Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 862-870

# EFFECT OF HEPARIN ON THE INHIBITION OF THROMBIN BY $\alpha_1$-PROTEINASE INHIBITOR

Isidore Danishefsky and Robin Pixley

Department of Biochemistry, New York Medical College
Valhalla, New York 10595

Received October 15, 1979

SUMMARY: Heparin interferes with the inhibition of thrombin by $\alpha_1$-proteinase inhibitor (αPI). The inhibitory effect of heparin is due to its binding to thrombin. Other glycosaminoglycans and carboxyl-modified heparin do not have the same effect as heparin. The results indicate that there are similarities in the structural requirements in heparin, for anticoagulant activity and for the inhibition of αPI interaction with thrombin.

## INTRODUCTION

Blood plasma is known to contain a number of proteinase inhibitors (1). Of these $\alpha_1$-proteinase inhibitor (also called $\alpha_1$-antitrypsin) is by far, the most abundant. In addition to inhibiting trypsin and several other proteolytic enzymes, αPI interferes with the action of thrombin (2-4). It was demonstrated that inhibition of thrombin by αPI is due to the formation of a complex involving equimolar amounts of the two proteins (3). Detailed studies of the kinetics of this process have been reported recently (5). In this communication we demonstrate that heparin inhibits the neutralization of the thrombin by αPI. Thus, with respect to the αPI-thrombin reaction, heparin effectively enhances coagulation.

## EXPERIMENTAL PROCEDURES

Materials - The products used in these studies were obtained as follows: BAPNA from Sigma Chemical Co.; Trypsin (Code TRTPCK) from Worthington Biochemical Corp.; pNPGP from ICN Chemical Co.; $\alpha_1$-Trypsin inhibitor quantitative immunodiffusion kit and chondroitin 6-sulfate from Miles Laboratories; polybrene from Polysciences, Inc.; PEG from K & K Laboratories; molecular weight standards from Pierce Chemical Co.; and chromogenic substrate S-2238 from Ortho Pharmaceuticals.

Supported by Grant HL-16955 from USPHS.

Abbreviations: αPI, $\alpha_1$-proteinase inhibitor ($\alpha_1$-antitrypsin); BAPNA, α-N-benzoyl-DL-arginine-p-nitroanilide; pNPGB, p-nitrophenyl-p-quanidino benzoate; PEG, polyethylene glycol-6000; S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide; SDS, sodium dodecyl sulfate; Tris/NaCl, tris-buffered saline (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5)

0006-291X/79/230862-09$01.00/0

Human fibrinogen purchased from Calbiochem, was purified according to Laki (6). Human thrombin prepared from Cohn fraction III (7) was a gift from Dr. John Fenton, II, of the New York State Department of Health, Albany, N.Y. Heparin from bovine lung (152 units per mg) was obtained from Upjohn International through the courtesy of Dr. L.L. Coleman. Dermatan sulfate was isolated from umbilical cord (8) and heparin methyl ester was prepared by treatment of heparin with diazomethane (9). Heparin preparations with 585 and 120 anticoagulant units per mg were obtained by fractionation of beef lung heparin on Biogel P-100 (10). Fractionation of heparin on the basis of antithrombin binding was performed by affinity chromatography (11) and activities were determined as described previously (12).

Assay Methods - αPI activity was determined during the fractionation steps by its effect in inhibiting the trypsin-catalyzed hydrolysis of BAPNA (13). Active site titration of the trypsin was performed with pNPGB (14). The isolated product was also identified by radial immunodiffusion assays (13). The concentration of αPI in solution was based on absorbance at 280 nm ($\varepsilon^{1\%}_{1\ cm}$ = 4.84) (15).

The action of αPI on thrombin was measured by assays for thrombin subsequent to preincubation with the inhibitor at 37° for specified periods. Thrombin activity was determined by its effect on the clotting of fibrinogen and by the rate of amidolysis of chromogenic substrate S-2238. The concentration of thrombin in solutions was determined by the absorbance at 280 nm ($\varepsilon^{1\%}_{1\ cm}$ = 18.3) (7). The clotting time was measured after mixing 100 µl of thrombin or test solution with 400 µl of 0.5% fibrinogen, in Tris/NaCl. The assays were performed in quadruplicate and compared with standard curves constructed in conjunction with each experiment. In the assays for the release of p-nitroaniline from S-2238, 350 µl of appropriately diluted thrombin or test solutions were mixed with 750 µl Tris/NaCl at 37° in a plastic cuvette. This was followed by addition of 200 µl 0.75 mM S-2238 containing 0.33 mg/ml polybrene and measurement of the absorbance at 405 nm on a recording spectrophotometer for 10 min. The amounts of thrombin in the solutions were determined by comparing the slopes ($\Delta A_{405}$ per min) with those obtained with known amounts of thrombin. The presence of polybrene did not affect the assay system with thrombin; it was employed, however, in order to neutralize the possible effects of heparin and other glycosaminoglycans on the chromogenic substrate.

Isolation of α-Proteinase Inhibitor - Blood plasma (100 ml), adjusted to pH 7.35 with $KHPO_4$ was stirred with 20 g PEG and the precipitate was removed by centrifugation. Another 15 g of PEG were mixed with supernatant and the resulting precipitate was dissolved in 30 ml Tris/NaCl, and dialyzed overnight against the same buffer. The dialyzate was then passed through a column of heparin-aminohexylsepharose (2.6 x 10 cm) to adsorb antithrombin III (16). Albumin was then removed from the effluent by adsorbtion on a column (5 x 40 cm) of Cibacron Blue F-3-GA (17). The fractions were monitored by their absorbance at 280 nm and by assays for trypsin inhibitory capacity (13). The albumin-free solution was concentrated by ultrafiltration on an Amicon PM 30 membrane, to approximately 25 ml. Successive chromatography on DEAE-cellulose (2.6 x 70 cm) at pH 8.8 and at pH 6.5 according to Pannell et al (18) yielded fractions with αPI. Some preparations at this stage contained a high molecular weight contaminant. The latter was removed by gel-filtration on Sephacryl S-200 equilibrated with 0.005 M $Na_2HPO_4$/0.1M NaCl, pH 7.0.

Electrophoresis - Polyacrylamide disc gel electrophoresis, pH 8.3, was carried out by standard procedures (19). SDS-polyacrylamide gel electrophoresis (20) was performed with 7.5% gels. The separations were allowed to proceed for approximately 3 h at 7mA/tube. Coomassie brilliant blue was used to stain for proteins.

## RESULTS AND DISCUSSION

The procedure for $\alpha_1$-proteinase inhibitor utilized in these studies allows for the concurrent isolation of this plasma protein and also for antithrombin III. During affinity chromatography the latter is bound to heparin-aminohexyl-sepharose (16) while the former is not adsorbed. The subsequent fractionations on DEAE-cellulose by gradient elution at pH 8.8 and 6.5 were the same as those described previously (18). The trypsin inhibitory capacity (13) as measured against active site titrated trypsin (14) was 80-90%. The product showed a single band on SDS-polyacrylamide gel electrophoresis and in disc gel electrophoresis. The molecular weight on the basis of SDS-gel electrophoresis

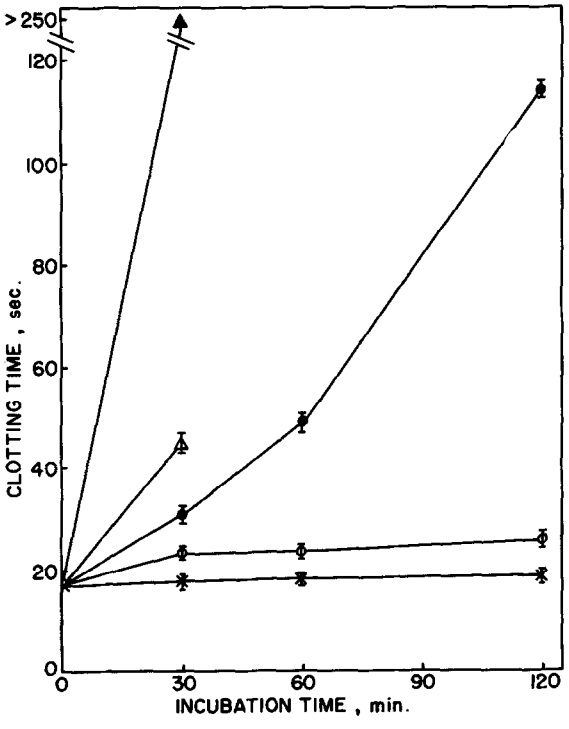



Fig. 1. Inhibition of thrombin by αPI and the effect of heparin on the process. Solutions consisting of 2.22 μM thrombin were incubated at 37° with different concentrations of αPI in Tris/NaCl with and without heparin. Immediately after mixing and after specific time intervals, the fibrinogen clotting time was determined on constant aliquots of the incubation mixture (see Experimental section). X thrombin only; ● plus 4.34 μM αPI; ▲ plus 8.95 μM αPI; ○ plus 4.34 μM αPI and 22.2 μg/ml heparin; △ plus 8.95 μM αPI and 19.7 μg/ml heparin. Each point represents the average of at least four determinations and the error bars indicate the S.D.

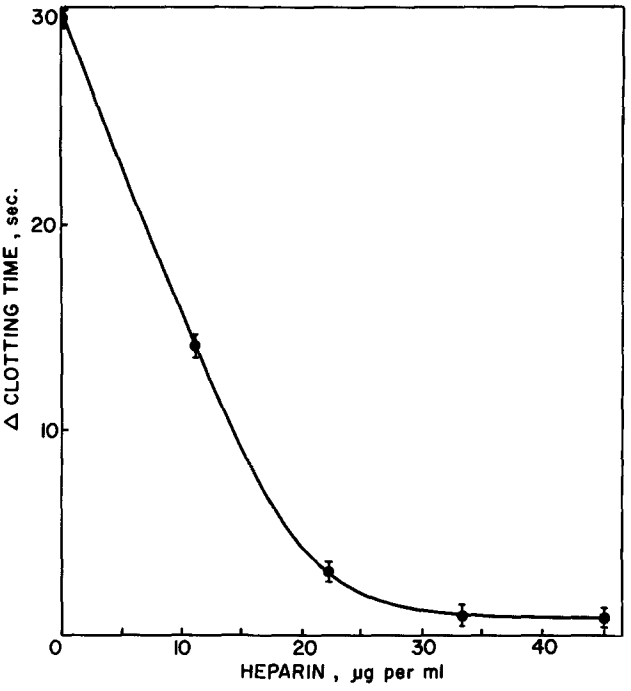
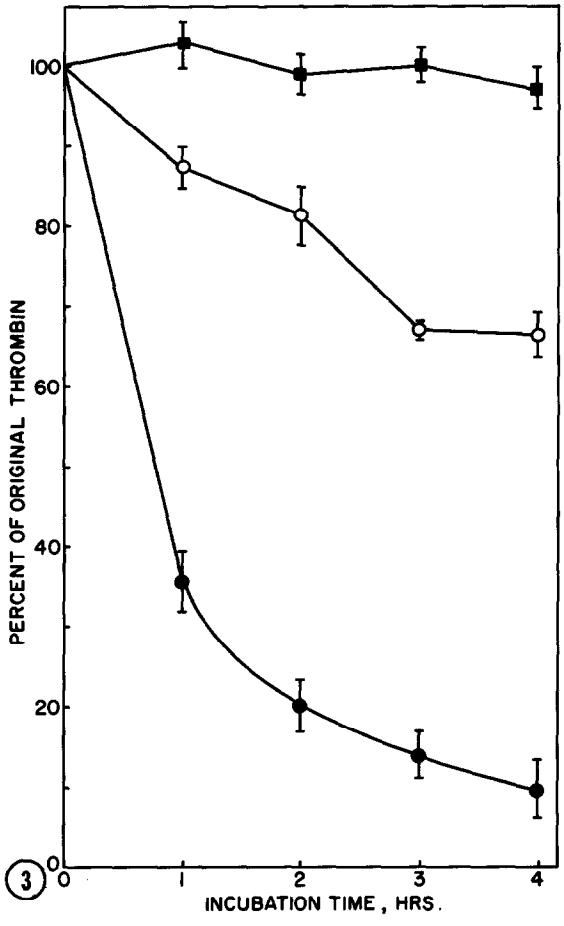



Fig. 2. Relationship between heparin concentration and thrombin-inhibitory action of αPI. Solutions of 2.22 μM thrombin, 4.34 μM αPI were incubated at 37° in Tris/NaCl for 60 min and aliquots were assayed for fibrinogen clotting activity, as described in Fig. 1. The increase in clotting time from that given by the same amount of thrombin (Δ clotting time) is shown on the plot. At least four determinations were done for each point and the standard deviations are shown.

was 54000 and the amino acid analyses agreed with those reported from other laboratories (15).

Human αPI was found to be effective in inhibiting the action of thrombin as reported previously (2-5). The rate is dependent on the concentrations and the relative amounts of thrombin and αPI. The results for 2:1 and 4:1 molar ratios of inhibitor to thrombin by the fibrinogen-clotting assay are shown in Figure 1. Of special interest was the finding that heparin relieves the inhibitory action of αPI (Fig. 1). The effect of different amounts of heparin when the concentrations of thrombin and inhibitor were constant, is described in Figure 2. Assuming an average molecular weight of 10,000 for heparin, the data indicate that almost all of the inhibition is relieved when the molar ratio of heparin to thrombin is 1:1.



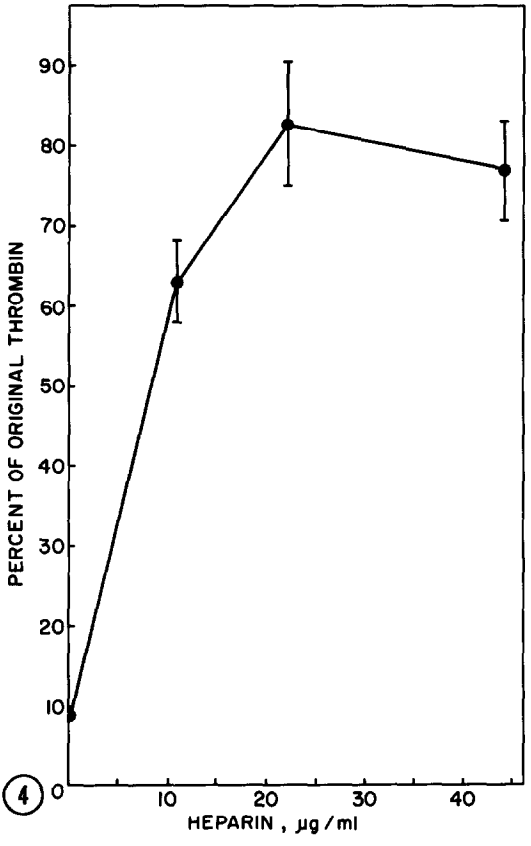




Fig. 3. Thrombin inhibition by αPI and the effect of heparin as determined by amidolysis of S-2238. Tris/NaCl solutions containing 2.22 µM thrombin and 4.38 µM αPI, with and without 44.4 µg/ml heparin, were incubated at 37°. Aliquots of the mixtures were then assayed for the rate of release of p-nitroaniline from S-2238 (See EXPERIMENTAL section) and the amount of thrombin was determined. ■ thrombin only, ● thrombin plus αPI, ○ heparin added. (Another control experiment on thrombin and heparin was also performed. This is not shown because the results were similar to that of thrombin alone.)

Fig. 4. Effect of heparin concentration on αPI inhibition of thrombin as determined by the rate of amidolysis of S-2238. The incubation mixture (37°) contained 2.22 µM thrombin, 4.38 µM αPI and different amounts of heparin in Tris/NaCl. Samples were analyzed at zero-time and after incubation for 2 h.

The effects of αPI on thrombin in the presence and absence of heparin were also determined by assays for the rate of the thrombin-catalyzed amidolysis of the p-nitroaniline amide of the synthetic polypeptide, S2238. Results of these studies (Figs. 3 and 4) agreed with those of the fibrinogen-clotting system.

The action of αPI on thrombin is due to the binding of inhibitor with thrombin to form an inactive complex (3). This can be demonstrated by SDS-poly-

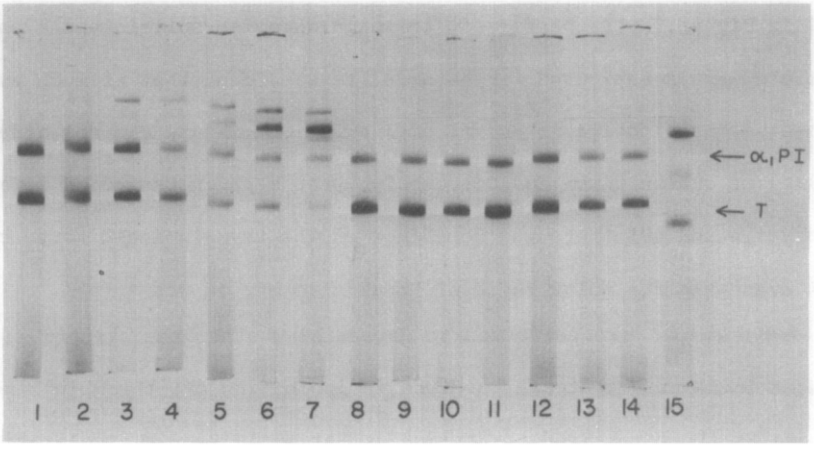



Fig. 5. SDS-polyacrylamide gel electrophoresis showing the interaction between $\alpha_1$-proteinase inhibitor with thrombin in the absence and presence of heparin. 175.8 µg αPI and 223.2 µg thrombin in 2 ml Tris/NaCl were incubated at 37°. In a concurrent experiment 64 µg of heparin was added to an identical mixture. At specific time intervals, 0.2 ml aliquots were withdrawn, mixed with 20 µl of 10% SDS and heated in boiling water for 2 min. Samples of 50 µl were then applied to each gel. Samples 8-14 contained heparin in the incubation mixture. Incubation times were as follows: 1 and 8, zero time, 2 and 9, 5 min; 3 and 10, 10 min; 4 and 11, 20 min; 5 and 12, 30 min; 6 and 13, 1 h; 7 and 14, 2 h; 15, molecular weight standards, starting from top, bovine serum albumin (68000), ovalbumin (43000), and chymotrypsinogen (25700). The positions of $\alpha_1$-PI and thrombin (T) with respect to the standards are indicated by arrows.

acrylamide gel electrophoresis (Fig. 5). Thrombin and αPI migrate as single bands with apparent molecular weights of 36500 and 54000, respectively (gel 1, Fig. 5). When incubated together in the absence of heparin, the two proteins form a complex with an apparent molecular weight of 92000 that eventually undergoes degradation to products with somewhat lower molecular weights (80000 and 74000). Degradation of the original complex is attributed to limited proteolysis by residual free thrombin. When thrombin was incubated with αPI in the presence of heparin (gels 8-14 in Fig. 5), the production of the complex was inhibited and only a minimal amount appeared after the reaction proceeded for an hour.

Studies were also conducted to determine whether the effect of heparin on the neutralization of thrombin by αPI is specific for heparin and thrombin, or whether it is a non-specific reaction that might be caused by other polyelectrolytes with similar structures. Thus, experiments identical to those

described in Figure 3 were performed, in which dermatan sulfate and chondroitin 6-sulfate were substituted for heparin. It was found that as much as 67 µg of these glycosaminoglycans per ml of incubation mixture did not affect the activity of αPI on thrombin. Conversely, when heparin was added to a mixture of trypsin and αPI there was no effect on the trypsin-inhibitory capacity (13) even when the molar ratio of heparin to enzyme was 4 to 1.

Several experiments were performed to determine whether the structural units or sequences in heparin that are involved in inhibiting αPI-thrombin binding, are the same as those required for anticoagulant activity via acceleration of the thrombin-antithrombin reaction. The preparations employed for these studies were: carboxyl-modified heparin (heparin methyl ester) which had almost no anticoagulant activity (9) and fractions with different anticoagulant activities obtained by gel-filtration of heparin on Biogel P-100 (10) and affinity chromatography on antithrombin-linked Sepharose (11). The results are summarized in Table I. The ineffectiveness of heparin methyl ester indicates a requirement of a free carboxyl group. For each of the fractionation systems, the components with higher anticoagulant activities are also more potent in inhibiting the binding of αPI to thrombin. However, the relationship between anticoagulant activity and αPI-inhibiting activity are not the same for the fractions from both separations methods. It is conceivable that the

Table I. Activity of Heparin Fractions in Inhibiting the Neutralization of thrombin by αPI.

| Heparin preparation | Anticoagulant units per mg | αPI inhibition,[a] % Residual thrombin |
|---|---|---|
| Heparin methyl ester | 0 | 0 |
| Unfractionated heparin | 152 | 40 |
| Fractionated heparin: | | |
| AT-Sepharose, bound | 605 | 60 |
| " , non bound | 17 | 40 |
| Biogel, high m.w. | 585 | 78 |
| " , low m.w. | 120 | 43 |

[a] Solutions consisting of 2.2 µM thrombin, 5.0 µM αPI were incubated with 1.0 µg/ml of heparin samples for 100 min at 37°. Aliquots were assayed at zero time and at end of incubation as described in the EXPERIMENTAL section.

molecular weight is also an important determinant in the inhibition of αPI-activity. This is consistant with the results for the Biogel components in which the more active fractions are those with higher molecular weights. This problem as well as detailed kinetic studies with characterized heparin fractions are the subject of current experiments.

In conclusion, these studies clearly demonstrate that heparin can protect thrombin from deactivation by αPI. It is proposed that the action of heparin on the thrombin-αPI reaction is due to the formation of a thrombin-heparin intermediate that cannot combine with αPI. The fact that thrombin binds with heparin has been reported from several laboratories (21-24). The inhibitor, αPI does not appear to react directly with heparin. This is evidenced by the findings that αPI does not bind to heparin that is linked to Sepharose. Additionally, heparin does not affect the action of αPI on trypsin. Although heparin functions to protect thrombin from neutralization by αPI, this reaction would not be expected to counteract its anticoagulant activity via potentiation of antithrombin under normal conditions. It may be an important consideration, however, when individuals with hereditary or drug-induced antithrombin deficiencies are treated with heparin.

## REFERENCES

1. Heimberger, N., Haupt, H. and Schwick, H.G. (1971) in Proc. Int. Conf. on Proteinase Inhibitors (Fritz, H. and Tschesche, H., Eds.) pp 1-22, W. De Gryter, New York, N.Y.
2. Matheson, N.R. and Travis, J. (1976) Biochem. J. 159, 495-502.
3. Downing, M.R. Bloom, J.W. and Mann, K.G. (1977) in The Chemistry and Biology of Thrombin (Lundblad, R. L., Mann, K.G. and Fenton, J.W., Eds.) pp 441-450, Ann Arbor Press, Ann Arbor, Mich.
4. Machovich, R., Borsodi, A., Blasko, G. and Orakzai, S.A. (1977) Biochem. J. 167, 393-398.
5. Downing, M.R., Bloom, J.W. and Mann, K.G. (1978) Biochemistry 17, 2649-2653.
6. Laki, K. (1951) Arch. Biochem. Biophys. 32, 317-324.
7. Fenton, J. II, Fasco, M. and Stackrow, A. (1977) J. Biol. Chem. 252, 3587-3589.
8. Danishefsky, I. and Bella, A. Jr. (1966) J. Biol. Chem. 241, 143-146.
9. Danishefsky, I. and Siskovic, E. (1972) Thromb. Res. 1, 173-182.
10. Danishefsky, I. and Radoff, S. (1979) Federation Proc. 38, 810.
11. Hook, M. Bjork, I., Hopwood, J. and Linhahl, U. (1976) FEBS Lett. 66, 90-93.

12. Danishefsky, I., Ahrens, M. and Klein, S. (1977) Biochim. Biophys. Acta 498, 215-222.
13. Dietz, A.A., Rubenstein, H.M. and Hodges, L. (1974) Clin. Chem. 20, 396-399.
14. Chase, T. and Shaw, E. (1967) Biochem. Biophys. Res. Commun. 29, 501-514.
15. Bloom, J.W. and Hunter, M.J. (1978) J. Biol. Chem. 253, 547-559.
16. Danishefsky, I., Zweben, A. and Slomiany, B.L. (1978) J. Biol. Chem. 253, 32-37.
17. Travis, J., Bowen, D., Tewksbury, D., Johnson, B. and Pannell, R. (1976) Biochem. J. 157, 301-306.
18. Pannell, D., Johnson, J. and Travis, J. (1974) Biochemistry 13, 5439-5445.
19. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427.
20. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
21. Li, E.H.H., Orton, C. and Feinman, R.D. (1974) Biochemistry 13, 5012-5017.
22. Machovich, R. (1975) Biochim. Biophys. Acta 412, 13-17.
23. Danishefsky, I., Tzeng, F., Ahrens, M. and Klein, S. (1976) Thromb. Res. 8, 131-140.
24. Griffith, M.J., Kingdon, H.S. Lundblad, R.L. (1978) Biochem. Biophys. Res. Commun. 1198-1205.