TABLE 10. DILUTIONS FOR FACTOR V ASSAY

TUBE	PLASMA (OR REFERENCE PLASMA) (ml)	FACTOR V-DEFICIENT SUBSTRATE (ml)	DILUTION	PLASMA CONCENTRATION %
1	0.1	1.9	1:20	5
2	0.1	0.9	1:10	10
3	0.1	0.4	1:5	20
4	0.2	0.3	1:2.5	40
5	0.3	0.3	1:2	50

6. Forcibly blow 0.1 ml of the diluted patient's plasma into a tube containing 0.2 ml of thromboplastin-calcium mixture and simultaneously start the stopwatch.
7. Mix the contents of the tube and stop the watch at the first indication of clot formation. Perform duplicate prothrombin times on each plasma dilution, average results, and record.
8. Repeat steps 2 through 7 substituting the reference plasma for the patient's plasma.
9. Calculation of results:
   A. Using two-cycle semilog graph paper, plot each average clotting time in seconds against the plasma concentration in percent. Use the logarithmic scale for the plasma concentration. Plot 1 to 10% on the first cycle, and 20 to 100% on the second cycle. There will be ten points plotted.
   B. Draw the straight line that best connects the five points of the reference plasma. This represents the normal activity curve.
   C. By interpolation, determine from the graph the concentrations of reference plasma that give the same clotting times as the different dilutions of patient's plasma. Multiply the resulting reference plasma dilutions by the patient plasma dilution factors. (The calculations are similar to those for the factor VIII and IX assay. See Figure 130 for an example.)
   D. Average the five values received for the percent activity of patient's plasma. This result represents the percent of normal activity of factor V present in the patient's plasma.

DISCUSSION

1. Pooled normal plasma may be used in place of the reference plasma.
2. Factor V-deficient plasma may be used in place of the factor V-deficient substrate. If this is employed, a prothrombin time greater than 60 seconds should be obtained on the factor V-deficient plasma before it is used. (Factor V-deficient plasma may be prepared by incubating normal plasma at 37°C for 24 hours or by refrigerating the normal plasma at 4 to 10°C for 2 weeks.)
3. An assay of factor VII or factor X may be performed according to the previously described procedure. Factor VII- (or factor X-) deficient substrate and reference plasma with a known factor VII (or factor X) assay are used in place of factor V-deficient substrate and reference plasma.

TWO-STAGE PROTHROMBIN TIME

The two-stage prothrombin test determines the concentration of prothrombin in the plasma. Normally, there are 300 to 360

units of prothrombin, per ml, in the plasma.

Warner, Brinkhous, and Smith Method
(Modified by Ware and Seegers)

REFERENCES

Miale, J.B.: *Laboratory Medicine: Hematology*, 5th Edition, C. V. Mosby Company, St. Louis, 1977.

Ware, A.J., and Seegers, W.A.: Two-stage procedure for the quantitative determination of prothrombin concentration, Am. J. Clin. Path., 19, 471, 1949.

REAGENTS AND EQUIPMENT

1. Water bath, 28°C, if possible.
2. Bacto-prothrombin two-stage reagent. May be obtained from Difco Laboratories, Detroit, Mi.
3. Thrombin, 1000 units. (Thrombin, Topical may be obtained from Parke, Davis & Company, Detroit, Mi.)
4. Bacto-AC globulin (bovine serum), obtainable from Difco Laboratories, Detroit, Mi.
5. Imidazole buffer, stock solution, pH 7.2 to 7.4.
   Imidazole 1.72 g
   (may be obtained from Edcan Laboratories, South Norwalk, Cn.)
   Hydrochloric acid, 0.1 N 90 ml
   Dilute to 100 ml with distilled water.
6. Bovine fibrinogen, obtainable from Armour Pharmaceutical Company, Chicago, Il.
7. Sodium chloride, 0.6% (w/v).
8. Working imidazole buffer.
   Stock imidazole buffer 0.5 ml
   Distilled water 9.5 ml
   Place the preceding mixture in a small beaker and sprinkle 0.2 g of bovine fibrinogen on the surface of the solution. Do not shake or stir. After 10 minutes, filter through No. 1 Whatman filter paper. This solution is effective for 2 hours.
9. Working thrombin solution.
   Add 5.0 ml of distilled water to the vial containing 1000 units of thrombin. Add this mixture to 5.0 ml of glycerol. Stable at 4°C for 1 month.
10. Working Bacto-AC globulin.
    Reconstitute the vial of AC globulin with 2 ml of distilled water. Stable at 4°C for several days.
11. Working Bacto-prothrombin two-stage reagent.
    Reconstitute contents of vial with 10 ml of 0.6% sodium chloride.
12. Sodium chloride, 0.85% (w/v).
13. Dilute Bacto-AC globulin.
    Working Bacto-AC globulin 0.5 ml. Dilute to 30 ml with 0.85% sodium chloride. This represents a 1:60 dilution of bovine serum.
14. Test tubes, 13 × 100 mm.
15. Stopwatch.

SPECIMEN

Citrated plasma: one part 0.11 M sodium citrate to nine parts whole blood. Obtain a similar blood specimen from a normal individual at the same time the patient's blood is obtained.

PRINCIPLE

Thrombin is added to the plasma in order to remove the fibrinogen present in the plasma. The clot is removed and after a short time the added thrombin is destroyed by the antithrombin present in the plasma. The prothrombin present in the plasma (unaffected by the preceding steps) is then converted to thrombin by the action of AC globulin and the two-stage reagent (containing thromboplastin, calcium ions, and acacia). Interference by antithrombin is avoided by diluting the plasma. The acacia increases the sensitivity of fibrinogen to thrombin (thus allowing the plasma to be diluted). The amount of thrombin formed

is measured with fibrinogen, by preparing a plasma dilution that will clot within 15 seconds. One unit of prothrombin gives rise to one unit of thrombin. In turn, one unit of thrombin is that amount which will clot 1 ml of a standardized fibrinogen solution in 15 seconds under the conditions of this test.

PROCEDURE

1. Pipet 0.4 ml of 0.85% sodium chloride into a 13 × 100-mm glass test tube.
2. Add 0.5 ml of control or patient plasma to the tube. Pipet 0.1 ml of thrombin solution into the diluted plasma in order to accomplish defibrination of the plasma.
3. Allow to sit at room temperature for 20 to 30 seconds.
4. Using an applicator stick, remove the fibrin clot that has formed. Wring the clot so that as much plasma as possible is expressed before removing the clot from the mixture.
5. Allow the plasma to sit for 10 minutes so that destruction of thrombin by antithrombin may occur.
6. If the prothrombin concentration in the plasma is thought to be normal, dilute the plasma 1:25 with dilute AC globulin (0.1 ml defibrinated plasma, plus 2.4 ml of dilute AC globulin). If the prothrombin concentration in the plasma is thought to be low, make a smaller dilution of the defibrinated plasma (1:20, 1:15, or lower). Mix the diluted plasma well. This mixture is relatively unstable and should be used as quickly as possible after diluting.
7. Pipet 0.1 ml of fibrinogen solution into each of six 13 × 100-mm test tubes, and place in the 28°C water bath.
8. Place 3 ml of the prothrombin two-stage reagent into a 13 × 100-mm test tube in the 28°C water bath. Pipet 1 ml of diluted plasma into this tube. Simultaneously start a stopwatch. During this time, the prothrombin present in the diluted plasma is being converted into thrombin. (Conversion to thrombin is usually complete in 4 to 6 minutes.)
9. At 1 minute intervals, pipet 0.4 ml of the diluted plasma-two-stage reagent mixture into one of the tubes containing 0.1 ml of fibrinogen. Simultaneously start a second stopwatch and mix the solution.
10. Using the tilt-tube method, note the first sign of cloudiness, or fine flocculation, and stop the watch. The first signs of fibrin formation are taken as the end point. Clot formation occurs a few seconds later.
11. Repeat steps 9 and 10 every minute, until the shortest time is obtained, and two readings alike are recorded. If the final clotting times are shorter than 13 seconds or longer than 17 seconds, a new dilution of the plasma must be made, and steps 6 through 11 repeated until the clotting time falls within 13 to 17 seconds.
12. Calculate the prothrombin units according to the following equation:

$$\text{Factor for dilution of plasma} \times \text{Thrombin units} = \text{Units of prothrombin}$$

A. Dilution of plasma.
   Steps 1 and 2: 1:2 dilution.
   Step 6: 1:25 dilution (or whatever dilution was used).
   Step 8: 1:4 dilution.
   Step 9: 4:5 dilution.
   Since the 1:2, 1:4, and 4:5 dilutions are routinely used, these may be combined to give a factor of 10, which should then be combined with the dilution used in step 6.

B. Refer to Table 11 in order to determine the number of thrombin units per ml of plasma.

TABLE 11. THROMBIN UNITS (TWO-STAGE PROTHROMBIN TEST)

CLOTTING TIME (SECONDS)	THROMBIN UNITS/ml OF PLASMA
13.0	1.20
13.5	1.15
14.0	1.10
14.5	1.04
15.0	1.00
15.5	0.96
16.0	0.92
16.5	0.88
17.0	0.85

C. If a plasma, diluted 1:25, gave a clotting time of 15.0 seconds, the units of prothrombin would be calculated as shown:

$$10 \times 25 \times 1.00 = 250 \text{ units of prothrombin/ml of plasma}$$

D. The results may also be reported as a percent of normal, by comparing the patient results with the control results as shown below:

$$\frac{\text{Prothrombin units/ml in patient's plasma}}{\text{Prothrombin units/ml in normal control plasma}} \times 100 = \text{Percent of normal}$$

DISCUSSION

1. For accurate results, a further correction should be made for the dilution of the blood with anticoagulant. Determine the hematocrit and then calculate the dilution of the plasma. If the hematocrit was 35%, 65% of the blood collected is plasma (65% of 4 ml, if 4 ml of blood was collected). Therefore, 2.6 ml of plasma was diluted with 0.5 ml of citrate to give a final plasma dilution of 2.6 : 3.1, or a factor of 1.19.
2. The reactivity of different preparations of fibrinogen varies. This causes a variation in the units of prothrombin. For this reason, it is desirable to report the results in percent of normal.
3. When the clotting times are done at 1-minute intervals, the time reaches a minimum and then slowly increases due to the action of antithrombin. The shortest clotting time achieved should, therefore, be used as the final result.
4. It is advisable to perform the routine one-stage prothrombin time before starting the two-stage procedure. In this way, it is possible to determine if there may be a decreased concentration of prothrombin. (However, remember that a prolonged prothrombin time may be caused by deficiencies other than prothrombin.)

STYPVEN TIME

The Stypven time is capable of detecting deficiencies in prothrombin and factor V and X. It therefore differs from the prothrombin time in that deficiencies in factor VII are not detected. The normal Stypven time is 20 to 25 seconds.

REFERENCE

Miale, J.B.: *Laboratory Medicine: Hematology*, 5th Edition, C. V. Mosby Company, St. Louis, 1977.

REAGENTS AND EQUIPMENT

1. Water bath, 37°C.
2. Stypven brand Russell's viper venom. (Obtainable from Burroughs Wellcome Co., Research Triangle Park, N.C.) Prepare according to directions on package.
3. Calcium chloride, 0.025 M.
   Anhydrous calcium chloride 1.38 g
   Distilled water 500 ml
4. Normal plasma control.
5. Test tubes, 13 × 100 mm.
6. Stopwatch.

SPECIMEN

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