# Increase in Fibrinogen and Fibrin-Related Antigen in Human Serum Due to In Vitro Lysis of Fibrin by Thrombin

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ABSTRACT In vitro lysis of fibrin, as indicated by increased fibrinogen-fibrin–related antigen (FR-antigen) in serum is usually seen when whole blood, or plasma, or highly purified fibrinogen prepared by several different procedures is clotted and kept at temperatures above 0°C. This increase is both time and temperature dependent, occurs despite the addition of various plasmin and cathepsin inhibitors, and is probably caused by thrombin evolved during clotting and/or added in vitro. In these experiments, the FR-antigen was measured by a sensitive, reproducible hemagglutination inhibition immunoassay adapted to the AutoAnalyzer. Serum from whole blood contained more than serum from plasma, and fibrin rather than fibrinogen proved to be essential for the in vitro lysis. The phenomenon was also caused by Arvin or Reptilase, suggesting that splitting of one or more arginine or lysine bonds in fibrin may be at least partially responsible. To obtain minimal levels of FR-antigen ($< 0.5$ μg/ml), plasma is clotted for 4 hr at 0°C with 1.0–5.0 U/ml thrombin, $CaCl_2$ (0.0125 mole/liter), and epsilon aminocaproic acid (0.05 mole/liter). Slightly higher levels, probably adequate for clinical diagnosis, are obtained by 10–30 min clotting at room temperature. Since endogenous and/or exogenous thrombin is essential for the collection of serum FR-antigen, all the FR-antigen found in normal serum probably results from an irreducible amount of in vitro lysis rather than from continuous intravascular clotting and fibrinolysis.

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## INTRODUCTION

Antigens reacting to antifibrin antibody, commonly called fibrinolytic digestion products (FDP),[1] have been identified in human serum by a number of immunological methods including electrophoresis, diffusion, and the tanned red-cell hemagglutination inhibition immunoassay (TRCHII), performed manually (1–3) or, as in the present studies, modified for use with the AutoAnalyzer. It has generally been assumed that the addition of fibrinolytic inhibitors, before or during clotting, prevents further in vitro lysis so that concentration of these antigens in serum does not exceed that which might occur in vivo. However, our studies have demonstrated a time-dependent in vitro increase in these antigens, despite the addition of fibrinolytic and cathepsin inhibitors, after normal or abnormal blood or plasma has clotted. The increase is attributed to thrombin added before and/or generated during coagulation. A similar effect is observed with purified fibrinogen. In contrast, the incubation of samples of normal blood or plasma before clotting usually has little effect on the amount of antigen found afterward.

When in vitro lysis is minimized, the FR-antigen level in clotted normal plasma is less than 1.0 μg/ml. Since thrombin appears essential to the preparation of serum

[1] *Abbreviations used in this paper:* DEAE, diethylaminoethyl-; EACA, epsilon aminocaproic acid; FDP, fibrinolytic digestion products; FR-antigen, fibrinogen-fibrin–related antigen; *p*-NPGB, *p*-nitrophenyl-*p'*-guanidino-benzoate HCl; PEG, polyethylene glycol; TLCK, *N*-α-tosyl-*l*-lysyl chloromethane HCl; TRCHII, tanned rd-cell hemagglutination inhibition immunoassay.

for measurement of FR-antigen, all the FR-antigen in normal serum may be caused by in vitro lysis.

## MATERIALS

The following materials were used:

Horse serum adsorbed with 1% (v/v) aluminum hydroxide suspension[2] for 30 min, containing heparin (10 U/ml).

Triton X-100 detergent 0.5% in water.[3]

Phosphate citrate buffer (Sorenson); 0.075 M phosphate, 0.075 M trisodium citrate, pH 7.8.

TES buffer: N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid.[4]

Other reagents for TRCHII as previously described (3).

Pancreatic,[5] soybean and lima bean trypsin inhibitors.[6]

Thrombin—four preparations tested: bovine thrombin[7] and three highly purified preparations of human thrombin[8] prepared by the same method (4) and diluted in 0.05 M sucrose.

Reptilase—two preparations tested[9] both diluted in 0.05 M sucrose.

Arvin, commercial grade,[10] diluted in 0.05 M sucrose.

Trasylol.[11]

Fibrinogen, fraction 1-0 of plasma (Blombäck procedure[12]) was employed as a source of partially purified fibrinogen. Other moderately pure and high purity fibrinogen preparations were made by cryoprecipitation or cryoethanol precipitation followed by successive precipitation with polyethylene glycol (PEG-4000 mol wt) at pH 6.1.[13]

Streptokinase (Streptase, Behringwerke[14]) diluted in 0.05% human serum albumin.

Plasminogen, fraction III of human plasma purified by a modified Oncley procedure and EACA extraction followed by DEAE-52 chromatography (5).

*AutoAnalyzer flow diagram and operation.* Uniform 2 mm I.D. glass tubing is used throughout. Dilutions of the standard or sample to be tested are mixed with a constant concentration of fibrinogen antiserum and preincubated during passage of the mixture through coil A (Fig. 1). At this stage the antiserum is wholly or partially neutralized by the antigen present in the plasma or serum sample. The previously coated, sensitized, red cells are introduced at B and agglutinated in coil C by any residual, unneutralized antiserum. Saline is added at D and the agglutinated cells which settle in coil E (1.5 turns, 21 inches) are removed by decanters F, G, and H. The residual solution is passed through the colorimeter I (420 mμ) where the optical density (OD) is recorded (J).

The tanned, coated red cells are suspended in phosphate citrate buffer containing 0.05% human serum albumin, and the cell concentration is adjusted to give a reading of approximately 0.8 OD when both sample and antiserum are omitted (approximately 1.5% red cells). When the antiserum is added, agglutination of the red cells should reduce the OD to approximately 0.10. The flask containing the red cells is kept in melting ice and gently stirred with a small magnetic stirrer. The tubing is cleaned for 30 min at the end of the day with a solution of 4.0% urea in 0.5% sodium hydroxide followed by continuous water rinses for a similar period.

When the net optical density is plotted against the fibrinogen concentration of plasma, a highly reproducible sigmoid curve is obtained which usually ranges from 0.05 to 0.7 OD. The system's sensitivity is largely determined by the antibody concentration used. The calibration curve is useful over about a fivefold range; carry-over contamination becomes a problem only when samples with a very high concentration of the antigen are followed immediately by samples with a very low concentration.

Assays obtained with simple equipment such as blood-grouping plates (3) correlate very well with those obtained by the AutoAnalyzer assay, but the automated method removes observer error, is much more precise, and much more rapid than manual methods.

*Sample preparation.* Blood was collected, usually without use of a tourniquet; when a tourniquet was unavoidable, it was released as soon as possible. Plasma was prepared from citrated blood (1 part 3.8% trisodium citrate to 9 parts blood). Platelet-rich plasma was obtained by centrifuging the blood at 750 *g* for 7 min at 21°C. Platelet-poor plasma was prepared by centrifuging twice at 12,000 *g* for 10 min at 4°C, or alternatively this platelet-poor plasma was recentrifuged in a Beckman preparative ultracentrifuge (Model L 2, No. 40 rotor) at 100,000 *g* for 120 min at 4°C. Blood and plasma were usually clotted and kept at 0°, 21°, and 37°C with and without the addition of thrombin; calcium chloride; epsilon aminocaproic acid (EACA); Trasylol; Kunitz soybean or pancreatic trypsin inhibitor or lima bean trypsin inhibitor; *N*-α-tosyl-*l*-lysyl chloromethane HCl (TLCK)[4] (6), an irreversible inhibitor of trypsin or plasmin; *p*-nitrophenyl-*p'*-guanidino-benzoate HCl (*p*-NPGB)[15] (7), a rapid active-site titrant for trypsin and plasmin; *N*-CBZ-α-glutamyl-*l*-tyrosine[16] and *N*-CBZ-α-*l*-glutamyl-*l*-phenylalanine[16] and 5% serum albumin, substrates for cathepsin A (8); benzoyl-*l*-arginine amide HCl,[16] substrate for cathepsin B (8); glycyl-*l*-phenylalanine amide acetate,[16] substrate for cathepsin C (8); and 5% purified human serum albumin, substrate for cathepsins D and E (9), all of these in buffered and unbuffered solutions. Since blood clotted at 0°C shows no clot retraction, whole blood specimens were left at 21°C for an additional 10 min before centrifugation to permit some clot retraction to occur. The fibrin was removed from plasma after clotting by pressing the clot against the wall of the tube with wooden applicator

[2] Cutter Laboratories, Berkeley, Calif.

[3] Alkylphenoxypolyethoxyethanol, Rohm and Haas Co., Philadelphia, Pa.; Cutscum, Fisher Chemical Co., New York.

[4] Calbiochem, Los Angeles, Calif.

[5] Worthington Biochemical Corp., Freehold, N. J.

[6] Mann Research Laboratories, New York.

[7] Parke, Davis & Co., Detroit, Mich.

[8] Prepared by the American National Red Cross with Dr. Kent Miller: (*a*) for the NIH and WHO, and (*b*) for the NIH Committee on Thrombolytic Agents; (*c*) prepared by Dr. B. Blombäck.

[9] Reagent grade, batch No. 00428, Pentapharm, Basel, Switzerland; highly purified material kindly supplied by Dr. B. Blombäck.

[10] Twyford Laboratories, London, N.W. 10, batch T 149; kindly supplied by Dr. W. R. Bell.

[11] Aprotinin (proteinase inactivator), Delbay Pharmaceuticals, Inc., Bloomfield, N. J.

[12] Antihemophilic globulin (human fraction 1-0) Lot No. 8-50, Michigan Department of Health, Lansing, Mich.

[13] Newman, J., and A. J. Johnson (unpublished data).

[14] Hoechst Pharmaceutical Co., Somerville, N. J.

[15] Cyclo Chemical Corp., Los Angeles, Calif.

[16] Mann Research Laboratories, New York.

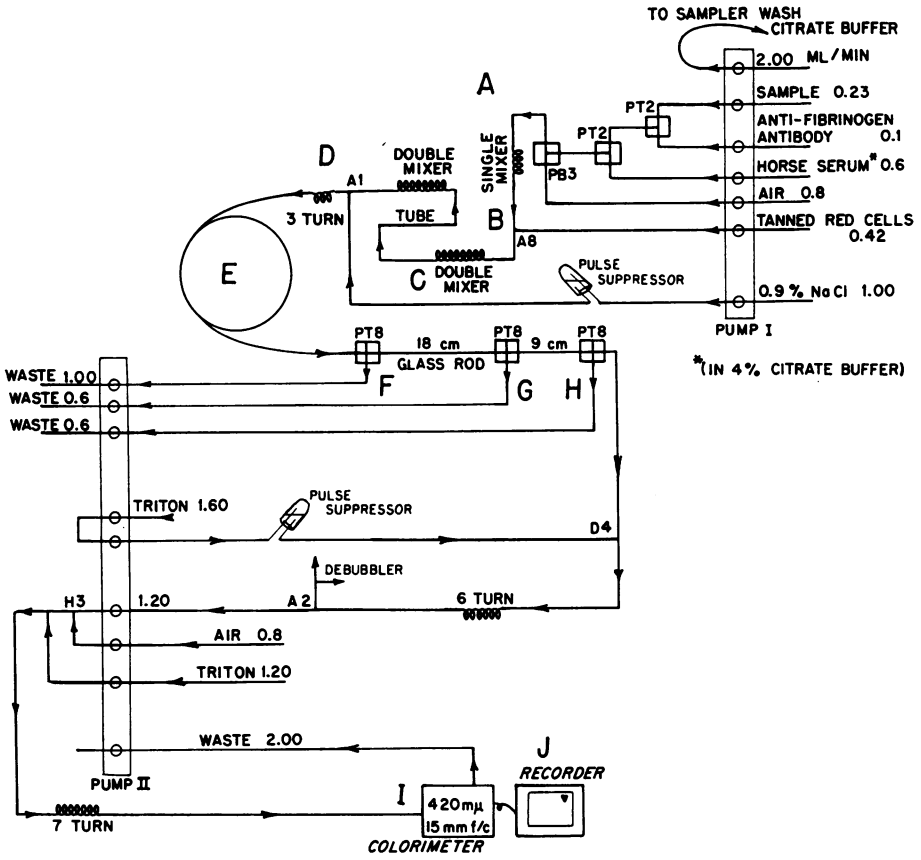


FIGURE 1 AutoAnalyzer flow diagram.

sticks and spinning the residual serum at 12,000 *g* for 10 min at 4°C. If not tested immediately, the serum was stored at 0°C or −20°C. Unless otherwise stated, samples of plasma and fibrinogen were clotted with an equal volume of a solution which contained bovine thrombin (20 U/ml), calcium chloride (0.025 mole/liter), and EACA (0.1 mole/liter). Before assay, plasma standards and serum or other samples were diluted to a constant protein concentration with phosphate citrate buffer and/or horse serum as previously described (3).

## RESULTS

When samples of blood with or without fibrinolytic inhibitors such as EACA were clotted and left at temperatures above 0°C, the level of FR-antigen in the serum increased progressively. In the 0°–37°C range, the higher the temperature and longer the storage time, the higher the FR-antigen level in the serum. However, when citrate plasma was incubated at 37°C for up to 72 hr *before* clotting, the FR-antigen level was about the same as that for the sample without preincubation, i.e., the increase was observed only *after* clotting. It was also dependent on at least two variables. (*a*) An unknown factor that appeared to be due to cells and which disappeared when the blood was centrifuged before clotting. Thus, 10 samples of serum from patients' whole blood clotted with 0.05 M EACA and thrombin (10 U/ml) at 23°C for 4 hr had a mean value of 5.30 ±1.78 SD μg/ml of FR-antigen as compared to 3.66 ±0.76 SD μg/ml for paired samples of recalcified citrate plasma clotted similarly ($P < 0.01$), the value for blood being higher in each instance. Similar, but smaller, differences were noted in another experiment using only 0.5 U thrombin per ml at either 0°C or 37°C. (Table I). No difference was found between clotted citrate plasma and clotted native plasma. The fact that plasma containing many white cells and platelets when spun slowly (5 min at 750 *g*) yielded levels similar to those for rapidly spun platelet-poor plasma (20 min at 10,000 *g*) or ultracentrifuged plasma (2 hr at 100,000 *g*) showed that the effect was relatively independent of white cells and platelets but not red cells. (*b*) A second factor appeared also to depend on clotting but to be independent of cells. In other words, the FR-antigen levels still increased markedly with time after this virtually cell-free plasma was clotted.

To demonstrate the importance of the fibrin clot, a number of samples of plasma were clotted at 0°C and

TABLE I

*FR-Antigen in Serum (μg/ml) from Whole Blood or Citrate Plasma*

| Hours at 37°C | Whole blood | Plasma |
|---|---|---|
| 0 | 2.12 (0.29) | 1.60* (0.32) |
| 1 | 3.13 (0.63) | 2.51 (0.84) |
| 4 | 3.31 (0.86) | 3.01 (2.17) |
| 6 | 3.63 (1.08) | 3.35 (2.15) |

Figures in the table represent mean value ±SD. The paired samples of blood or plasma from 12 patients were clotted with 0.05 M EACA, thrombin (0.5 U/ml), and also with 0.0125 M $CaCl_2$ for citrate plasma so that the final dilution of the serum was similar. After 1 or 4 hr at 37°C specimens were transferred to 0°C for the balance of the 6 hr period. * $P < 0.01$. The value for citrate plasma was lower than whole blood in 39 instances, higher in 7 and the same in 2.

left at this temperature for 2 hr; the fibrin was then removed from half the specimens from each plasma. Some samples were kept at 0°C for 24 hr; others were incubated at 37°C for varying shorter periods (Fig. 2) then returned to 0°C for the remainder of the 24 hr of storage. An increase in FR-antigen was found only in those samples from which the fibrin clot had not been removed and which had been incubated for prolonged periods at temperatures above 0°C.

*Calcium, pH, and ionic strength.* In general, more FR-antigen is found when citrate plasma is clotted with thrombin if calcium is omitted completely from the clotting system. The optimal pH for minimal residual FR-antigen in clotted plasma lay between 7.0 and 8.0; the addition of sodium chloride to increase the ionic strength of the mixture, making it hypertonic, also increased the residual FR-antigen in serum. These effects may all be due to mild inhibition of clotting under nonphysiologic conditions; for example, with a low or high pH, further fibrin was often observed when the pH was brought to 7–8.

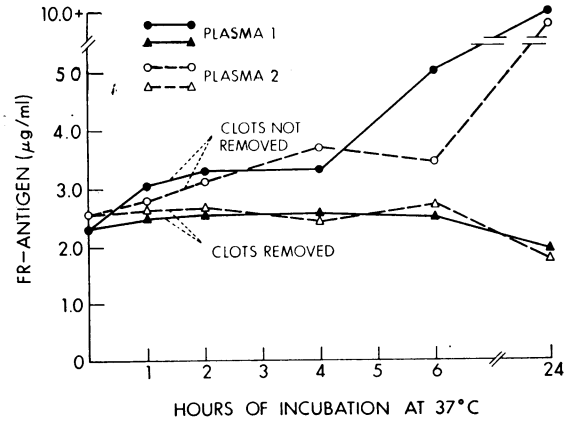


FIGURE 2 Two plasmas were clotted at 0°C for 2 hr, with 0.05 M EACA, 0.0125 M $CaCl_2$, and thrombin 10 U/ml, pH 7.8. The fibrin clot was removed from some samples of each and left undisturbed in others. Samples were incubated at 37°C for various periods of time.

*Inhibitors of plasminogen activation and of plasmin (fibrinolysis).* While the FR-antigen values noted in serum from clotted normal blood or plasma at 0°C were not affected by fibrinolytic inhibitors, this was not always true for patients' blood or plasma. Thus, 18 samples of citrate plasma clotted with 10 U thrombin/ml but without EACA had a mean value of FR-antigen of 2.56 ±1.16 SD μg/ml as compared to 2.01 ±0.72 SD μg/ml with 0.01 M EACA ($P < 0.01$) and 1.95 ±0.85 SD μg/ml with 0.05 M EACA ($P < 0.01$); the difference between the two concentrations of EACA was not statistically significant. When the EACA concentration was increased to 0.5 mole/liter, or Trasylol (250 KIU/ml) or 0.5–1.0 mg/ml of Kunitz pancreatic or soybean trypsin inhibitor or lima bean trypsin inhibitor was added, alone or together (since each inhibitor might have a slightly different substrate binding site and a different binding constant), there was no additional inhibitory effect. Two other plasmin inhibitors were also utilized: the slow, irreversible histidine-specific chloromethyl ketone, *N*-α-tosyl-*l*-lysyl chloromethane HCl (TLCK) (6) and the very rapid *p*-nitrophenyl-*p'*-guanidino-benzoate HCl (7) (*p*-NPGB), the active-site inhibitor derived from benzene. The TLCK (0.02 mole/liter) was incubated with fibrinogen for up to 4 hr at pH 6.8 before clotting and for up to 24 hr at 37°C after clotting. Since *p*-NPGB may act as a substrate for both plasmin and thrombin (7) up to 0.001 mole/liter was incubated with fibrinogen; the excess amino acid ester was dialyzed out by changing the dialysis fluid six times within 6 hr, then thrombin, EACA, and calcium were added. The clot was left for 12 or 24 hr at 0°C and also at 37°C before centrifugation and assay. As there was no evidence of inhibition with either TLCK or *p*-NPGB, the degradation seemed not to be due to any preformed plasmin or possible activation of contaminating plasminogen during the period of incubation.

*Cathepsin inhibitors.* Purified fibrinogen was incubated with synthetic substrates or natural substrates for cathepsins A, B, C, D, or E. The fibrinogen was clotted and kept at 0°C or 37°C; samples were removed at suitable time intervals for assay of FR-antigen. Besides the natural, high molecular weight, fibrin substrate in this experiment, a low molecular weight specific synthetic substrate with a greater affinity for one or more of the cathepsins was used, in an effort to compete for the active site of the enzyme and specifically inhibit the

release rate of the FR-antigen. The substrates employed were *N*-CBZ-α-glutamyl-*l*-phenylalanine and *N*-CBZ-α-glutamyl-*l*-tyrosine, both relatively specific for cathepsin A, benzoyl-*l*-arginine amide HCl for B, glycyl-*l*-phenylalanine amide acetate for C (8) and 5% purified human serum albumin for D and E (9) in buffered and unbuffered solutions. The release rate of the FR-antigen from highly purified fibrinogen (fibrin) was relatively unaffected by any of them singly or together. Addition of the reducing agent cysteine, needed to activate some of these enzymes, did not increase the amount of FR-antigen released on incubation of the purified fibrinogen or plasma clot at 37°C.

*Effect of fibrinogen purification.* Normal plasma contains many proteolytic enzymes any of which could contaminate fibrinogen, even material purified by various procedures. Different fractionation methods used in an attempt to eliminate or concentrate possible contaminating enzymes in the substrate fibrinogen included precipitation by ethanol, cold (cryoprecipitation), ethanol plus cold, polyethylene glycol (PEG), and glycine with or without EACA. The use of several sequential purification procedures resulted in very highly purified fibrinogen preparations. The various fractionation methods were unavailing, however, as FR-antigen was released in every preparation after clotting and incubation at 20°–37°C.

To rule out degradation of the fibrinogen by intrinsic contaminating enzymes, it was incubated for 24 hr at 37°C, then clotted with thrombin, EACA, and $CaCl_2$ at 0°C. No increase in FR-antigen was observed. However, the FR-antigen increased in the control when this fibrinogen was similarly incubated and clotted, then incubated further at 37°C. Moreover, the FR-antigen increase was not eliminated when the fibrinogen was heated to 53°C before adding EACA, clotted with thrombin, and incubated at 37°C.

*Plasminogen contamination.* The following experiments were performed to exclude the possibility that the fibrinolysis observed was due to activation of plasminogen contaminating the purified fibrinogen or thrombin.

(*a*) Assays for plasminogen in preparations of fibrinogen and thrombin. Two samples of purified fibrinogen prepared by cryoprecipitation, cryoethanol precipitation, and subsequent successive precipitation with PEG and a sample of purified human thrombin (prepared by Dr. Kent Miller) were assayed for plasminogen by a fibrinolytic assay (10) capable of detecting $< 0.01$ CTA caseinolytic unit of human plasminogen. The thrombin contained no measurable plasminogen; both fibrinogen samples contained $< 0.001$ CTA unit/ml plasminogen.[17]

[17] The fibrinolytic assays of fibrinogen and thrombin were performed by Dr. H. Lackner.

(*b*) Effect of adding purified streptokinase and purified plasminogen. Each material was added separately to fibrinogen (containing 0.05 M EACA and 100 KIU/ml Trasylol), and clotted with 5.0 or 0.5 units of human thrombin. The samples were then left for 18 hr at either 0°C or 37°C; the fibrin was separated, and the FR-antigen measured (Table II). At 37°C the higher concentration of thrombin yielded higher FR-antigen levels but the addition of varying amounts of streptokinase (0.125–250 U/ml) caused no increase in FR-antigen. This finding led to the conclusion that possible activation of trace amounts of contaminating plasminogen in fibrinogen and thrombin could not be responsible. Furthermore, while there was an increase in FR-antigen in the experiment with added plasminogen, the increase appeared to be due to intrinsic contamination of the plasminogen with FR-antigen; thus the antigen was present in the control where fibrinogen was omitted and the level was actually lower in the specimens containing fibrinogen presumably due to the adsorption of antigen on fibrin.

These experiments would appear to exclude the effects of small amounts of contaminating plasminogen or plasmin, as well as the activation of plasminogen to plasmin by thrombin. They would also seem to preclude the protective effect of plasmin inhibitors on fibrinogen,

TABLE II

*FR-Antigen Levels in Human Fibrinogen Containing Fibrinolytic Inhibitors and Clotted with Human Thrombin after the Addition of either Plasminogen or Streptokinase*

| | Temperature | | | | |
|---|---|---|---|---|---|
| | 0°C | 37°C | 0°C | 37°C | 0°C |
| Plasminogen (*CTA U/ml*) | | | | | |
| 3.0 | 2.7 | 20.0 | 3.5 | 6.4 | 9.0 |
| 1.0 | 0.6 | 14.0 | 1.0 | 3.3 | 2.9 |
| 0.3 | 0.4 | 13.6 | 0.7 | 3.3 | 2.7 |
| 0.1 | 0.4 | 10.0 | 0.7 | 2.1 | 1.6 |
| 0.0 | 0.0 | 7.2 | 0.4 | 1.1 | 0.5 |
| Streptokinase (*U/ml*) | | | | | |
| 250* | 0 | 2.4 | 0 | 0 | 0 |
| 0 | 0 | 3.1 | 0 | 0 | 0 |
| Thrombin (*U/ml*) | 5.0 | 5.0 | 0.5 | 0.5 | 0.5 Fibrinogen omitted |

All tubes were left at designated temperature for 18 hr before fibrin was separated.

* Streptokinase concentrations of 50, 2.5, and 0.125 U/ml yielded similar results.

TABLE III

*FR-Antigen (μg/ml) after Clotting of Purified Fibrinogen with Different Concentrations of Various Agents*

| | Initial concn. | Storage temperature | Number of doubling dilutions of enzyme | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | (*U/ml*) | | | | | | | | | | | | |
| Thrombin* | 100.0 | 0°C | 0 | 0 | 0 | 0 | 0 | 0 | 2.2 | 4.2 | 22.0 | 64.0 | 75.0 |
| Arvin | 10.0 | 0°C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9.8 | 14.0 | 76.0 |
| Reptilase‡ | 1.0 | 0°C | 4.1 | 1.8 | 0.3 | 0.3 | 0.3 | 0.2 | + +§ | + + | + + | + + | |
| Thrombin | 100.0 | 37°C | 108.0 | 15.0 | 10.4 | 6.5 | 2.2 | 2.2 | 3.6 | 4.6 | 26.0 | 18.0 | 172.0 |
| Arvin | 10.0 | 37°C | 78.0 | 58.0 | 38.0 | 96.0 | 10.8 | 27.6 | 14.6 | 10.0 | 10.8 | 8.4 | 16.0 |
| Reptilase | 1.0 | 37°C | + + | + + | 9.2 | 7.0 | 7.3 | 6.6 | + + | + + | + + | + + | |

* Purified human thrombin, Lot H-1, WHO.

‡ Highly purified, prepared by Dr. B. Blombäck.

§ >10.0, not tested further.

All tubes, which contained 0.05 M EACA and 0.0125 M $CaCl_2$, were left for 18 hr before the fibrin was separated.

rather than fibrin, in plasma as shown by Mullertz (11) and Ratnoff (12), since fibrin was formed in all the experiments including those with added SK and plasminogen and there was no evidence for the free plasmin.

*Effect of purified human thrombin.* Table III shows the effect of varying concentrations of: (*a*) purified human thrombin, (*b*) Arvin, and (*c*) Reptilase on purified human fibrinogen in the presence of EACA and calcium. With each of these clotting agents, FR-antigen is released on incubation of the samples at 37°C. Although the amount of antigen released varied with each of the agents, a concentration of each enzyme was found which resulted in little or no release of FR-antigen at 0°C; with too low a concentration of each enzyme much, but not all, of the excess FR-antigen could usually be removed when more thrombin was added.

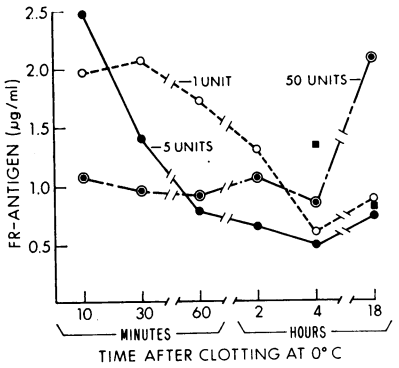


FIGURE 3 Effect of variation of thrombin concentration. Six normal plasmas were clotted at 0°C with different concentrations of thrombin together with calcium and EACA. Values for 25, 12.5, and 0.5 U/ml (not shown) were intermediate. The value obtained when thrombin was omitted is indicated (■).

*Effect of varying the thrombin concentration and elapsed time before removing the fibrin clot.* Samples of platelet-poor citrate plasma from six normal controls were clotted at 0°C with 0.5, 1.0, 5.0. 12.5, 25.0, and 50.0 U bovine thrombin per ml, and the fibrin was removed at different times after clotting. With 25–50 U thrombin per ml, relatively low levels of FR-antigen were obtained in 10 min, but the level rose again between 4 and 18 hr (Fig. 3). Still lower levels were obtained after 2–4 hr with lesser quantities of thrombin. Since thrombin concentrations of 10 U/ml or higher may have a small but significant blank (on the order of 0.5 μg/ml), the OD caused by this blank (when present) has been deducted from the values shown in the figure.

When samples of platelet-poor citrate plasma from normal females (not on progestational drugs) and from normal males were clotted for 4 hr at 0°C with varying concentrations of thrombin (0.5–10.0 U/ml), the FR-antigen content of serum was < 1.0 μg/ml and commonly < 0.5 μg/ml. The optimal amount of thrombin was found to be 1.0 U/ml. Levels of FR-antigen in the 3rd trimester of normal pregnancy were slightly higher than normal (1.3 μg/ml). Plasma from patients with factor VIII, IX, or X deficiency sometimes required slightly higher concentrations of thrombin (> 1.0 U/ml) to achieve the lowest level of FR-antigen in serum, the latter value on occasion also being higher than normal. A slightly higher level of FR-antigen was achieved more rapidly when plasmas were clotted with 25 U thrombin/ml at room temperature for 30 min, i.e., a mean value of 1.2 μg/ml as compared to 0.7 μg/ml when the same plasmas were clotted for 4 hr with 1.0 U thrombin/ml at 0°C. The magnitude of the difference is probably acceptable for clinical purposes, plasma being preferred to whole blood. Incubation at 37°C (for

even 10 min) gives a higher value and should be avoided.

## DISCUSSION

The present study: (*a*) demonstrates the importance of in vitro proteolysis which may increase the FR-antigen value in serum from controls or patients unless special precautions are taken; (*b*) suggests that in sera from normal individuals, at least, this proteolysis may be due primarily to thrombin; and (*c*) establishes a new base line value for normal sera where these precautions are observed. Since differences between normal and abnormal sera may depend on quantitation at a very low level of FR-antigen, it is obviously essential to control as many variables as possible during sample preparation.

The in vitro variables which affect the FR-antigen content of serum include the following:

*Temperature and time of incubation of the whole blood or plasma clot.* The importance of fibrin, rather than fibrinogen, is illustrated in various experiments. There was very little if any increase in FR-antigen before clotting of normal whole blood or plasma or of purified fibrinogen. On the other hand, the FR-antigen in samples of whole blood or plasma from normal controls or patients nearly always increased after incubation of fibrin clots at temperatures above 0°C. As the increase in FR-antigen can be 20-fold or more after 24 hr incubation at 37°C, it is advisable to clot the specimen at 0°C.

*Effect of whole blood as compared with plasma.* Serum prepared from clotted whole blood has more FR-antigen than serum from clotted plasma. Thus, it should be obtained from clotted plasma (citrated or native) rather than from whole blood. Centrifugation of plasma at different speeds appears to have little effect on the FR-antigen content of the serum; results were essentially the same after low and high speed centrifugation or ultracentrifugation. The platelet count in the plasma sample seemed to be of little importance, and the results were also unchanged by freezing platelet-rich or platelet-poor plasma before clotting. Centrifugation at the speeds used removes most of the red cells as well as many white cells. The causes for the excess of FR-antigen in serum from whole blood as compared with serum from plasma is unknown but might be ascribed to a plasmin-like enzyme found in red cells (13), to plasmin activator, or to erythrokinase (14), but direct proof has not been sought.

*Fibrinolytic inhibitors.* In normal plasma clotted at 0°C, fibrinolytic inhibitors appear to be unnecessary; but if they are omitted in patient plasma clotted under similar circumstances the serum may contain significantly more FR-antigen. Since there is usually little difference in FR-antigen with use of 0.01 M and 0.05 M EACA, the higher concentration is preferred. However, fibrinolytic inhibitors did not block most of the increased antigen associated with the incubation of normal clotted blood, plasma or purified fibrinogen. This was true for EACA (0.01–0.5 mole/liter), Trasylol, Kunitz soybean and pancreatic inhibitors, and lima bean inhibitor, either singly or combined, as well as the plasmin inhibitors TLCK and *p*-NPGB. The synthetic ester and naturally occurring protein substrates for cathepsins A, B, C, D, and E also failed to inhibit the release of the FR-antigen; cysteine, which is essential for the action of cathepsins B and C, as well as some endopeptidases in plasma, not only failed to accelerate the phenomenon but actually seemed to inhibit it. Thus, peptide release usually occurred with low pH (below 6.5) which is consistent with cathepsins A, B, C, D, or E, or with high pH (above 8.0) which is inconsistent with all of them and probably reflects the activity of an entirely different enzyme.

*Effect of thrombin.* Blood clots slowly at 0°C, and the addition of an optimal quantity of thrombin before clotting helps to decrease the amount of FR-antigen in the resulting serum; there is usually little effect if thrombin is added after fibrin has been separated. The thrombin concentration is critical; if thrombin is omitted or used in too high a concentration, increased values of FR-antigen are observed. The optimal amount is 0.5–5.0 U/ml; plasma from patients with coagulation defects may require the higher concentration.

Highly purified human thrombin seems no better for this purpose than most commercially available bovine thrombin. Whether the thrombin produces more rapid and therefore more complete clotting of the blood is not yet known, but the fact that large amounts increase the amount of FR-antigen suggests that some contaminating enzyme or thrombin itself is primarily responsible for the increased FR-antigen in normal sera.

The increase in FR-antigen is not due to a contaminant introduced in vitro since it is evident in whole blood or recalcified plasma to which nothing has been added but calcium. Furthermore, the deliberate addition of varying amounts of plasminogen to purified fibrinogen had little or no effect.

One might speculate that a proteolytic enzyme in plasma is activated by, or associated with, the third phase of blood coagulation, i.e., the formation of fibrin by thrombin. The effect of such an activator was negligible in the presence of fibrinolytic inhibitors, however, when varying amounts of SK were deliberately added to purified fibrinogen and the mixture was clotted with thrombin. Since the FR-antigen effect is temperature dependent and is consistent with an enzymatic mechanism, and the increased levels are found in

whole blood or native plasma without additives, thrombin or some other enzyme activated during clotting must be responsible for it.

The principal findings which led us to conclude that FR-antigen release is probably caused by the enzyme thrombin are: (*a*) inhibitors of various other plasma proteolytic enzymes fail to inhibit antigen release; (*b*) large amounts of antigen are released from native or recalcified citrated plasma (with nothing else added); (*c*) similar amounts are released with use of highly purified substrate fibrinogen prepared by a number of methods—with ethanol, cold (cryoprecipitation), ethanol plus cold, PEG, or glycine, alone or in combination, contaminated with, or containing only trace amounts of, plasminogen; (*d*) release also occurs with a wide variety of highly purified human and animal thrombin preparations with or without contaminating plasminogen; and (*e*) it also occurs with both Arvin and Reptilase. The comparison between thrombin, Arvin, and Reptilase is of special interest.

In plasmin-digested fibrinogen, FR-antigen peptides of greater than 50,000 mol wt react with anti-fibrinogen antisera in the TRCHII (15, 16); peptides which are smaller probably react less well, if at all. It therefore seems unlikely that the low molecular weight peptides, rapidly released after splitting of the Gly-Arg linkages of fibrin-fibrinopeptide A or B by thrombin, Arvin, or Reptilase activity, are responsible. Recent studies have shown, however, that thrombin may also split other peptide bonds in fibrin(ogen) as exemplified by the Blombäcks' studies on the disulfide knot, notably the Arg-Val No. 19–No. 20 bond of the A $\alpha$-chain (17); and at least one additional unknown linkage of the B $\beta$-chain.[18] In other studies on the disulfide knot in human fibrinogen, both Arvin and Reptilase slowly split the Arg-Gly bond No. 14–No. 15, linking the B peptide to the rest of the B $\beta$-chain (18); Arvin and Reptilase also split, unlike thrombin,[18] the Arg-Ala No. 42–No. 43 bond of the B $\beta$-chain. In addition, Arvin split the Arg-His No. 23–No. 24 bond, while thrombin (17) and Reptilase split the Arg-Val No. 19–No. 20 bond of the A $\alpha$-chain (18). Thus it is highly probable that thrombin, Arvin, or Reptilase can split fibrin(ogen) slowly into large, immunologically competent fragments at a sensitive Arg or Lys bond, in addition to the very rapid splitting associated with the Gly-Arg bond linking fibrinopeptide A.

The idea that thrombin is fibrinolytic was suggested by Nolf in 1908 (19) and demonstrated by Guest and Ware (20) and Kowarzyk (21). The question of contamination by plasmin or plasminogen activator was not excluded, however, since the need for specific inhibitors of these enzymes was not fully appreciated at that time.

The increase in FR-antigen is of clinical importance since it may influence the amount found in human serum. The optimal conditions for achieving the lowest FR-antigen level appear to be as follows. Citrated plasma is clotted on ice with an equal volume of a mixture containing 0.1 M EACA, 0.025 M $CaCl_2$, and two concentrations of thrombin, 5.0 and 1.0 U/ml (at the 1.0 unit level, the thrombin blank can be ignored). The clot is left undisturbed for 4 hr, then pushed against the side of the tube with a wooden applicator stick to press out the serum. This serum, with the fibrin removed, is centrifuged. At this stage, the 1:2 diluted sample can be tested or stored. Plasma from patients with gross coagulation defects may require the higher concentration of thrombin to achieve the lowest value. Values about 1½–2 times the lowest value (probably adequate for clinical use) are obtained by clotting plasma at room temperature for 10–30 min with 5–25 U thrombin per ml.

The mean level of FR-antigen in normal serum is usually $< 1.0$ $\mu$g/ml (commonly $< 0.5$ $\mu$g/ml). Levels of FR-antigen reported in many publications, including our own, almost certainly overestimated the in vivo values; lack of proper precautions in obtaining serum probably resulted in in vitro lysis during clotting of the samples. Indeed, since endogenous and/or exogenous thrombin is essential for the collection of serum FR-antigen, thrombin may contribute a small, perhaps irreducible amount of in vitro lysis which could be responsible for all the FR-antigen found in normal human serum.

By providing an alternative explanation for the presence of most of the FR-antigen found in normal serum, these data cast additional doubt on the concept of continuously recurring in vivo clotting and fibrinolysis in normal individuals, the "dynamic equilibrium" between clotting and lysis in the circulating blood.

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