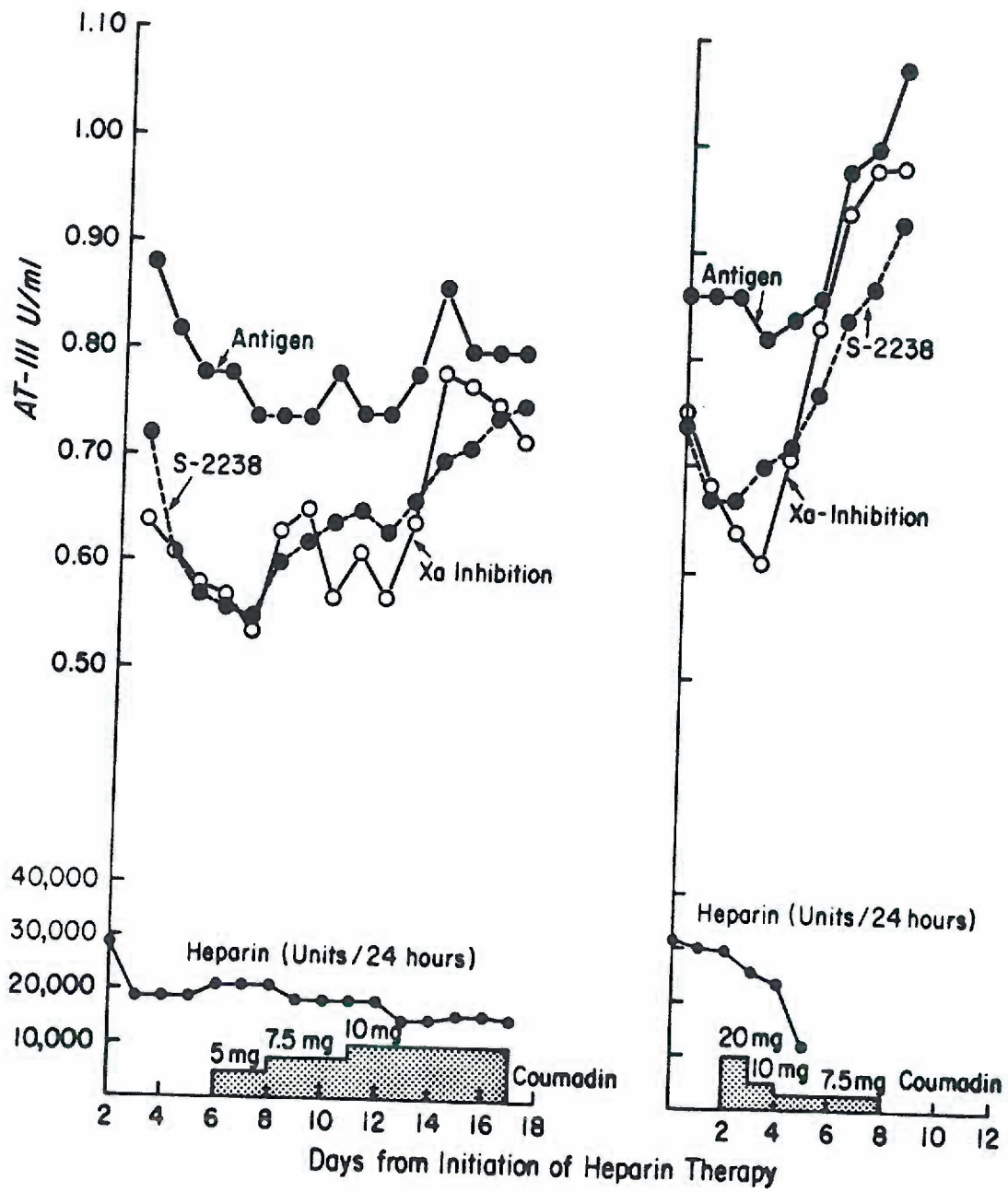   tions infused and Coumadin dosages.

Figure 21.   Correlation of plasma heparin concentration
              by S-2222 and aPTT during heparin infusion.
              (Least squares analysis.)

aPTT (Seconds) vs Plasma Heparin Concentration (U/ml)

Figure 22.   Patients #1, #2, and #3: Plasma heparin
concentrations determined using S-2222
plotted against aPTT values during and
after heparin infusion.
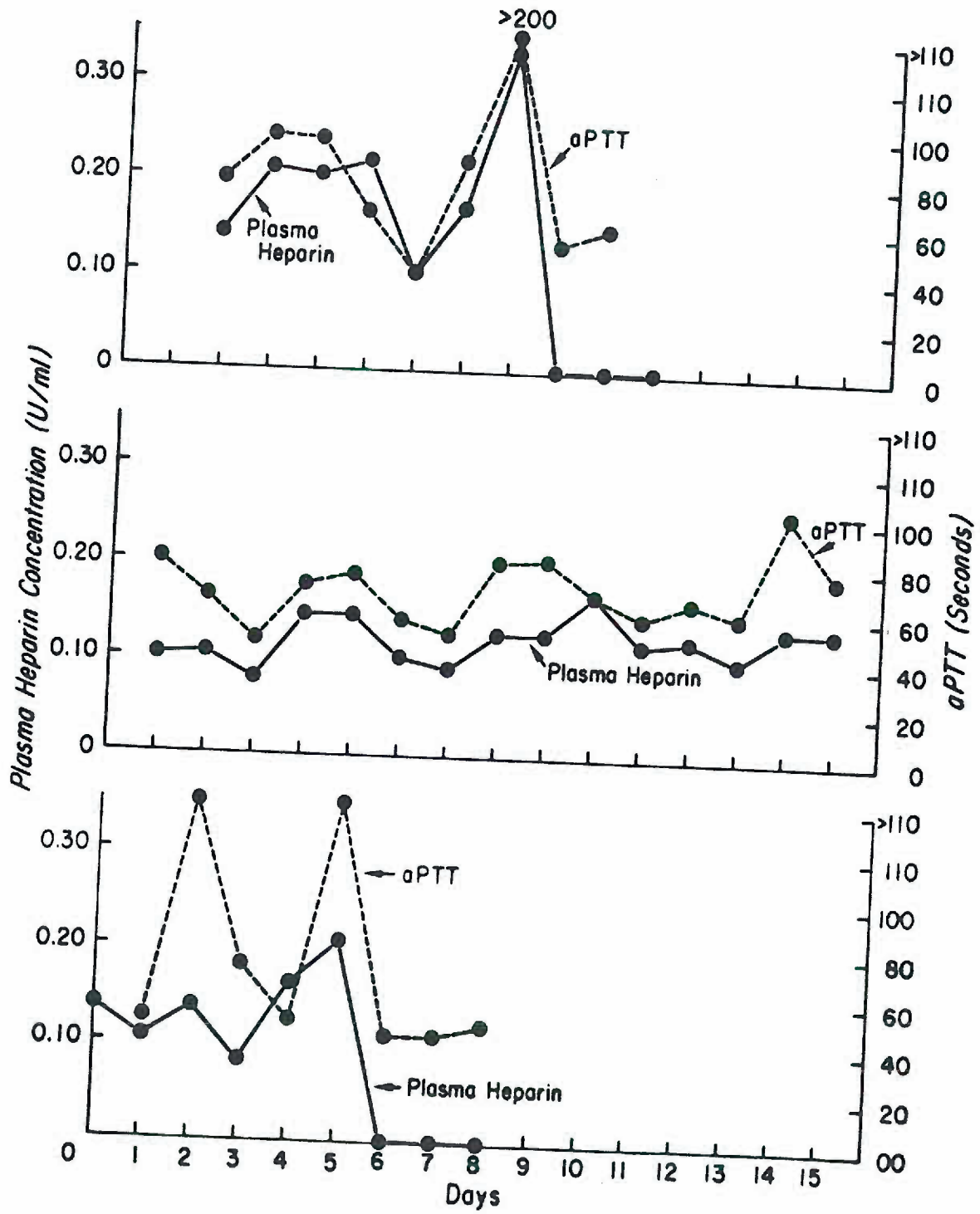
Figure 23.   Patients #1, #2, and #3: AT III levels by
                S-2238 are shown plotted against plasma
                heparin concentration determined by S-2222
                and heparin concentration infused.