Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 3028–3032, July 1977
Medical Sciences

In vitro and *in vivo* correlation of clotting protease activity: Effect of heparin

(coagulation/antithrombin III/animal model)

SANFORD N. GITEL, RONALD C. STEPHENSON, AND STANFORD WESSLER

Department of Medicine, New York University School of Medicine, New York, New York 10016

Communicated by Saul Krugman, May 2, 1977

ABSTRACT The thrombogenicity of three highly purified proteases (thrombin, activated Factor X, and activated Factor IX) was determined quantitatively in an animal model. The minimal amounts required to produce a standard score 4 thrombus were 1.1 nmol for thrombin, 0.12 nmol for activated Factor X, and 0.018 nmol for activated Factor IX. After the administration of heparin at 5, 10, and 20 units/kg in rabbits, the thrombogenicity of each of these proteases decreased progressively. The heparin-induced inhibition of thrombosis decreased in the order, activated Factor IX > activated Factor X > thrombin at each heparin concentration. These differences were statistically significant.

These *in vivo* data provide support for the following hypotheses originally developed from *in vitro* experiments: (*i*) activation of the blood coagulation system, which proceeds through a cascade mechanism, incorporates biochemical amplification; (*ii*) the inhibition of activated Factor IX by antithrombin III in the presence of heparin is an important reaction in the prevention of thrombosis; (*iii*) less heparin is required to inhibit thrombosis prior to thrombin generation than afterward; (*iv*) an increase in the reactivity of antithrombin III reflects a decreased tendency to thrombosis while a decrease in this reactivity reflects an increased tendency to thrombosis.

An animal model can be defined as a living organism with an inherited, naturally acquired, or induced pathological process that closely resembles, in one or more respects, the same phenomenon occurring in man. In this sense, animal models never provide final answers but offer only approximations: no single model can ever duplicate a disease in man. For a model to be a good one, it must provide a new insight, have relevance to a particular problem, and respond predictably (1).

There are special reasons for wanting animal models in the area of thrombosis. Currently there is an explosion of biochemical knowledge in the field of coagulation, and animal models can help separate the trivia from the observations of intrinsic biological importance. These models, moreover, can serve as a bridge between an understanding of molecular biology and the conquest of disease.

Whereas there is a paucity of natural animal models of thrombosis, there is a plethora of the induced varieties. One standard preparation that in the past has been an effective bridge between *in vitro* data and findings in man is the stasis model of thrombosis (2). This preparation has appropriateness, simplicity, and reproducibility when applied to venous thrombosis (3).

In the present investigation the stasis model was used to quantitate the thrombogenicity of highly purified thrombin (Factor IIa), activated Factor X (Xa) and activated Factor IX (IXa) and to measure the antithrombotic effect of heparin against each of these proteases as well as Factor X coagulant protein (X-CP) that is isolated from Russell's viper venom and can convert Factor X to Xa. The data obtained in these experiments have provided *in vivo* support for some of the key biochemical hypotheses in blood coagulation that may be relevant to venous thrombosis in man.

Abbreviations: Xa, activated Factor X; IXa, activated Factor IX; X-CP, Factor X coagulant protein of Russell's viper venom.

MATERIALS AND METHODS

Russell's viper venom (*Vipera russelli*), DEAE-cellulose, and bovine serum albumin were obtained from Sigma Chemical Co. Sephadex G-100 and QAE Sephadex were products of Pharmacia Fine Chemicals, Inc. Sodium pentobarbital, 50 mg/ml, was obtained from Abbott Laboratories. Porcine gut heparin (lot 45584) as a solution containing 1000 units/ml was from Riker Laboratories, Inc. Male New Zealand White rabbits weighing 2.0 kg were obtained from Camm Research Institute.

Rabbit blood was collected by venipuncture from the marginal ear vein, and plasma was harvested as described (4).

Parke, Davis topical thrombin was freed from contaminating Xa by established procedures (5). The purified preparation of bovine thrombin, 2400 NIH units/mg, was dialyzed against isotonic saline. Xa was prepared by activation of the barium sulfate eluate of bovine plasma with crude Russell's viper venom in the presence of 10 mM Ca^{2+}. The Xa was purified by chromatography on DEAE-cellulose (5) and dialyzed against isotonic saline. Highly purified bovine IXa, 1.3 mg/ml, was generously provided by Earl Davie, Department of Biochemistry, University of Washington, Seattle, WA. X-CP was isolated by chromatography on Sephadex G-100 and QAE Sephadex (6) and dialyzed against isotonic saline. Rabbit antithrombin III was purified to homogeneity by established procedures (7). The Xa inhibitory activity of rabbit plasma samples was determined as described previously (4). The assay for X-CP was performed as described by Jackson *et al.* (8).

Inhibition of X-CP by Antithrombin III.

(*a*) *In the absence of heparin.* To 0.1 ml of X-CP was added 0.3 ml of 0.02 M Tris·maleate, pH 7.5, and the mixture was incubated at 37°. After 1 min, 0.1 ml of purified antithrombin III, with an activity equivalent to that of normal human plasma, or 0.1 ml of physiological saline (control sample) was added. The resulting solution was mixed and incubated at 37°. At 2-min intervals, 0.1-ml aliquots were removed and assayed for X-CP activity.

(*b*) *In the presence of heparin.* To 0.1 ml of X-CP was added 0.2 ml of 0.02 M Tris·maleate, pH 7.5, and 0.1 ml of heparin, 1 unit/ml, and the assay was continued as described above.

Assay for Thrombogenicity. The stasis assay (2) was modified for pretreatment of rabbits with heparin as follows. After injection of 1.5 ml of pentobarbital into a marginal ear vein, a blood sample was taken from another ear vein. The jugular veins were exposed and freed from surrounding tissue. Two sutures were placed under a jugular vein, 1 cm apart. A stopwatch was started and 0.5 ml of a solution containing heparin was injected into a marginal ear vein at 1 min. At 2 min, a sec-

Table 1. Effect of heparin injection on Xa inhibitory activity (XaIA) and heparin levels in rabbit plasma

Heparin dose, units/kg	XaIA increase,* %	Heparin, unit/ml		Equivalent heparin in human plasma, unit/ml
		Expected	Found*	
0	0	0	0	0
5	10	0.17	0.025	0.01
10	60	0.33	0.12	0.04
20	120	0.67	0.25	0.075

* The mean from eight rabbits at each heparin concentration.

ond blood sample was obtained from the ear contralateral to that used for heparin injection. At 6 min, 0.5 ml of solution containing a procoagulant was injected into a marginal ear vein. A segment of the contralateral jugular vein was isolated by ligating the sutures 10 sec after injection of the protease. At 16 min, the isolated segment was removed, placed in 5% (wt/vol) trisodium citrate, and opened. The enclosed thrombus was graded on a scale of 0 to 4 (2) with 0 representing no thrombus and 4 representing a complete thrombotic cast of the isolated segment.

Preparation of Stock Solutions of Thrombin, Xa, IXa, and X-CP. Each of the proteins was diluted in physiological saline containing 10 mg of bovine serum albumin per ml and buffered to pH 7.5 with 0.02 M Tris-maleate such that infusion of 0.5 ml of the stock solution in the absence of heparin resulted in a score 4 thrombus and 0.5 ml of a 50% dilution of the stock solution yielded a score 2 thrombus. The stock solutions were stored at −20° and thawed at 37° immediately before use.

Heparin Assay. Plasma heparin concentrations were determined as described (4).

Statistical Analysis of the Data. The data were analyzed for statistical significance by the Wilcoxon rank sum test (9, 10).

RESULTS

Dilution Curves for Thrombotic Potential of Thrombin, Xa, IXa, and X-CP. Fig. 1 illustrates the thrombotic potential, on the 0 to 4 scale, for thrombin, Xa, IXa, and X-CP as a function of dilution in the stasis model assay. For each of the proteins the 100% point was the stock solution used in subsequent experiments. The dilution curves for the proteins were nearly superimposable, indicating that the stock solutions of each protein were closely matched for thrombotic potential.

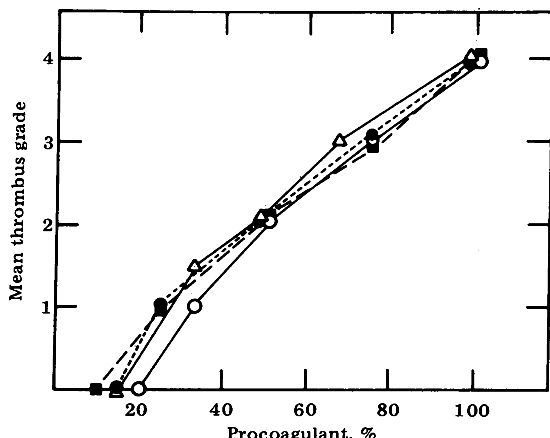

FIG. 1. Thrombotic potential of thrombin (△), Xa (○), IXa (■), and X-CP (●) as a function of concentration of procoagulant. The concentrations of each of the proteases in its stock solution (100% point) were: IIa, 2.2 μM; Xa, 0.24 μM; and IXa, 36 nM. Each point is the mean of at least three assays.

Thrombogenicity of Activated Clotting Factors. The minimal amount of thrombin, Xa, and IXa needed to produce a score 4 thrombus was determined to be 40 μg of thrombin, 5.5 μg of Xa, and 800 ng of IXa. These weights are equivalent to 1.1 nmol of thrombin, 0.12 nmol of Xa, and 0.018 nmol of IXa, respectively. Thus, IXa is two orders of magnitude more thrombogenic than thrombin on a molar basis.

Xa Inhibitory Activity of Rabbit Plasma after Injection of Heparin. The Xa inhibitory activity of rabbit plasma was determined before and after pretreatment of rabbits with heparin. Pretreatment with physiological saline resulted in no change in the Xa inhibitory activity of rabbit plasma (Table 1). The Xa inhibitory activity of rabbit plasma increased as a function of the amount of heparin injected. The expected heparin concentration, the measured heparin concentration in rabbit plasma based on published data (4), and the concentration of heparin needed in human plasma to obtain the same increase in Xa inhibitory activity are also shown (4).

The discrepancy between the expected and observed heparin concentrations can be explained by the observation that there is an appreciable binding of heparin to the endothelium (11) or by the possibility that the volume of distribution for heparin is greater than the plasma volume (12). Thus, all of the heparin injected into the animal will not be found in the plasma. Moreover, the amount of heparin removed from the plasma is dependent upon the initial heparin concentration (Table 1).

Effect of Heparin on Thrombotic Potential of IIa, Xa, and IXa. For each of the three activated clotting proteases, injections of 0.5 ml of the stock solution, after pretreatment of the rabbits with 0.5 ml of isotonic saline, yielded score 4 thrombi. The assay was performed 20 times with each of the three proteins and induced a score 4 thrombus each time.

The effects of pretreating rabbits with 5, 10, and 20 units of heparin per kg on thrombus score are shown in Fig. 2. The thrombotic potential decreased progressively for each procoagulant as the heparin concentration increased. The extent of this decrease varied depending on the protein being tested. Although the proteases were equally thrombogenic in the absence of heparin, in the presence of heparin their relative thrombogenicity decreased in the order, thrombin > Xa > IXa at each of the concentrations studied (Table 2). The differences in thrombogenicity between activated clotting factors were statistically significant, $P < 0.05$, at all of the heparin concentrations tested except that the difference between Xa and IXa with 20 units of heparin per kg was significant at $P < 0.10$ (Table 2).

Effect of Antithrombin III and Heparin on Activity of X-CP *In Vitro*. Antithrombin III either in the absence of or in the presence of heparin had no effect on the activity of X-CP.

The Effect of Heparin on Thrombotic Potential of X-CP. In rabbits pretreated with isotonic saline rather than heparin, injection of 0.5 ml of the X-CP stock solution produced score 4 thrombi. Pretreatment of the rabbits with heparin caused a decrease in the thrombogenicity of the X-CP (Fig. 2). Table 2

FIG. 2. Thrombotic potential of thrombin (△), Xa (○), IXa (■), and X-CP (●) as a function of the amount of heparin used to treat the rabbit and the resulting Xa inhibitory activity (XaIA). The concentrations of each of the proteases injected were: thrombin, 2.2 μM; Xa, 0.24 μM; and IXa, 36 nM. The concentration of X-CP was not determined. % NHP, % normal human plasma.

presents the data for the thrombotic potential of X-CP in rabbits pretreated with 5 and 10 units of heparin per kg. In each case the X-CP was inhibited to a lesser degree than IXa, and overall the decrease in thrombotic potential of X-CP as a function of the amount of heparin injected resembled the response curve of Xa more closely than that of IXa.

DISCUSSION

The data obtained in this study provide *in vivo* support for four hypotheses originally developed from *in vitro* experiments.

(1) Activation of the Blood Coagulation System Incorporates Biochemical Amplification. In 1964, Davie and Ratnoff (13) and Macfarlane (14) proposed that blood coagulation proceeds via a cascade mechanism in which, sequentially, one serine protease activates a succeeding proenzyme to a protease until prothrombin is converted to thrombin. Thrombin converts fibrinogen to fibrin which polymerizes to form a gel. Based on the increasing amounts of each proenzyme present in plasma as prothrombin is approached in the sequence, Macfarlane (14) proposed that biochemical amplification occurred during the activation of the coagulation system. Thus, activation of the coagulation sequence should lead to the elaboration of increasingly greater amounts of serine proteases as the end point of fibrin formation is approached. This concept implies that the further removed an activated clotting factor is from fibrin formation, the fewer are the number of molecules of that protease required to produce a thrombotic event of specified severity.

The hypothesis of amplification was tested, in rabbits, with three activated clotting factors (IXa, Xa, and thrombin) at known concentrations. The amount of each activated clotting factor, on a molar basis, necessary to induce equivalent responses in the stasis assay varied in the order, thrombin > Xa > IXa. This order of thrombogenicity was observed at each of the measured end points (Table 3). Thus, small amounts of IXa represent a potent thrombogenic stimulus such that as little as 200 ng of IXa is thrombogenic in 2-kg rabbits. This observation may account for the observed thrombogenicity of some Factor IX concentrates administered to human subjects (15–20).

Table 2. Effect of injection of heparin on the thrombotic potential (score) of thrombin(IIa), Xa, IXa, and X-CP*

Protein	Heparin, units/kg	Score†	Mean score
IIa	5	2; 3,3,3,3,3; 4,4,4,4,4	3.4
Xa	5	2,2,2; 3,3,3,3,3,3,3,3,3,3,3	2.8
IXa	5	1; 2,2,2,2,2,2,2,2,2; 3	2.0
X-CP	5	2; 3,3,3,3,3,3	2.9
IIa	10	2,2,2,2,2,2,2,2; 3,3,3,3,3,3	2.4
Xa	10	1; 2,2,2,2,2,2,2,2	1.9
IXa	10	1,1,1,1,1,1,1; 2	1.1
X-CP	10	1,1,1,1,1,1; 2,2,2,2	1.4
IIa	20	1,1,1,1; 2,2,2,2	1.5
Xa	20	0,0,0; 1,1,1,1,1,1	0.7
IXa	20	0,0,0,0,0; 1,1	0.3

* The injected solutions contained IIa, Xa, and IXa at concentrations of 2.2 μM, 0.24 μM, and 36 nM, respectively. The concentration of X-CP was not determined.

† Each number represents the result from a single rabbit. 4 = largest thrombus, 0 = no thrombus.

(2) Inhibition of IXa by Antithrombin III in the Presence of Heparin Is an Important Reaction in the Prevention of Thrombosis. Antithrombin III is a naturally occurring plasma protease inhibitor that neutralizes the serine proteases IIa (21–24) and Xa (25–28) in a slow, progressive manner. In the presence of heparin, however, the inhibition of these proteases is nearly instantaneous (21–28). Recently, Rosenberg and associates (29–33) have shown, *in vitro*, that antithrombin III in the presence of heparin is a broad-spectrum serine-protease inhibitor capable of rapidly neutralizing activated Factor XII, activated Factor XI, IXa, and plasmin.

The importance of these latter observations *in vivo* might be questioned because antithrombin III inhibition of IXa is slow compared to the inhibition of thrombin or Xa in the bovine system (34). This finding is consistent with the presence of IXa and activated Factor XI in serum, whereas Xa and thrombin are absent.

At each of the heparin concentrations examined (Fig. 2), thrombosis induced by IXa was inhibited to a significantly greater extent than thrombosis induced by Xa or thrombin, even though in the absence of heparin all three proteases were of equal thrombotic potential. Thus, our data demonstrate that heparin cofactor (antithrombin III and heparin) can inhibit thrombosis induced by IXa.

To support this thesis further, the thrombogenicity of X-CP was investigated. X-CP was not inhibited by antithrombin III,

Table 3. Amounts of activated clotting factors required to cause thrombosis in the stasis assay

Thrombus grade	Amount of activated clotting factor, nmol*		
	Thrombin	Xa	IXa
4	1.1 (20)	0.12 (20)	0.018 (20)
3	0.74 (4)	0.09 (4)	0.014 (4)
2	0.55 (4)	0.06 (4)	0.09 (4)
1.5	0.3 (4)	—	—
1	—	0.04 (4)	0.004 (4)
0†	0.17 (3)	0.03 (2)	0.002 (3)

* The numbers in parentheses are the number of experiments done at each concentration.

† The maximum amount of each activated clotting factor which produced no thrombosis in the stasis assay.

in either the presence or absence of heparin; it initiates thrombosis at a position in the clotting sequence equivalent to IXa. The experiments showed, however, that when X-CP of equal thrombotic potential to IXa and Xa in the absence of heparin was used as the thrombogenic stimulus, the heparin-induced inhibition of thrombosis was indistinguishable from that obtained when Xa was used as the substrate. This observation is best explained by the fact that, when X-CP is infused into a heparinized rabbit, the antithrombotic effect of the drug is expressed at the Xa step. Because IXa and X-CP initiate thrombosis at an equivalent position in the coagulation sequence, the capacity of heparin to inhibit IXa to a greater extent than X-CP is indicative of the *in vivo* inhibitory effect of heparin cofactor on IXa and the absence of an effect on X-CP.

(*3*) **Less Heparin Is Required to Inhibit Thrombosis Prior to Thrombin Generation than Afterward.** Based on the concept of biochemical amplification in blood coagulation and the ability of antithrombin III to inhibit Xa rapidly in the presence of small quantities of heparin, it was proposed that less heparin is required to inhibit thrombosis prior to thrombin formation than afterward (35). This hypothesis is one of the main concepts underlying the prophylactic use of low-dose heparin therapy in surgical patients.

Although the general validity of this hypothesis has not previously been demonstrated *in vivo*, data obtained from clinical trials in man are consonant with this thesis. Thrombosis is significantly decreased in patients treated preoperatively and postoperatively with subcutaneous injections of 10,000 units of heparin per day (36). In contrast, once thrombosis has occurred, doses of 25,000 to 50,000 units of heparin per day are commonly used for the prevention of thrombus extension (36). The former situation is usually defined as a state in which thrombin is not present in the circulation, and the latter situation is interpreted as a state in which thrombin is or has been present in the circulation. However, due to the disparity in the two clinical situations, the deduction of the validity of the hypothesis that less heparin is required to inhibit thrombosis prior to thrombin formation than afterward can only be conjectural.

The data presented herein provide direct support for this hypothesis. Thus, to cause a 50% reduction in thrombosis induced by thrombin, 36 units of heparin are required. In contrast, to cause a 50% reduction in thrombosis prior to thrombin formation, at the Xa or IXa step, only 18 units or 9 units of heparin, respectively, are required. This observation would explain why trace amounts of heparin would make Factor IX concentrates containing small amounts of IXa nonthrombogenic (16).

(*4*) **An Increase in the Reactivity of Antithrombin III Reflects a Decreased Tendency to Thrombosis, and a Decrease in this Reactivity Reflects an Increased Tendency to Thrombosis.** Congenital antithrombin III deficiency has been described in man (37, 38). In general, the quantity of antithrombin III is decreased to levels of approximately 40% of normal with a parallel decrease in the reactivity of this inhibitor, frequently resulting in multiple clinical episodes of thrombosis. A single case of congenitally decreased reactivity of antithrombin III associated with a normal quantity of this protein has been reported (39). Similarly, drug-induced changes in the reactivity of antithrombin III (Xa inhibitory activity) have been observed. Women taking oral contraceptives, a population that has an increased tendency toward thrombosis (40), have been found to have a normal quantity of antithrombin III with impaired reactivity (41). On the other hand, patients on warfarin therapy have been found to have normal quantities of antithrombin III while the reactivity of this inhibitor is enhanced (42, 43). However, except for the congenitally deficient patients, no correlation in man can be made between Xa inhibitory activity and the inhibition of thrombosis.

It has been postulated that changes in Xa inhibitory activity reflect either an increased or a decreased tendency to thrombosis (41, 42). Heparin can increase Xa inhibitory activity (4, 41). Table 1 presents the average increase in the Xa inhibitory activity of rabbit plasma after heparin injection. The data presented in Fig. 2 show that there is an inverse relationship between the Xa inhibitory activity and the extent of intravascular coagulation induced by each of the thrombotic stimuli tested. Thus, each increase in Xa inhibitory activity is paralleled by a comparable decrease in thrombosis. These data imply that a change in plasma Xa inhibitory activity is one marker for a decreased or increased risk of thrombosis.

This study was supported by National Heart, Lung and Blood Institute Grant 2 R01 HL18333, National Institutes of Health, Department of Health, Education, and Welfare, and the Sara Chait Foundation Fund for Vascular Diseases.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. Frenkel, J. K. (1969) *Fed. Proc.* **28,** 160–161.
2. Wessler, S., Reimer, S. M. & Sheps, M. D. (1959) *J. Appl. Physiol.* **14,** 943–946.
3. Wessler, S. (1962) *Am. J. Med.* **33,** 648–666.
4. Gitel, S. N., Wessler, S. & Medina, V. M. (1977) *Circ. Res.* **41,** in press.
5. Yin, E. T. & Wessler, S. (1968) *J. Biol. Chem.* **243,** 112–117.
6. Kisiel, W., Hermodson, M. A. & Davie, E. W. (1976) *Biochemistry* **15,** 4893–4906.
7. Yin, E. T., Eisenkramer, L. & Butler, J. V. (1975) in *Heparin: Structure, Function, and Clinical Implications*, eds. Bradshaw, R. A. & Wessler, S. (Plenum Press, New York), pp. 239–242.
8. Jackson, C. M., Gordon, J. G. & Hanahan, D. J. (1971) *Biochim. Biophys. Acta* **252,** 255–261.
9. Wilcoxon, F. (1945) *Biometrics* **1,** 80–83.
10. Wilcoxon, F. (1947) *Biometrics* **3,** 119–122.
11. Hiebert, L. M. & Jaques, L. B. (1976) *Thromb. Res.* **8,** 195–204.
12. Teien, A. N. & Bjørnson, J. (1976) *Scand. J. Haematol.* **17,** 29–35.
13. Davie, E. W. & Ratnoff, O. (1964) *Science* **145,** 1310–1312.
14. Macfarlane, R. G. (1964) *Nature* **202,** 498–499.
15. Loeliger, E. A., Hensen, A., Mattern, M. J., Veltkamp, J. J., Bruning, P. F. & Hemker, H. C. (1967) *Folia Med. Neerl.* **10,** 112–125.
16. Kingdon, H. S., Lundblad, R. L., Veltkamp, J. J. & Aronson, D. L. (1975) *Thromb. Diath. Haemorrh.* 33, 617–631.
17. Kasper, C. K. (1973) *N. Engl. J. Med.* **289,** 160.
18. Steinberg, M. & Breiling, B. J. (1973) *N. Engl. J. Med.* **289,** 592.
19. Marchesi, S. L. & Burney, R. (1974) *N. Engl. J. Med.* **290,** 403–408.
20. Blatt, P. M., Lundblad, R. L., Kingdon, H. S., McLean, G. & Roberts, H. R. (1974) *Ann. Intern. Med.* **81,** 766–770.
21. Brinkhous, K. M., Smith, H. P. & Warner, E. D. (1939) *Am. J. Physiol.* **125,** 683–687.
22. Waugh, P. F. & Fitzgerald, M. A. (1956) *Am. J. Physiol.* **184,** 627–639.
23. Monkouse, F. C., France, E. S. & Seegers, W. H. (1955) *Circ. Res.* **3,** 397–402.
24. Abildgaard, U. (1968) *Scand. J. Clin. Lab. Invest.* **21,** 89–91.
25. Yin, E. T. & Wessler, S. (1970) *Biochim. Biophys. Acta* **201,** 378–390.

26. Seegers, W. H. & Marciniak, E. (1962) *Nature* **193**, 1188–1190.
27. Biggs, R., Denson, K. W. E. & Akman, N. (1970) *Br. J. Haemotol.* **197**, 283–305.
28. Yin, E. T., Wessler, S. & Stoll, P. J. (1971) *J. Biol. Chem.* **246**, 3694–3702.
29. Rosenberg, R. D. & Damus, P. S. (1973) *J. Biol. Chem.* **248**, 6490–6506.
30. Damus, P. S., Hicks, M. & Rosenberg, R. D. (1973) *Nature* **246**, 355–357.
31. Rosenberg, R. D. (1974) *Circulation* **49**, 603–605.
32. Highsmith, R. F. & Rosenberg, R. D. (1974) *J. Biol. Chem.* **249**, 4335–4338.
33. Rosenberg, R. D. (1975) *N. Engl. J. Med.* **292**, 146–151.
34. Kurachi, K., Fujikawa, K., Schmer, G. & Davie, E. W. (1976) *Biochemistry* **15**, 373–377.
35. DeTakats, G. (1950) *J. Am. Med. Assoc.* **142**, 527–534.
36. Wessler, S. & Gitel, S. N. (1976) in *Progress in Hemostasis and Thrombosis*, ed. Spaet, T. H. (Grune and Stratton, Inc., New York), pp. 311–329.
37. Egebert, O. (1965) *Thromb. Diath. Haemorrh.* **13**, 516–530.
38. Marciniak, E., Farley, C. H. & DeSimone, P. A. (1974) *Blood* **43**, 219–231.
39. Sas, G., Pepper, S. & Cash, J. D. (1975) *Thromb. Diath. Haemorrh.* **33**, 564–572.
40. Vessey, M. P. & Doll, R. (1968) *Br. Med. J.* **2**, 199–205.
41. Wessler, S., Gitel, S. N., Wan, L. S. & Pasternack, B. S. (1976) *J. Am. Med. Assoc.* **236**, 2179–2182.
42. Wessler, S., Gitel, S. N. & Bank, H. (1976) *Circulation Suppl. II* **54**, 201 (Abstr.).
43. Ødegard, O. R. & Teien, A. N. (1976) *Thromb. Res.* **8**, 167–171.