3. Allow to incubate for 30 seconds.
4. Pipet 0.1 ml of adenosine diphosphate solution into the tube and simultaneously start a stopwatch.
5. Agitate the mixture vigorously while keeping it in the 37°C water bath. Examine the mixture every 5 to 10 seconds for macroscopic agglutination. Normal clumping should occur within 30 seconds after the addition of adenosine diphosphate. Record the degree of platelet aggregation (1+, 2+, 3+, or 4+) and the time, after the addition of adenosine diphosphate, before clumping occurred.
6. Repeat steps 2 through 5, using the patient's platelet-rich plasma. If no aggregation with the patient's platelets has occurred after 2 minutes, place a small drop of the mixture on a slide. Examine the plasma mixture under the microscope for small clumps of platelets. (This may also serve as a check to determine if there are sufficient platelets in the plasma to give valid results.)

DISCUSSION

1. Failure of the normal control plasma to show normal platelet aggregation may be due to improperly prepared platelet-rich plasma. Therefore check the plasma microscopically for platelets. If the platelet-rich plasma shows a decrease in platelets, both control and patient bloods must be redrawn and the procedure repeated.
2. A more complete procedure for platelet aggregation is outlined in Chapter 7, Automation (Bio/Data Platelet Aggregation Profiler).

TEST FOR INACTIVATORS AND INHIBITORS (ANTICOAGULANTS)

Naturally occurring anticoagulants may be divided into two classes: (1) *inhibitors*, which act immediately to block the reaction between coagulation factors, and (2) *inactivators*, which progressively destroy the factors. Most cases of coagulation defects are due to deficiencies of one or more clotting factors. The presence of inactivators or inhibitors is rare but must be properly diagnosed when present. Inhibitors have been observed in certain chronic illnesses such as lupus erythematosus and chronic nephritis. Inactivators have been demonstrated in patients with hemophilia and Christmas disease, in patients with congenital deficiencies of other coagulation factors, and in some women soon after childbirth.

REFERENCE

Hardisty, R.M., and Ingram, C.I.C.: *Bleeding Disorders, Investigation and Management*, Blackwell Scientific Publications, Oxford, 1965.

REAGENTS AND EQUIPMENT

1. Water bath, 37°C.
2. Calcium chloride, 0.025 M.

Anhydrous calcium chloride	1.38 g
Distilled water	500 ml

3. Partial thromboplastin (platelet substitute).
4. Normal control plasma.
5. Test tubes, 13 × 100 mm.
6. Stopwatch.

SPECIMEN

Citrated plasma: one part 0.11 M sodium citrate to nine parts whole blood; or oxalated plasma: one part 0.1 M sodium oxalate to nine parts whole blood.

PRINCIPLE

Inactivators may be detected by performing an activated PTT on the patient's plasma, normal control plasma, and 1:1 mixtures of the patient and control plasmas at specifically timed intervals. These results are compared with each other and with a freshly made 1:1 mixture of patient and control plasma, to determine if the clotting time is progressively prolonged (inactivator present), or if the clotting time

shows no appreciable increase after the first set of tests is performed (inhibitor or factor deficiency present). If an inhibitor or factor deficiency is present, varying dilutions of the patient's plasma with normal control plasma may be made. In the presence of an inhibitor, all dilutions are prolonged. With a factor deficiency, dilutions containing only a small amount of normal control plasma may show prolonged clotting times (tubes No. 5, No. 6, and also No. 7 in the procedure description following).

PROCEDURE

1. Place tube of blood in a cup containing crushed ice immediately after the blood is drawn.
2. Centrifuge blood at 2500 RPM for 10 minutes. Immediately remove the plasma and place the tube in a cup containing crushed ice.
3. Perform an activated PTT on each of the following:
   A. Patient's plasma.
   B. Normal control plasma.
   C. 1:1 mixture of patient's plasma and normal control plasma (0.1 ml of each).
4. Record results on time chart. (See below.)

Time (min)	No. 1 Patient's plasma (seconds)	No. 2 Normal control plasma (seconds)	No. 3 Incubated patient & control plasmas (seconds)	No. 4 Patient & control plasmas (seconds)
0				
30				
60				
120				

5. Pipet 1.0 ml of patient's plasma into a 13 × 100-mm test tube (No. 1) and place in 37°C water bath.
6. Pipet 1.0 ml of normal control plasma into test tube No. 2 and incubate at 37°C.
7. For the incubated patient plus control plasmas, pipet 0.5 ml of each plasma into tube No. 3, and incubate at 37°C.
8. Incubate preceding three tubes for 30 minutes at 37°C.
9. At the end of 30 minutes, perform an activated PTT on the contents of tubes No. 1, No. 2, and No. 3. Record the results.
10. At the same time, perform an activated PTT using 0.1 ml of the patient's plasma from tube No. 1 and 0.1 ml of normal control plasma from tube No. 2. Record results in the indicated column (No. 4).
11. Allow tubes No. 1, No. 2, and No. 3 to incubate for an additional 30 minutes.
12. At the end of the 30 minutes, repeat steps 9 and 10. Record results on the chart.
13. Allow the tubes to incubate for an additional 60 minutes.
14. At the end of one hour, repeat steps 9 and 10 and record the results.
15. To interpret the results of this procedure, compare the clotting times of tubes No. 1, No. 2, No. 3, and No. 4 at the different time intervals. It must be kept in mind, however, that as the plasmas incubate, there is normally a slight increase in the clotting times due to some loss of labile components in the plasma. For this reason, attention must be paid to the clotting times of the normal control plasma (tube No. 2). Before the clotting times of the patient's plasma and the patient-control mixture are considered prolonged, the degree of prolongation from the previously run test must be greater than that shown by the normal control plasma (tube No. 2). If an inactivator is present, the clotting times of tubes No. 1 and No. 3 will become progressively prolonged as the time interval increases. The clotting time of tube No. 4, although

TABLE 15. PLASMA DILUTIONS (TEST FOR INHIBITORS)

TUBE NO.	PATIENT'S PLASMA (ml)	NORMAL CONTROL PLASMA (ml)	PATIENT PLASMA DILUTION	ACTIVATED PTT (seconds)
1	0.0	1.0	0	
2	0.2	0.8	1:5	
3	0.4	0.6	2:5	
4	0.5	0.5	1:2	
5	0.6	0.4	3:5	
6	0.8	0.2	4:5	
7	1.0	0.0	—	

longer than the original, should not be as prolonged as that of tubes No. 1 and No. 3. It generally shows a clotting time closer to that of the control plasma. In the presence of an inhibitor, or factor deficiency, tubes Nos. 1, 2, 3, and 4 will not show more than a normal increase in the clotting time as the time interval increases. The clotting time of tube No. 4 will more closely parallel that of tube No. 3.

16. If an inactivator has been found to be present, the procedure may be stopped here. However, to distinguish between an inhibitor and factor deficiency, continue with the procedure as follows.
17. Label seven 13 × 100-mm test tubes, No. 1 through No. 7, and carefully prepare the indicated dilutions of patient's plasma with normal control plasma, as shown in Table 15.
18. Perform an activated PTT, in duplicate, on each of the preceding seven tubes. Average the results and record.
19. Interpretation of results: In the presence of an inhibitor, all tubes containing the patient's plasma (tubes No. 2 through No. 7) will show a prolonged clotting time. If a coagulation factor deficiency exists, normal clotting time results will occur on the contents of tubes Nos. 2, 3, and 4. (The clotting time from tube No. 1 must always be normal.) The clotting time on the plasmas from tubes No. 5 and No. 6 may or may not be prolonged, depending on the severity of the factor deficiency.

EUGLOBULIN CLOT LYSIS TIME

The euglobulin clot lysis time is a screening procedure for the measurement of fibrinolytic activity and is more sensitive than the clot lysis time. The euglobulin fraction of plasma contains plasminogen, fibrinogen, and activators capable of transforming plasminogen to its active state, plasmin. Normally occurring inhibitors of this reaction (plasminogen to plasmin) are not present in this fraction of the plasma. Once a clot is formed, clot lysis occurs more quickly than in whole blood. Increased fibrinolytic activity has been associated with circulatory collapse, adrenalin injections, sudden death, pulmonary surgery, pyrogen reactions, and obstetric complications. Normally clot lysis does not occur in less than 2 hours but is usually complete within 4 hours. Clot lysis in less than 2 hours is indicative of abnormal fibrinolytic activity.

REFERENCE

Bucknell, M.: The effect of citrate on euglobulin methods of estimating fibrinolytic activity, J. Clin. Path., *11*, 403, 1958.

REAGENTS AND EQUIPMENT

1. Calcium chloride, 0.025 M.
   Anhydrous calcium chloride 1.38 g
   Distilled water 500 ml
2. Acetic acid, 1% (v/v).