Action of Heparin on the Inhibition of Thrombin by α_1-Proteinase Inhibition†

Robin Pixley‡ and Isidore Danishefsky*

ABSTRACT: α_1-Proteinase inhibitor inhibits the action of thrombin while heparin is effective in relieving this inhibition. Kinetic analyses of the inhibition in the absence and presence of heparin were conducted by employing the *p*-nitroanilide of a synthetic peptide, S-2238, to determine residual thrombin activity. Under pseudo-first-order conditions, the rate of thrombin neutralization was proportional to α_1-proteinase inhibitor concentration up to the highest concentration of inhibitor employed, 94.6 μM. The second-order rate constant under the conditions studied was 6.64×10^3 M^{-1} min^{-1}, and heparin was found to cause a decrease in the rate constant. On the basis of the concentrations used, the kinetics of thrombin inhibition, both in the absence and in the presence of heparin, can be described by a one-step reaction. The effect of heparin is due to its binding to thrombin. The degree to which heparin interferes with the rate of thrombin inhibition depends on its molecular weight but not on its anticoagulant activity. Thus, in the presence of 11.7K and 22K heparins the respective rate constants are 3.5×10 and 1.0×10^3 M^{-1} min^{-1}. Additionally, the concentration of heparin required to produce a maximal decrease in reaction rate depends on the molecular weight of the heparin employed.

Thrombin is a serine protease that is generated from its zymogen, prothrombin, during the blood coagulation process. In addition to catalyzing the conversion of fibrinogen to fibrin, thrombin also modifies certain procoagulant proteins and induces various platelet responses (Davie & Fujikawa, 1975; Martin et al., 1975). Thrombin can be neutralized by a number of inhibitory proteins that are normally present in the circulatory system. Most prominent among these are antithrombin III, α_1-proteinase inhibitor (αPI),[1] and α_2-macroglobulin (Heimburger, 1975). Although the plasma concentration of αPI exceeds that of the other inhibitors, it is apparent from kinetic data that antithrombin III contributes about 80% of the thrombin-inhibitory action while αPI contributes about 15% (Downing et al., 1978).

It was reported from this laboratory (Danishefsky & Pixley, 1979) and subsequently confirmed by others (Long & Williamson, 1981; Takahara & Sinohara, 1982) that heparin inhibits the neutralization of thrombin by αPI. This is in contrast to the accelerating effect of heparin on the neutralization of thrombin by antithrombin III (Rosenberg, 1977). The present report attempts to define the mode action of heparin on the thrombin–αPI reaction. This involved studies on the kinetics of thrombin inhibition by αPI and the manner in which heparin influences the reaction rates. Also, in order to determine whether there is a common structural characteristic in heparin that is responsible for its effects on thrombin neutralization by αPI and antithrombin III, additional studies were performed on heparin fractions with different molecular weights and anticoagulant activities.

Materials and Methods

Proteins. Human α-thrombin was kindly provided by Dr. John W. Fenton (Fenton et al., 1977). The preparation had over 98% active sites as determined by titration with pNPGB (Chase & Shaw, 1970). The antithrombin III, employed in the assays for heparin anticoagulant activity, was prepared from human plasma as described previously (Danishefsky et al., 1978; Villanueva & Danishefsky, 1979). α_1-Proteinase inhibitor was isolated from human plasma by the method of Kurechi et al. (1979) after preliminary separation of antithrombin III (Danishefsky & Pixley, 1979). The activity of the αPI as assayed according to Dietz et al. (1974) against active site titrated trypsin (Chase & Shaw, 1970) was over 95%.

Protein concentrations were determined from the absorbance at 280 nm and corrections for light scattering. The extinction coefficients used were 1.83 mL mg^{-1} cm^{-1} for thrombin (Fenton et al., 1977) and 0.484 mL mg^{-1} cm^{-1} for αPI (Bloom & Hunter, 1978). Molar concentrations were calculated on the basis of molecular weights of 36 500 and 54 000 for thrombin and αPI, respectively.

Heparin Fractions. Beef lung heparin with an activity of 152 USP units/mg was a gift from the Upjohn Co., Kalamazoo, MI. High-activity heparin that is heterodisperse with respect to molecular weight, termed HA-heparin in this paper, 450 units/mg, was prepared by affinity chromatography of the commercial material on antithrombin-linked Sepharose (Radoff & Danishefsky, 1982).

Commercial heparin was also fractionated according to size on Bio-Gel P-100 to yield components with weight-average molecular weights of 22.0K, 15.9K, 13K, 11.7K, and 8.6K as determined by sedimentation equilibrium procedures (Pixley & Danishefsky, 1982). Each of these were fractionated according to anticoagulant activity on antithrombin-linked CH-Sepharose as described previously (Pixley & Danishefsky, 1982). The specific fraction employed in some of the present studies were 22K heparin with 38, 520, and 870 units/mg and 11.7K heparin with 5, 382, and 516 units/mg. The activities of the heparin preparations were based on their ability to accelerate the neutralization of thrombin by antithrombin III utilizing the chromagenic substrate S-2238 (Radoff & Danishefsky, 1981).

Thrombin Determinations. Assays for thrombin were based on its effect in catalyzing the release of *p*-nitroaniline from

† From the Department of Biochemistry, New York Medical College, Valhalla, New York 10595. *Received March 8, 1983.* This investigation was supported by Grant HL-16955 from the National Institutes of Health.

‡ Present address: Thrombosis Research Center, Temple University, Philadelphia, PA 19140.

[1] Abbreviations: αPI, α_1-proteinase inhibitor (α_1-antitrypsin); HA-heparin, high-activity heparin that is heterodisperse with respect to size; PEG, poly(ethylene glycol), M_r 6000; pNA, *p*-nitroaniline; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate; S-2238, D-phenylalanylpipecolyl-L-arginyl-*p*-nitroanilide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/83/0422-4810$01.50/0

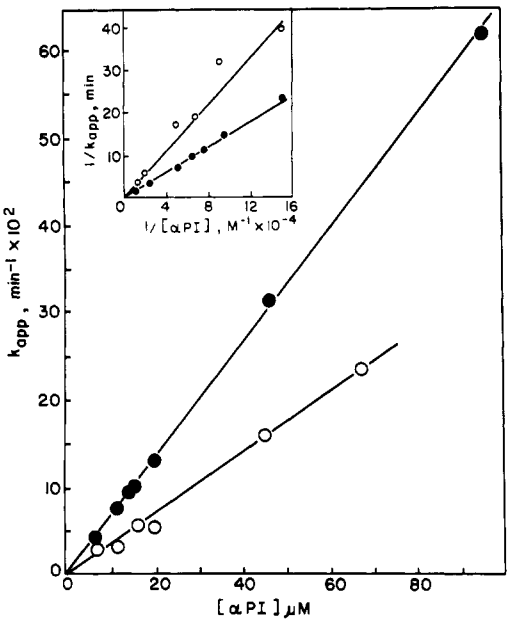

FIGURE 1: Relation between the k_{app} values for thrombin inhibition and the concentrations of αPI, in the absence and presence of heparin. Solution of 2.2 μM thrombin in 0.15 M NaCl–0.01 M Tris, pH 7.5, containing the indicated concentrations of αPI were incubated at 37 °C, and samples were assayed for thrombin after a series of intervals. Incubation mixture with heparin also contained 28.8 μg of HA-heparin/mL. All determinations were made in triplicate. The second-order rate constant was obtained from the slopes of the graphs. The inset shows reciprocal plots of the same data: (○) with heparin; (●) no heparin.

the chromogenic substrate S-2238. The reactions were performed at 37 °C. The thrombin sample in 300 μL of 0.15 M NaCl–0.05 M Tris-HCl, pH 7.5, containing 8.25 mg of PEG/mL, was mixed with 800 μL of the above buffer, and the absorbance at 405 nm was set to zero. The solution was then mixed with 200 μL of 0.65 mM S-2238, and the absorbance was recorded automatically for at least 5 min. The reaction rate, as obtained from the linear section of the recorder tracing, was converted to concentration of pNA released per minute. The value of the extinction coefficient of pNA for the conditions of the assay was 9500 M^{-1} cm^{-1}. All determinations were performed in triplicate.

Measurement of Thrombin–αPI Interaction. The rate of reaction between thrombin and αPI was determined by incubation of the two proteins in 0.15 M NaCl–0.05 Tris, pH 7.5, at 37 °C, for different time intervals. Aliquots of the incubation mixture were diluted at least 100-fold with the buffer in order for the thrombin concentration to be within the range of the assay system, and appropriate samples were determined by means of S-2238 as described above. The dilution also served to prevent further reaction as could be seen by the linearity of the rate of release of pNA with time.

Results

Assays. The kinetics of the action of αPI in the presence and absence of heparin was determined by assays for residual active thrombin after the two proteins were incubated for specified times. The concentration of thrombin was measured by its effect on the rate of release of pNA from the synthetic substrate S-2238. The presence of heparin in concentrations as high as 5.4 μg/mL did not interfere with thrombin measurements. Similar findings were also reported from other laboratories (Nordenman & Bjork, 1978; Takada et al., 1979).

Kinetics of Inhibition. Studies were conducted on the kinetics of inhibition by a range of molar excesses of αPI, and

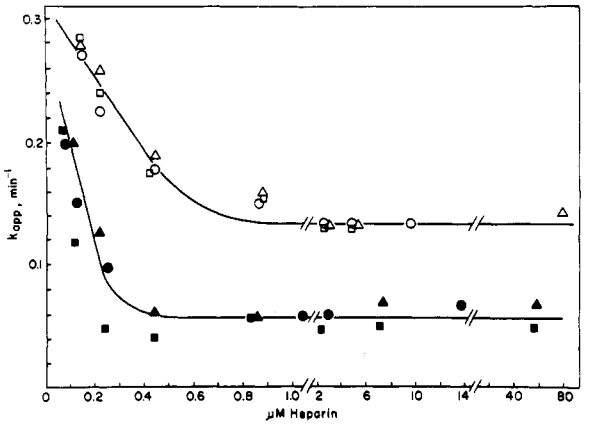

FIGURE 2: Effect of concentration of heparin with different molecular weights and anticoagulant activities, on the pseudo-first-order rate constant (k_{app}) of the thrombin–αPI reaction. Incubation mixtures for determination of k_{app} contained 2.0 μM thrombin, 45.3 μM αPI, and the indicated concentrations of heparin in 0.15 M NaCl–0.01 M Tris at 37 °C. Assays for residual thrombin activity were performed on samples taken after 4, 8, and 12 min. The molecular weights and anticoagulant units per milligram of the heparin samples were the following: (●) 22K, 870; (■) 22K, 520; (▲) 22K, 38; (○) 11.7K, 516; (□) 11.7K, 382; (△) 11.7K, 5.

it was found in all the experiments that the reactions followed pseudo-first-order kinetics for at least three half-lives. When HA-heparin was included in the incubation mixture, the reactions were still pseudo first order, but the rate of thrombin inhibition decreased considerably. Plots of the respective pseudo-first-order constants (k_{app}) vs. the related concentrations of αPI and the respective double-reciprocal plots are shown in Figure 1. It is seen that there is no saturation of the rate of thrombin inhibition when the concentration of αPI is as high as 94.6 μM. Thus, if the reaction proceeds via a reversible intermediate, the binding must be extremely weak (Kitz & Wilson, 1962). The second-order rate constant for the reaction in the absence of heparin obtained from these experiments is 6.64×10^3 M^{-1} min^{-1}. Second-order rate constants for the reaction were also calculated directly according to the procedures of Downing et al. (1978). The value thus obtained, i.e., 6.65 (±0.08) $\times 10^3$ M^{-1} min^{-1}, compares well with the above value and with that reported by Downing et al. When the experiments were performed in the presence of 28.8 μg/mL HA-heparin, the rate constant was decreased to 3.33×10^3 M^{-1} min^{-1}.

Effect of Fractionated Heparins. Since heparin is heterodisperse with respect to molecular weight and anticoagulant activity (Radoff & Danishefsky, 1982, and references cited therein), it was of interest to determine whether any of these physical or chemical properties are related to its action in inhibiting the neutralization of thrombin by αPI. Generally, heparin fractions with greater chain length also have a higher anticoagulant activity as measured by the acceleration of the inhibition of thrombin by antithrombin III (Laurent et al., 1978; Lane et al., 1978; Thunberg et al., 1979; Andersson et al., 1979; Radoff & Danishefsky, 1981). In order to determine which of the two factors, anticoagulant activity or chain length, contributes to the inhibition of the thrombin–αPI reaction, studies were conducted with heparins, M_r 22K and 11.7K, that were subfractionated according to anticoagulant activity on antithrombin-linked Sepharose (Pixley & Danishefsky, 1982). In these experiments, the concentrations of αPI and thrombin in the reaction mixture were kept constant at a molar ration of about 20:1, and the heparin concentration was varied over a wide range. The effect of each of the fractions on the rate of thrombin inhibition is plotted in Figure 2. It is seen that

Table I: Rate Constants for the Reaction between Thrombin and αPI in the Presence of Heparin with Different Molecular Weights[a]

heparin fraction M_r	activity[b] (units/mg)	k_{app}[c] (min^{-1})	k'[d] (mM^{-1} min^{-1})
22 000	350	0.043	1.0
15 900	238	0.102	2.3
13 000	186	0.134	3.0
11 700	114	0.159	3.5
8 600	59	0.198	4.4
unfractionated	152	0.114	2.5
no heparin		0.307	6.8

[a] Incubation mixtures consisted of 2.0 μM thrombin, 43.3 μM αPI, and 104 μg/mL heparin. Samples were removed at 0, 4, 8, and 12 min, and appropriate dilutions were assayed for residual thrombin activity. [b] Anticoagulant activity of fraction as measured by acceleration of the action of antithrombin III on thrombin. [c] Pseudo-first-order rate constant for rate of decrease in thrombin activity. [d] Apparent second-order rate constant calculated from the relationship $k' = k_{app}/[\alpha PI]$.

the effects of the heparins are dependent on their size and that subfractions with different anticoagulant activities are similar as long as they have the same molecular weight.

The results in Figure 2 also show that when the heparin concentrations are relatively low, the reaction rates are dependent on heparin concentrations. However, the effect of heparin reaches a maximum and further increases do not produce greater effects on the rate of thrombin neutralization. Thus, heparin does not inhibit the thrombin–αPI reaction completely. The maximum attainable effect is dependent on the molecular weight of the heparin sample and is not influenced by the anticoagulant properties of the heparin fraction. Specifically, heparin with a higher chain length decreases the rate of thrombin neutralization to a greater degree than does the smaller size heparin.

Another difference between the heparins with different molecular weights is the amount that can produce the respective maximal decrease in the thrombin–αPI reaction rate. Thus, for the experiments described in Figure 2, 22K heparin reaches its maximal effect at a concentration of about 0.3 μM whereas 11.7K heparin produces maximal action when its concentration is about 0.6 μM. Conceivably, the concentrations required for maximal activities may be related to the number of heparin oligosaccharide units or concentration by weight rather than to the overall chain length or molar concentration.

Since the action of heparin reaches a maximum and remains constant above certain concentrations (Figure 2), the effect of chain length on activity could be determined by comparing the effects of a given saturating concentration of different molecular weight fractions on the rate of the thrombin–αPI reaction. The results of such a study on five heparin fractions are shown in Table I. The pseudo-first-order rate constants as well as the apparent second order constants for the thrombin–αPI reaction clearly demonstrate a direct relationship between chain length of the heparins and their effect in decreasing the reaction rate.

Discussion

The inhibition of thrombin by αPI has been shown to result from the formation of a tight complex between the two proteins (Downing et al., 1977; Danishefsky & Pixley, 1979). If the process is analogous to that of various trypsin inhibitors (Laskowski & Kato, 1980), the sequence may be described by the equation

$$\text{thrombin} + \alpha\text{PI} \rightleftharpoons \text{thrombin}\cdot\alpha\text{PI} \rightarrow \text{thrombin}-\alpha\text{PI} \quad (1)$$

where, thrombin·αPI is a loose intermediate that transforms to a stable tight complex, thrombin–αPI. Should this be the case, the action of heparin may be on the formation of the labile complex or on its conversion to the final product. The finding that the reaction rate in the absence of heparin does not saturate under pseudo-first-order conditions even when the concentration of αPI is 94.6 μM argues against the two-step process. Addition of heparin results in a decrease of the rate of inactivation of thrombin by αPI, but the reaction still follows pseudo-first-order kinetics over the whole range of αPI concentrations. There is thus no evidence for the formation of an intermediate labile complex in the thrombin–αPI reaction, whether heparin is present or not. Therefore, on the basis of the present data, the action of heparin cannot be on a putative rapid preequilibrium step depicted by eq 1. Instead, the present results suggest that there is a direct reaction between thrombin and αPI (eq 2) and that heparin decreases the rate of this reaction.

$$\text{thrombin} + \alpha\text{PI} \rightarrow \text{thrombin}-\alpha\text{PI} \quad (2)$$

It is possible that saturation of the rate of thrombin neutralization does occur when the concentration of αPI is considerably greater than 96 μM. However, the reaction with such high concentrations is too rapid to allow for accurate assays. Conceivably, such measurements could be achieved by stopped-flow fluorometry as demonstrated for the reaction between thrombin and antithrombin III (Olson & Shore, 1982). In that reaction, it was shown that the kinetics are consistent with a simple bimolecular process when the antithrombin III concentration is 20 μM (Downing et al., 1978; Jesty, 1979; Jordan et al., 1979) but that at levels greater than 70 μM the data indicate that a two-step process is involved (Olson & Shore, 1982). By analogy with the thrombin–antithrombin III reaction and with those of various other protease inhibitors (Laskowski & Kato, 1980), it is conceivable that the reaction between thrombin and αPI proceeds via a loose intermediate (eq 1), although this has not been demonstrated in the present study.

It is proposed that the action of heparin is via the binding to thrombin rather than to αPI. This is evidenced, first, by the finding that interference by heparin cannot be overcome by excess αPI. Second, αPI does not bind to heparin that is linked to Sepharose (Danishefsky & Pixley, 1979) whereas thrombin does bind (Li et al., 1974; Machovich, 1975; Danishefsky et al., 1976; Griffith et al., 1978). Third, heparin does not inhibit the interaction of αPI with trypsin (Danishefsky & Pixley, 1979). Similarly, although αPI inhibits factor X_a (Ellis et al., 1982), the reaction rate is not decreased by heparin (Long & Williamson, 1981; B. Lahiri and I. Danishefsky, unpublished results).

The kinetic data lead to the conclusion that heparin-bound thrombin does react with αPI; however, the reaction rate is considerably less than that with free thrombin. This is consistent with the data showing that at specific concentrations of heparin, the reaction rate attains a value that remains constant; that is, it does not change with increasing heparin concentration. This limiting value reflects the rate of reaction between the thrombin–heparin complex and αPI.

The degree to which heparin decreases the rate constant for the thrombin–αPI interaction depends on the molecular weight of the heparin. Specifically, complexes of thrombin with heparin of higher molecular weights react at a slower rate with αPI than do complexes containing heparin with lower molecular weights. However, there is no significant difference between heparins with different anticoagulant activities when fractions within the same size range are compared to each

other. This relationship between the molecular weight of heparin and inhibition of thrombin–αPI complex formation was also found in studies utilizing gel electrophoresis (not shown). It is noteworthy nonetheless, that the inhibitory effect on the thrombin–αPI reaction is dependent on various structural features of the heparin macromolecule so that chondroitin sulfates or dermatan sulfate has no measurable activity (Danishefsky & Pixley, 1979; Long & Williamson, 1981).

In addition to modifying the rate of thrombin neutralization to a different extent, the heparin fractions differ from each other with respect to the molar concentrations required to exert their maximal effect (Figure 2). Thus, a higher concentration of 11.7K heparin than of the 22K heparin is required to produce the maximum effect specific for that heparin fraction. The data also suggest that each heparin molecule modifies the reactivity of several thrombin; however, the results of the kinetic experiments do not allow for rigorous conclusions regarding the thrombin–heparin binding stoichiometry. Preliminary data from experiments on the binding of thrombin and heparin in 0.01 M Na_2SO_4–0.07 M NaCl, as measured by the displacement of the thrombin peak in HPLC, show that 22K heparin does indeed bind at least four thrombins (B. Lahiri and I. Danishefsky, unpublished results). The problem of the binding stoichiometry is currently under study since it may be a factor in the mechanism of action of heparin in decreasing the interaction between thrombin and αPI as well as in accelerating the neutralization of thrombin by antithrombin III.

Registry No. α_1-Proteinase inhibitor, 9041-92-3; heparin, 9005-49-6; thrombin, 9002-04-4.

References

Andersson, L. O., Barrowcliffe, T. W., Holmer, E., Johnson, E. A., & Soderstrom, G. (1979) *Thromb. Res. 15*, 531–541.

Bloom, J. W., & Hunter, M. J. (1978) *J. Biol. Chem. 253*, 547–559.

Chase, T., & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.

Danishefsky, I., & Pixley, R. (1979) *Biochem. Biophys. Res. Commun. 91*, 862–870.

Danishefsky, I., Tzeng, F., Ahrens, M., & Klein, S. (1976) *Thromb. Res. 8*, 131–140.

Danishefsky, I., Zweben, A., & Slomiany, B. (1978) *J. Biol. Chem. 253*, 32–37.

Davie, E. W., & Fujikawa, K. (1975) *Annu. Rev. Biochem. 44*, 799–829.

Dietz, A. A., Rubenstein, H. M., & Hodges, L. (1974) *Clin. Chem. (Winston-Salem, N.C.) 20*, 396–399.

Downing, M. R., Bloom, J. W., & Mann, K. G. (1977) in *The Chemistry and Biology of Thrombin* (Lundblad, R. L., Mann, K. G., & Fenton, J. W., Eds.) pp 441–450, Ann Arbor Press, Ann Arbor, MI.

Downing, M. R., Bloom, J. W., & Mann, K. G. (1978) *Biochemistry 17*, 2049–2053.

Ellis, V., Scully, M., Macgregor, I., & Kakkar, V. (1982) *Biochim. Biophys. Acta 701*, 24–31.

Fenton, J. W., Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finlayson, J. S. (1977) *J. Biol. Chem. 252*, 3587–3598.

Griffith, M. J. Kingdon, H. S., & Lundblad, R. L. (1978) *Biochem. Biophys. Res. Commun. 83*, 1198–1205.

Heimburger, N. (1975) in *Proteinase and Biological Control* (Reich, E., Rifkin, D. B., & Shaw, E., Eds.) pp 367–386, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Jesty, J. (1979) *J. Biol. Chem. 254*, 10044–10050.

Jordan, R., Beeler, D., & Rosenberg, R. (1979) *J. Biol. Chem. 254*, 2902–2913.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Kurechi, T., Kress, L. F., & Laskowski, M., Sr. (1979) *Anal. Biochem. 99*, 415–420.

Lane, D. A., Macgregor, I. R., Michalski, R., & Kakkar, V. (1978) *Thromb. Res. 12*, 257–276.

Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593–626.

Laurent, T., Tengblad, A., Thunberg, L., Hook, M., & Lindahl, U. (1978) *Biochem. J. 175*, 691–701.

Li, E. H. H., Orton, C., & Feinman, R. D. (1974) *Biochemistry 13*, 5012–5017.

Long, W. F., & Williamson, F. B. (1981) *Br. J. Pharmacol. 73*, 505–509.

Machovich, R. (1975) *Biochim. Biophys. Acta 412*, 13–17.

Martin, B. M., Feinman, R. D., & Detwiler, T. C. (1975) *Biochemistry 14*, 1308–1314.

Nordenman, B., & Bjork, I. (1978) *Thromb. Res. 12*, 755–765.

Olson, S. T., & Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891–14895.

Pixley, R., & Danishefsky, I. (1982) *Thromb. Res. 26*, 129–133.

Radoff, S., & Danishefsky, I. (1981) *Thromb. Res. 22*, 353–365.

Radoff, S., & Danishefsky, I. (1982) *Arch. Biochem. Biophys. 215*, 163–170.

Rosenberg, R. D. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26*, 10–18.

Takada, A., Koide, T., & Takada, Y. (1979) *Thromb. Res. 16*, 59–68.

Takahara, H., & Sinohara, H. (1982) *Thromb. Res. 27*, 45–50.

Thunberg, L., Lindahl, U., Tengblad, A., Laurent, T., & Jackson, C. (1979) *Biochem. J. 181*, 241–243.

Villanueva, G., & Danishefsky, I. (1979) *Biochemistry* 18, 810–817.