A reliable and sensitive test for both hypo- and hypercoagulability would be very desirable for clinical and research purposes. Rapid detection of hypercoagulability would be of value in conditions such as spontaneous clotting fluctuations which occur during stress in patients with congenital or acquired valvular heart diseases and with previous myocardial infarction.

It is difficult to find a test with equal sensitivity for both hypo- and hypercoagulability. Thrombelastography, the thromboplastin generation test, and earlier thrombin generation tests do not fulfill these requirements completely. The thrombelastograph undoubtedly can detect marked hypercoagulability but it is less suited for recording finer deviations from the normal; it is also a rather expensive device. The thromboplastin generation test has been used in the past to detect hypercoagulability. It is inferior to thrombin generation although recently a modification designed for hypercoagulability has been described.

The thrombin generation test was originally devised for studies on hemophilia. The test was subsequently used to study clotting abnormalities in cirrhosis of the liver and other diseases, iron loading deficiencies, and Hageman factor deficiency. We have used it in the method described below to study the neutralizing effect of the human procoagulant from urine on circulating anticoagulants. It was instrumental in the discovery of spontaneous clotting fluctuations in certain patients including those subjected to moderate treadmill stress.

We have found that thrombin generation, after considerable modification and strict standardization, is a promising test for detecting various degrees of both hypo- and hypercoagulability. Hypercoagulability is defined here as an increase of the in vitro-clotting kinetics brought about by endogenous variations of the patient's clotting system. These variations may or may not reflect in vivo hypercoagulability predisposing to intravascular clotting. In the present investigation, thrombin generation was used to study clotting deviations in cardiac patients subjected to controlled stress.

**Methods**

**PRINCIPLE**

The concentration of thrombin, which rises and then falls during the clotting of recalcified citrated plasma, is measured by transferring serial samples from the recalcification mixture to a fibrinogen solution and recording the resulting clotting time of this solution. The speed with which the fibrinogen solution clots depends upon the amount of thrombin present in the aliquot of recalcification mixture transferred into it.

**THROMBIN GENERATION TEST**

**Equipment**

1. International centrifuge, clinical model CL.
2. All-glass thermostated waterbath without stirrer set at 37°C and equipped with a rack to hang the test tubes in the water. 3. Multifit syringes with straight metal tip, 5 ml and 10 ml (Luer-Lok is unsatisfactory). 4. Yale disposable hypodermic needles, 20 gauge, 1/2 inches long. 5. Two stop watches. 6. Wooden applicator sticks. 7. Centrifuge tubes, 12 ml, heavy wall. 8. Test tubes with rim, 10 × 75 mm, and 13 × 100 mm. 9. 1 ml/100 and 0.2 ml/1000 pipettes, serological. 10. Tissue paper. 11. Surgical sponges, 4" × 4".

**Reagents**

1. Sodium citrate, USP, 3.8%. 2. 0.5 M calcium chloride. 3. Buffered saline, pH 7.42, consisting of four parts 0.95% sodium chloride and one part...
barbital acetate buffer, pH 7.42.10 4) Fibrinogen (bovine fraction I, Armour) solution, 1%, in buffered saline. For each ml solution, 1 mg BaSO₄ is added and the solution is stirred several times at room temperature, centrifuged clear, and the supernatant stored in a freezer in aliquots of 1 ml.

**Performance of the Test**

**Step One.** Blood is drawn by a two-syringe technique. A 4 × 4 gauze pad is placed on the arm where the hub of the needle will be. Immediately after the tourniquet has been applied, a venipuncture is performed, a few ml of blood withdrawn, the needle left in the vein, and the syringe removed. The blood should now flow freely from the hub of the needle. The test sample is withdrawn to precisely the 10 ml mark of a 10 ml syringe which has previously been filled with 2 ml of sodium citrate, 3.8%, with exclusion of air. Syringe and needle are removed from the vein, the needle taken off the syringe, the barrel moved one-quarter inch back, and the contents of the syringe mixed by tilting it gently back and forth. All these steps are performed in rapid succession. If difficulty is encountered with the venipuncture, the tourniquet is released for about 30 seconds after the first syringe is in place and then reapplied before the second syringe is filled. The patient must not "pump" his hand but only close his fist tightly during the procedure.

Thrombin generation must be carried out within 30 minutes after shedding of blood. The fresh, noncentrifuged specimens are kept at room temperature until they are tested.

**Step Two.** Centrifugation. The content of the second syringe is carefully emptied into a siliconized 12 ml centrifuged tube and centrifuged for exactly five minutes at full speed (1470 R.C.F.). Since the test is also sensitive to changes in platelet concentration, correct centrifugation is a critical procedure.

**Step Three.** Preparation for thrombin generation. In the center of the test tube rack suspended in the prewarmed thermostated waterbath are placed seven 10 × 75 mm tubes. One-tenth ml of fibrinogen solution is pipetted into each tube. Which has been rinsed previously with distilled water and dried, is placed in one corner of the rack. One ml of plasma from the upper third of the plasma phase of the centrifuged specimen is carefully pipetted into this tube with the 1 ml nonsiliconized pipette without allowing the plasma to run down the side of the tube. The plasma is incubated for one minute (not longer).

**Step Four.** Thrombin generation. After this minute, using a 0.2 ml pipette, 0.1 ml 0.5 × CaCl₂ is carefully blown into the citrated plasma, and a stop watch is started. After exactly four minutes, 0.1 ml of the recalcified plasma is transferred with a 0.2 ml pipette into one of the small test tubes previously filled with fibrinogen solution. To prevent technical error it is essential to wipe off the end of the pipette with tissue paper after the aliquot has been drawn up and to avoid excessive blowing when expressing the sample from the pipette.

The pipette should reach the bottom of the tube and at this very instant a second stop watch is started, always leaving the first watch running. The time required by the fibrinogen solution to clot is determined by slowly and rhythmically tilting the tube which is dipped again into water at 37°C during each back and forth motion. The endpoint, when the first fibrin strands are visible, is quite sharp. A globular mass forms rapidly when the time interval is short. The procedure is repeated at the 8th, 12th, 16th, 20th, 24th, and 28th minutes of incubation.

When a visible fibrin clot is formed in the recalcified plasma, a wooden applicator stick is pierced into the clot and the clot wrapped around the stick by rotating it with some pressure along the wall of the tube. Clot and stick must be left in the tube. At the beginning and the end of the incubation period, very small amounts or no thrombin at all might be present in the recalcified plasma, hence, transfer of this material to the fibrinogen solution results in a very long or indefinite clotting time. For practical reasons, the clotting time of the fibrinogen solution is not followed beyond 120 seconds. The peak thrombin generation in normal plasma occurs around the 12th or 16th minute, producing a clotting time of the fibrinogen solution of 20 seconds or less.

In plasma with poor thrombin generation, as with hemophilia or with inhibitors interfering with intrinsic thromboplastin (formation), a clot may not appear in the recalcified plasma; however, some more or less retarded thrombin generation will result.

**Precautions**

Strict adherence to the details outlined above is essential to avoid faulty results. The main sources of error are storage, uncontrolled contact with glass, alterations of the platelets, and silicone-contaminated recalcification test tubes. It is not necessary to perform the test in the fasting patient but fatty meals or exercise are to be avoided.

**Method of Graphing. Normal Thrombin Generation Curve**

The thrombin generation curve representing the average values from 25 normal individuals is shown in figure 1. The typical pattern is that of an increasing yield of thrombin, indicated by shortening of the clotting times of the fibrinogen solution to which an aliquot of the recalcification
A clot is always formed in the recalcified plasma before the maximum thrombin yield is obtained, except with very pathological specimens. We feel that the initial descending portion of the thrombin generation curve is the most important for diagnostic purposes since its course is determined predominantly by the rapidity and quantity of thrombin formed, and to some extent by removal of thrombin. The diagnostic significance of the subsequent ascending portion, which is determined predominately by the rate of disappearance of the generated thrombin, is less clear at the present time. We have noted, however, that the disappearance rate is frequently much faster in samples in which a high yield of thrombin is generated quickly.

We do not interpret rapid removal of thrombin from the generation mixture, after a large thrombin yield has been generated previously, as an indication of hypocoagulability. The high thrombin concentration is present for a certain length of time (permitting its assay) during which it interacts with fibrinogen. Its subsequent removal has no bearing on the process of fibrin formation. In contrast, thrombin removal, occurring so rapidly that its concentration remains low, could be the cause of hypocoagulability.

**Effect of Dilution**

The thrombin generation test is most sensitive when the sample is not diluted. With the method described here, the dilution of the sample by recalcification is only 10%, in contrast to previous methods in which the dilution was 50% or greater. The differences in thrombin generation are quite marked; more thrombin is generated faster in the diluted sample. Two examples shown in figure 2 demonstrate that the dilution effect on thrombin generation masks the one important deviation from the normal which the thrombin generation test...
should detect, the faster and greater thrombin generation with hypercoagulability.

**Effect of Storage**

Blood or plasma samples for thrombin generation cannot be stored, even in siliconized tubes. The changes in the thrombin generation pattern which occur on standing of the sample vary considerably from plasma to plasma but, in any case, are markedly enhanced by contact with glass. In general, there is more rapid thrombin generation, except for longer preincubation at 37°C. Freezing, followed by thawing, induces extreme shifting of the generation pattern. This particular condition is shown in figure 3 which represents the delayed and deficient thrombin generation pattern of a thrombocytopenic (platelet count of 40,000) plasma and the marked alterations induced by freezing and thawing of the sample. The fresh sample reflects correctly the defective thrombin generation as compared with the frozen sample in which the defect is entirely masked.

**Reproducibility**

The reproducibility of the thrombin generation pattern is excellent both with regard to duplicates from the same plasma sample and tests done at intervals in the same individual with a stable clotting system. Figure 4 shows the thrombin generation curve obtained from two normal individuals on separate occasions. The graph at the left includes duplicate tests performed on the same day which are practically identical.

**Hypercoagulability**

The combination of accelerated generation and increased yield of thrombin as a form of hypercoagulability results in a very characteristic pattern. The accelerated thrombin generation is manifested by a relatively short fibrinogen clotting time at the 4th minute and the peak values (shortest clotting times of fibrinogen solution) at the 8th minute. This is in contrast to normal samples in which very long (over 100 seconds) or no values are obtained at the 4th minute and in which the peak values occur at the 12th or 16th minute. Figure 5 represents some characteristic thrombin generation patterns of patients with suspected in vivo hypercoagulability associated with various diseases. The patterns are distinctly different from normal curves. The reproducibility of thrombin generation is again demonstrated by the curves 1a and 1b obtained on different days from a patient with idiopathic pulmonary hypertension.

**Subjects**

It is possible to demonstrate transitory hypercoagulability patterns by thrombin generation, provided the samples are properly timed. In our initial attempt to study variations in coagulability in humans under physiologic conditions, stress was applied in the form of a standardized treadmill exercise. Individuals with cardiac diseases were selected because we have previously noted that they frequently have an unstable clotting system. Most of these patients were evaluated by the Work Classification Unit of the Colorado Heart Association. Their functional reserve was assessed by observing the effect of a mild treadmill stress program.
Hypercoagulability patterns of thrombin generation of patients with severe primary (idiopathic) pulmonary hypertension (1a, 1b); thrombocythemia (800,000) with recurrent thrombophlebitis (2); recurrent myocardial infarction (3); thrombocythemia (500,000) (4); thrombocythemia (480,000) with recurrent pulmonary infarction (5). Platelet count for 1a, 1b, and 3 was normal. Note early onset of thrombin formation, early peak of maximum thrombin yield.

on cardiovascular function. Serial thrombin generation tests were carried out simultaneously. The duration and intensity of the treadmill exercise were adapted to the individual's capacity, but the original protocol was designed as follows: six minutes at three miles per hour at a gradient of 4°; six minutes rest; six minutes at three miles per hour at a gradient of 8°; six minutes rest; six minutes at three miles per hour at a gradient of 12°. Thrombin generation tests were performed generally before the treadmill exercise, within one minute after completion of each phase, and, in addition, thirty or fifty minutes after performance of the last phase.

Results

TREADMILL; NORMAL INDIVIDUALS

Serial thrombin generation curves from a normal healthy male before, during, and after treadmill stress are shown in figure 6 as a representative example. There are at no time gross deviations from the normal as are found in patients described below. There is a suggestion of an earlier onset of thrombin generation after the second treadmill phase and one hour later. The rapid disappearance rate of thrombin from the pretreadmill control sample, which is occasionally seen in normal individuals, is slowed down by stress.

TREADMILL; EBSTEIN'S ANOMALY

Serial thrombin generation curves from a patient with Ebstein's anomaly are shown in figure 7. In this instance a pattern of normal coagulability which existed before the stress is converted stepwise into patterns of a marked
hypercoagulability during the stress; the changes persisted for at least thirty minutes after termination of the exercise.

**TREADMILL; TETRALOGY OF FALLOT**

Serial thrombin generation curves from a patient with tetralogy of Fallot during treadmill stress are shown in figure 8. They provide an instructive example of an unstable clotting system as reflected by thrombin generation. This patient initially exhibited a delayed and reduced thrombin generation. After the first treadmill phase, no measurable thrombin was generated; after the second phase, thrombin generation was nearly normal. After the third phase, there was a swing back to marked hypocoagulability which was more pronounced fifty minutes after the third phase. The patient also exhibited considerable prolongation of the thrombin time and a markedly increased fibrinolytic activity throughout the test. Other patients with tetralogy of Fallot did not exhibit such marked alterations of thrombin generation.

**TREADMILL; RHEUMATIC HEART DISEASE**

Changes in the coagulability of human blood induced by mild treadmill stress are well demonstrated in individuals with a reduced ability to generate thrombin. The following two examples were obtained from patients with rheumatic heart disease. Figure 9 shows serial thrombin generation curves from a patient with aortic insufficiency. Thrombin generation was delayed markedly before the treadmill exercise. After each of the two phases the thrombin generation curve was progressively shifted toward normal; thirty minutes after treadmill exercise the curve was entirely normal. The thrombin generation pattern in this patient during and after treadmill

This observation applies also to patients being treated with prothrombin-depressing agents (fig. 10). Serial thrombin generation curves were determined on a patient with mitral valvulitis and pulmonary hypertension. The patient’s prothrombin activity was reduced therapeutically to 17% of normal (one-stage method). This reduction is reflected in a diminished thrombin generation (I). The patient walked only two minutes on the treadmill before he was forced to stop because of a pounding heart. These two minutes of moderate exercise, however, were enough to induce a marked shift of the thrombin generation pattern toward normal values (II). Treatment with Coumadin did not prevent the shift.

TREADMILL; MYOCARDIAL INFARCTION

In figure 11 are reproduced serial thrombin generation curves obtained during treadmill stress from two patients who had recovered from myocardial infarctions. Neither was treated with prothrombin-depressing agents. Patient A, on the left side of the figure, did not exhibit marked changes of his thrombin generation pattern following exercise. This is in contrast to the thrombin generation pattern of patient B, on the right side, who exhibited a moderate delay of thrombin generation before treadmill exercise; the curve shifted already with the first treadmill phase into the normal range and remained there after the second and final phase.

Patients with previous myocardial infarction
Two patients with previous myocardial infarction. Treadmill stress. Serial thrombin generation tests. 1: control before treadmill; 2: after first phase; 3: after second phase; 4: after third phase. For further legend see figure 1.

who are treated with prothrombin-depressing agents, such as Coumadin, are definitely not protected, in all instances, against the development of hypercoagulability as defined in the introduction. Figure 12 shows serial thrombin generation curves during treadmill stress in a patient with previous myocardial infarction who was treated inadequately with Coumadin. The prothrombin activity was 42% and, consequently, thrombin generation was not very markedly reduced. Thrombin generation was not altered to any extent during the three treadmill phases; in particular, no hypercoagulability was induced by the stress despite inadequate reduction of prothrombin activity.

The patient whose thrombin generation curves are shown in figure 13 behaved quite differently during exercise. He also had had a previous myocardial infarction and was treated with Coumadin, the prothrombin activity being reduced to 33%. As a result, the patient's thrombin generation was moderately delayed and lessened. With the treadmill stress, however, the thrombin generation is accelerated and the thrombin yield increased. Immediately after the third treadmill phase (IV), there was a clearcut picture of hypercoagulability with the peak thrombin activity occurring at the 8th minute; at this time, the patient experienced angina. Thirty minutes after the third treadmill phase, there was a shifting back of the thrombin generation pattern toward the pretreadmill hypocooagulability values. It should be emphasized that, in contrast, serial prothrombin determinations with the conventional one-stage method did not show any changes of the prothrombin time before, during, and after this treadmill experiment, not even at the time of the accelerated and increased thrombin generation. This is a good example showing that the prothrombin time does not reflect the actual clotting condition.

Discussion

Thrombin generation is a screening test,
Counudin-treated patient with previous myocardial infarction. Prothrombin 33%. Treadmill stress. Serial thrombin generation tests. I: before treadmill; II: after first treadmill phase; III: after second phase; IV: immediately after third phase; V: 30 minutes after third phase. N: Average normal thrombin generation curve from 25 individuals. For further legend see figure 1.

with the outcome determined by the activities of many clotting factors. One of its distinct advantages is that the results are expressed as a curve based upon a series of measurements. Thus, a more comprehensive analysis of an individual clotting situation is obtained than when a single measurement is used. Another decisive advantage of the thrombin generation test is that the relationship between the factors promoting and inhibiting clot formation is not changed. The test circumvents the potential physicochemical alterations of clotting factors which might occur when plasma has to be diluted, incubated, stored, absorbed, and otherwise handled. Consequently, variations in coagulability can be measured which cannot be detected by other methods in which the very technique employed may artificially produce hypercoagulability. We have repeatedly observed that uncontrolled glass contact, dilution, and storage induce alterations which make comparison of minor changes, from sample to sample, difficult. Similar observations have already been reported with a less sensitive thrombin generation technique.20 The acceleration of coagulation by dilution of the sample is a well established fact; the enhancement of thrombin generation by dilution is a particular example of this general rule. Dilution of plasma with equal amounts of saline increases the thrombin yield of the mixture two- to threefold.21 It has been suggested that the protein concentration in undiluted plasma prevents maximum thrombin generation, thereby acting as a nonspecific protection against intravascular coagulation.21

Our study has shown by serial thrombin generation tests that fluctuations of the clotting system occur in certain cardiac patients. Causes for the fluctuations are unknown, but might be clarified in the future by additional studies during the course of the disease.

Hypercoagulability in patients with previous myocardial infarctions is of great practical interest. Based on our limited experience, there are two kinds of hypercoagulability in these patients: a) that which is already present at the time of the test (which is performed under resting conditions in most instances), and b) that not present at the time of the test but which is induced by physical exercise (or other stimuli). Hypercoagulability at the time of the test (which was not always performed under resting conditions, but in absence of exercise) was found in patients with myocardial disease by several investigators who were using primarily the heparin-retarded clotting time or the thrombelastograph.3 Hypercoagulability, however, was not present in all instances. The use of other methods, such as the thromboplastin generation test, was less revealing.22 The coagulability of patients with previous myocardial infarction after standardized physical exercise has not been studied previously. Yet there are compelling reasons for intensive investigations in this regard. First, it is known that the antihemophilic globulin, AHG, might be present in abnormally higher concentrations in patients with coronary artery disease, thus predisposing them to thrombosis at the site of local vascular disease.21 The average higher level of AHG in patients with coronary atherosclerosis is not influenced by prothrombin-depressing agents.24 Second, the antihemophilic globulin concentrations in normal persons increase substantially after short but marked physical stress.25 The platelet count increases after physical stress.26, 27 The effect of exercise on other clotting factors is known to be more erratic.28
The increase of AHG and platelets might play a role in the hypercoagulability observed by us with the thrombin generation test in patients with myocardial infarction. The increase in either or both of these clotting components would explain at least partially the absence of protection against the development of hypercoagulability in some of these patients treated with prothrombin-depressing agents, because these agents have no influence on AHG and platelets and their accelerating effect on prothrombin conversion. One might speculate whether a clearcut hypercoagulability has to be obtained in order to set the stage for the formation of intravascular clots or whether a rapid shift of the coagulability from the low range to normal as in the patient of figure 10 or the patient B of figure 11 has the same potential effects. We are inclined to believe in the latter possibility.

Summary
A refined, reliable thrombin generation test with good sensitivity for both hypo- and hypercoagulability is described. When serial thrombin generation curves were obtained in cardiac patients subjected to standardized treadmill stress, fluctuations of the clotting system were observed in some and a marked tendency to hypercoagulability in others. The relationship of in vitro hypercoagulability as reflected by thrombin generation to an increased tendency to thrombosis remains to be determined.

References
Thrombin Generation in Normal Subjects and Cardiac Patients
KURT N. VON KAULLA and EDITH VON KAULLA

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