Outstanding Characteristics of Thrombokinase Isolated from Bovine Plasma

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ABSTRACT Thrombokinase has been isolated from bovine plasma by a procedure which begins with the highly purified product of a previously described method, chromatographs it on DEAE-cellulose, and then fractionates it by continuous flow electrophoresis, yielding 0.2 mg per liter of oxalated plasma. The electrophoretic fraction has shown a single boundary in the ultracentrifuge; and its esterase activity on toluenesulfonylarginine methyl ester has been about the same as that of thrombokinase previously isolated by repeated electrophoretic fractionations. Thrombokinase is a euglobulin with minimum solubility near pH 5.0. It is most stable within the pH range 7.5 to 9.5; but there is also a peak in the stability curve near pH 1.8. A few micrograms of thrombokinase per milliliter can activate prothrombin in the presence of EDTA. A few thousandths of a microgram causes rapid production of thrombin in the system: prothrombin, thrombokinase, calcium chloride, phosphatide, "accelerator." But, thrombokinase has less than 1/175 the proteolytic activity of crystallized trypsin.

Thrombokinase was first isolated from bovine plasma by a series of three electrophoretic fractionations (1). Later, it was reported (2, 3) that chromatography on DEAE-cellulose offered a less arduous means for attaining a comparable specific activity. However, it has been found that the chromatographic preparation is not homogeneous electrophoretically. Now, one chromatographic step and one electrophoretic step have been incorporated into the procedure for isolation of thrombokinase.

Thrombokinase so prepared, has about the same esterase activity on toluenesulfonylarginine methyl ester (TAMe) as thrombokinase prepared by the more laborious procedure; and it sediments in the ultracentrifuge with a single, grossly symmetrical boundary. A few of its outstanding enzymatic and physicochemical properties are herein delineated.

EXPERIMENTAL

Methods and Materials

COAGULATION METHODS AND MATERIALS As described (4).

ASSAY OF THROMBOKINASE Working assay, as described (4), except that inosithin has been substituted for Howell's cephalin. All assays for activity were performed by this method unless otherwise specified.

INOSITHIN A commercial preparation of alcohol-insoluble phosphatides derived from soybeans (5–11). Supplied by Associated Concentrates, Inc., 57–01 32nd Avenue, Woodside, New York.

ASSAY OF TAME ESTERASE Method of Sherry and Troll (12) with the following minor modifications: A control incubation of TAMe without added enzyme has given a small increase in formol titration, which has been subtracted from the enzyme tests of the day. Assays with thrombokinase were more consistent, and generally a little higher, when dilutions of thrombokinase were made with buffered albumin.

BUFFERED ALBUMIN FOR COAGULATION TESTS AND FOR TAME ESTERASE ASSAY A 1 per cent solution of crystallized bovine plasma albumin was dialyzed against saline buffered with 0.02 M veronal, pH 7.4 (13) for at least 3 days. It was then diluted 1 to 10 with the same buffer for coagulation tests. For esterase assays it was diluted 1 to 10 with 0.5 M tris buffer, pH 9.0. In each case, it was used as a diluent for thrombokinase. It was not used as a diluent for trypsin, nor for thrombokinase when it was to be tested in direct comparison with trypsin.

CASEINOLYSIS Method of Kunitz (14), with minor modifications.

CRYSTALLIZED TRYPSIN Twice crystallized, salt-free, lyophilized. Supplied by Worthington Biochemical Corporation, Freehold, New Jersey.

ESTIMATION OF PROTEIN (*a*) Method of Lowry *et al.* (15), with crystallized bovine albumin as standard, for Table I, and with thrombokinase as standard, for Figs. 2, 3, and 5. (*b*) By optical density at 280 mμ, with thrombokinase as standard for Fig. 4. (*c*) By dry weight. Thrombokinase, product 8, dried to constant weight in a vacuum oven at 107–111°C, for standardization of methods *a* and *b*, above.

DEAE-CELLULOSE *N*,*N*-Diethylaminoethylcellulose, Eastman Organic Chemicals, Rochester, New York. Suspension prepared as described (3).

BUFFER FOR ULTRACENTRIFUGAL ANALYSIS 0.18 M NaCl: 0.0066 M Na_2HPO_4: 0.0002 M KH_2PO_4, pH 8.01, $\Gamma/2 = 0.2$.

0.1 M EDTA 0.930 gm. dihydrated, disodium salt of EDTA plus 0.1 M NaOH to 25.0 ml. The pH of the solution was 7.52.

Method of Isolation

Chromatography on DEAE-cellulose and continuous flow electrophoresis, previously studied in detail (1–3), are now added to the method described in 1959 (4).

At one point of that procedure, the precursor of thrombokinase is adsorbed on barium sulfate, along with prothrombin. Much of the adsorbed prothrombin is eluted by 0.1 M phosphate, pH 6.6, and discarded. Then, more protein is eluted by 0.4 M phosphate, pH 6.6. From this second eluate, protein precipitated by ammonium sulfate between 0.4 and 0.5 saturation is concentrated and allowed to activate "spontaneously" at 4°C. The activated concentrate is stored at −23°C. At this point the yield of protein is now 0.032

TABLE I

LATER STEPS IN THE PREPARATION OF THROMBOKINASE FROM 212 LITERS OF OXALATED PLASMA

Product	Volume	Yield of activity	Yield of protein	Purification index
	ml	*per cent of product 5*	*per cent of product 5*	*activity/protein*
5. Activated concentrate	220	100	100	1.0 *x*
6. Redissolved isoelectric precipitate	212	76	10.2	7.5 *x*
7. Chromatographic fraction	101	55	3.2	17.2 *x*
8. Electrophoretic fraction	49	29	0.89	32.6 *x*

x, the purification index of product 5 with respect to plasma. See text.
Each numerical entry is an average value derived from at least three preparations.

per cent. Because of the difficulty of comparing activated product with inactive precursor, the yield of clotting factor cannot yet be stated; and the purification index at this point is designated as x, where x would be obtained by dividing yield of clotting factor by yield of protein. If the yield of clotting factor were 10 per cent, x would be $\frac{10.000}{0.032} = 310$. If the yield were 1 per cent, x would be 31.

Once thrombokinase is fully activated, it becomes possible at each subsequent step to determine the yield of activity in relation to product 5, as shown in Table I. At the next step the isoelectric precipitate contains 76 per cent of the activity, but only 10.2 per cent of the protein. Among the proteins removed in the supernate are those which would otherwise be hard to separate from thrombokinase in the ensuing chromatographic step.

A column 19 mm in diameter and about 27 cm high is prepared with a suspension of 6 gm DEAE-cellulose, and operated at 4°C. The redissolved isoelectric precipitate from ten batches (212 liters) of oxalated plasma is run into the column at the rate of 110 ml per hour and is followed by 260 ml 0.12 M phosphate, pH 8.0, at the same rate. The effluent is set aside. Then, 0.40 M phosphate, pH 8.0, is introduced in three portions of 40 ml, 10 ml, and 100 ml, respectively; and the effluent is collected in three corresponding fractions. The last fraction usually contains the entire yield of thrombokinase; and it is stored at −23°C. One or two such batches are fractionated electrophoretically.

To each 100 ml of chromatographic fraction, 233 ml saturated ammonium sulfate is added, to reach 0.7 saturation. The precipitate is collected by centrifugation and dissolved in 10 ml. 0.4 M phosphate, pH 8.0. The turbid, amber solution is dialyzed for 36 to 41 hours at 4°C against veronal $\Gamma/2 = 0.02$, pH 8.6. The dialyzed concentrate is transferred to the 30 mm sample vessel of a Spinco model CP continuous flow paper electrophoresis cell. The cell is operated in a refrigerator at 5–6°C. The instrument settings are: reservoir 4.2; left wick siphon 8.4; right wick siphon 9.9. This results in a "tilt to the left;" *i.e.*, the entire pattern is pushed toward the cathode while migration occurs toward the anode. The sample feed rate scale is set at 1.20 to deliver about 0.46 ml per hour to the extreme left tab near the top of the curtain. The electrolyte is veronal $\Gamma/2 = 0.02$, pH 8.6; and it is stored in tubs within the refrigerator. Care is taken to maintain constant temperature; and the tubs are replenished with precooled buffer.

First, the buffer is allowed to flow for several hours. Then, the electric current is turned on and the entire system allowed to stabilize overnight. The next morning, the sample is started down the curtain; and the collection of fractions is begun 3 to 4 hours later. Under these conditions, and with a constant current of 50 ma, the voltage is kept within a narrow range, *e.g.* 780 to 795. In one such run, the three best fractions were collected in tubes 19, 20, and 21 of the set of 32 tubes. Protein impurities occurred, in low concentration, on both sides of the peak fractions.

The three best fractions of a run, taken together, constitute the "electrophoretic fraction" of this paper. Its purification index is twice as high as that of the chromatographic fraction. This difference is greater than would have been expected from results obtained 2 or 3 years ago, and may reflect a lower quality of present chromatographic fractions.

The use of a frozen working standard has been helpful in comparing thrombokinase activity of different preparations from month to month; and this was the basis for the activity data of Table I. However, the working standard has not been sufficiently stable at −18 to −23°C to be dependable over a period of years. For this purpose the TAMe esterase assay has been more reliable, provided that comparisons were made after removal of thrombin; *i.e.*, on products 7 and 8.

Values for TAMe esterase units per milligram of the present electrophoretic fractions have usually been in the same range as the best values reported

in 1960 and since, namely between 296 and 359, but sometimes a little higher. If product 5 represents a yield of clotting factor between 1 and 10 per cent, then the value of x in Table I is between 31 and 310, and the purification index of product 8 is between 1,000 and 10,000. In recent preparations, product 8 has yielded 0.2 mg per liter of oxalated plasma.

Enzymatic Tests

When he introduced the term, thrombokinase, in 1904, Morawitz remarked that it behaved like an enzyme in many respects (16). Especially since the first activation experiments with crystallized trypsin (17), it has been anticipated that the activator of prothrombin would be a trypsin-like enzyme. However, reports from this laboratory (18, 3) have indicated that the proteolytic activity of thrombokinase would, at most, be slight, whether measured by fibrinolysis or by caseinolysis.

On the other hand, TAMe esterase assays had revealed that thrombokinase definitely possessed this enzymatic activity in addition to its prothrombin-activating property. It then became of interest to inquire what the caseinolytic activity might be in relation to the esterase activity. This is shown in Fig. 1, where given amounts of enzyme, as measured by esterase activity, have been incubated with casein to determine their proteolytic activity.

As seen from Fig. 1, trypsin had more than 7 times as much caseinolytic effect as product 6 when both preparations were used at concentrations near 100 esterase units per ml; and it had more than 25 times as much as product 7. The low level of caseinolytic activity remained about the same for product 8. Other tests disclosed that trypsin had more than 7 times as much esterase activity per mg as product 8. Hence trypsin had at least $25 \times 7 = 175$ times as much caseinolytic activity per mg as did thrombokinase at step 8.

The small amount of proteolytic activity remaining at steps 7 and 8 may well reflect a property of the thrombokinase molecule. However, it remains to be proven that even this is not due to a minute amount of impurity. Meanwhile it is concluded that the caseinolytic activity of thrombokinase is less than $\frac{1}{175}$ that of crystallized trypsin.

Stability in Relation to pH

At different points along the course of purification, brief tests had shown that thrombokinase was most stable in slightly alkaline solution. The concentrate was allowed to activate at pH 8.8 (4); the redissolved isoelectric precipitate was stored at pH 7.7 – 7.8, chromatography was performed near pH 8.0 (2), and electrophoretic fractionation at pH 8.6 (1).

The chromatographic fraction, product 7 of Table I, was found to be most stable near pH 8.0, but a second peak of fair stability occurred near pH 1.6. This second peak was in evidence when activity was measured as TAMe

esterase as well as when it was estimated by thrombin production in the working assay.

The electrophoretic fraction, product 8 of Table I, showed a similar pH stability pattern.

Portions of electrophoretic fraction, 0.5 ml each, were mixed with 0.5 ml of 0.4 M NaCl, then with enough distilled water plus HCl or NaOH solution to make the total volume 2.0 ml. The concentration of protein was 0.34 mg/ml; and NaCl was 0.1 M. The pH value plotted in Fig. 2 is the glass electrode reading, (corrected when necessary for sodium ion effect), of a sample taken immediately after mixing. The mixture was incubated at the specified pH for 2 hours at 37°C. Then a sample was immediately diluted 1/250 in buffered albumin, which brought the pH into the range, 7.0 to 7.4. This, or a further dilution was assayed for thrombokinase activity,

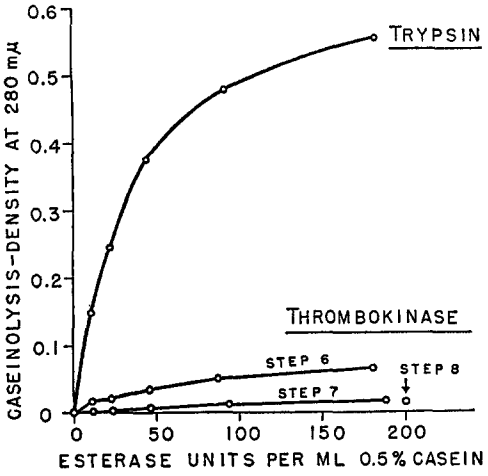

FIGURE 1. Low caseinolytic activity of thrombokinase as compared with trypsin. Each curve shows the caseinolysis produced by different amounts of enzyme preparation. The amount of added enzyme is indicated in TAMe esterase units. Steps 6, 7, and 8 refer to Table I.

in comparison with dilutions made directly from the electrophoretic fraction into buffered albumin at the beginning of the experiment, and stored at 5°C.

The results are shown in Fig. 2. Thrombokinase was most stable between pH 7.5 and pH 9.5; but there was again a peak near pH 1.85. The mixtures were cloudy from pH 5.68 down through pH 3.99. The mixture at pH 2.92 was quite clear; nevertheless it lost almost all its activity. All the more acid mixtures were clear, except the one at pH 0.62, which was again cloudy.

The peak near pH 1.85 invites further investigation. The experiment has been repeated several times. When tested, the pH has usually been about the same at the end of the 2 hour incubation as at the beginning. The same general pattern appeared when thrombokinase was only $\frac{1}{25}$ as concentrated, at 0.014 mg/ml, and all mixtures were clear in the gross. The loss of activity near pH 4 was not instantaneous; it was not necessary to pass

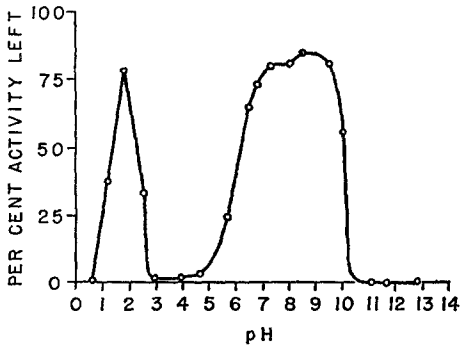

FIGURE 2. Stability of thrombokinase in relation to pH.

through this range very quickly in order to demonstrate the greater stability near pH 1.8. And near pH 5, the decrease was slow enough to permit isoelectric precipitation to occur for 1 hour at 4–6°C without noticeable loss of activity.

The foregoing results, although unusual, are not unique in every respect. Under special conditions, trypsin shows a secondary rise in stability near

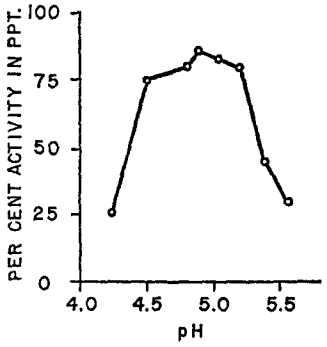

FIGURE 3 *a*

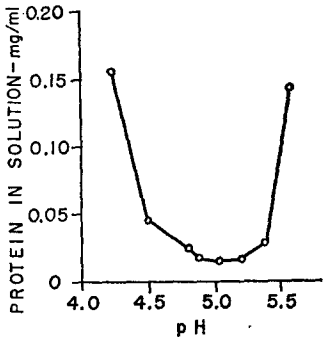

FIGURE 3 *b*

FIGURE 3. Isoelectric precipitation of thrombokinase. (*a*) Activity recovered in precipitate. (*b*) Protein left in solution.

pH 13 (19). Various degrees of stability in the region of pH 2 to pH 3 have been reported for trypsin (19), ribonuclease (20), lysozyme (21), and plasminogen (22, 23).

Thrombokinase as Euglobulin

The isolectric precipitation of partially purified thrombokinase at step 6 apparently depends on the properties of thrombokinase, *per se*; for a similar precipitation can be performed with thrombokinase isolated electrophoret-

ically (1). And it has often been convenient to use this method to concentrate thrombokinase from electrophoretic fractions.

For the data of Figs. 3 *a* and 3 *b*, 1 ml of electrophoretic fraction was diluted to 3 ml with cold distilled water plus 0.1 M acetic acid in varying proportions. The mixture was allowed to stand for 30 minutes in a centrifuge at 4°C, and then centrifuged at 4°C for 30 minutes. The supernate was decanted; and its pH and protein content were determined. The precipitate was dissolved in 1 ml veronal $\Gamma/2 = 0.02$, pH 8.6; and its thrombokinase was assayed in comparison with the electrophoretic fraction.

Fig. 3 *a* shows that most thrombokinase was recovered in precipitates formed at pH 4.8 to 5.2; and Fig. 3 *b* shows that the protein was at its minimum solubility between pH 4.9 and 5.2. Further experiments revealed that isoelectric precipitation of thrombokinase would give fair yield with concentration of sodium chloride as high as 0.05 M. In a series of precipitations with acetate buffer, pH 4.8, the solubility of the protein increased sharply when the molarity of the buffer rose above 0.5. Essentially similar, though quantitatively different, results were obtained at pH 5.0 to 5.1. Thus, thrombokinase could be precipitated, or contrariwise, "salted in," in the manner typical of a euglobulin.

Ultracentrifugal Patterns

Thrombokinase, as isolated by repeated electrophoretic fractionations, made a close approach to electrophoretic homogeneity at pH 8.6 (1). The present electrophoretic fraction gave a single, grossly symmetrical boundary in the analytical ultracentrifuge, as illustrated in Fig. 4.

37 ml. of electrophoretic fraction was mixed with 37 ml cold distilled water and 20 ml 0.1 M acetic acid, which brought the pH to 4.8 as judged by brom cresol green on a spot plate. After 30 minutes at 4°C, the precipitate was collected by centrifugation for 30 minutes at 4°C and dissolved in 2.0 ml 0.4 M phosphate, pH 7.98. The solution was dialyzed at 4°C against sodium chloride, $\Gamma/2 = 0.18$: phosphate $\Gamma/2 = 0.02$, pH 8.0. Ultracentrifugal runs were made with the undiluted solution, and also with dilutions of the protein solution in the equilibrated buffer. Temperature, which was maintained constant for each run, was 18.6°C during the run of Fig. 4. With thrombokinase varying between 0.20 and 0.82 mg/ml, sedimentation was faster with higher concentrations of protein, which makes it necessary to consider the possibility of association-dissociation phenomena.

No conclusions with regard to ultracentrifugal homogeneity are yet drawn. However, the single, grossly symmetrical boundaries were observed repeatedly.

Action of Thrombokinase in Presence of EDTA

Evidence that thrombokinase can function without ionic calcium was presented many years ago (13, 24). Nevertheless, it was desirable to test thrombokinase in the presence of EDTA, because of the latter's capacity to form unusually stable complexes with calcium (25) and other ions (26). It has been used as an anticoagulant (27–31), and the amount added has been

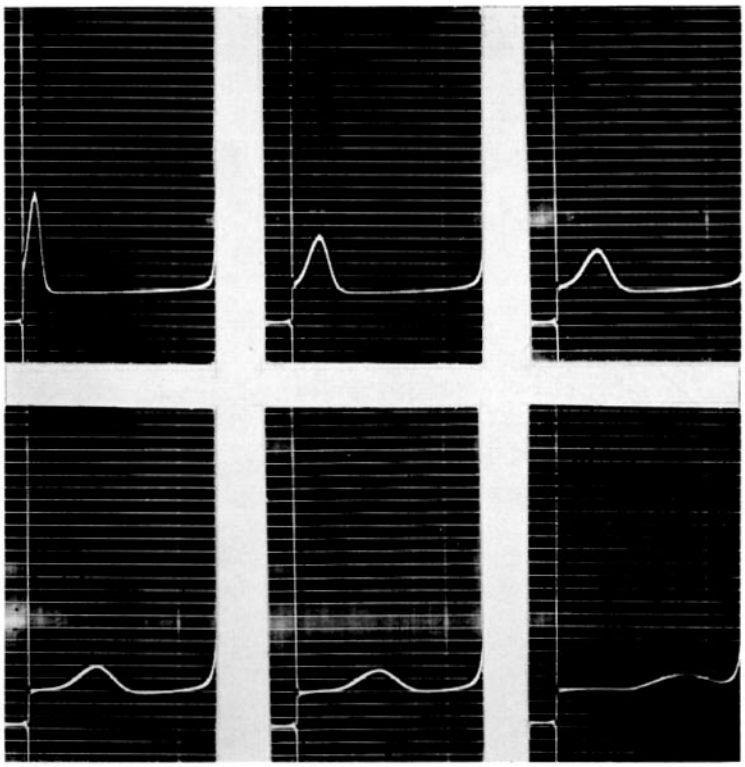

FIGURE 4. Ultracentrifuge patterns of thrombokinase. Concentration of protein 0.82 per cent. Angle of phaseplate, 70°. Photographs at 11, 28, 43, 73, 88, and 136 minutes after rotor reached speed of 59,780 RPM. Direction of sedimentation is to the right.

0.5 to 1.5 mg of disodium salt per ml of whole blood. The present studies used 0.01 M EDTA, or 3.72 mg of the dihydrated disodium salt per ml of prothrombin activation mixture.

It was verified that 0.01 M EDTA suppressed production of thrombin in the system used for the working assay of thrombokinase. For the present test, the mixture contained: 0.5 ml prothrombin (34 units); 0.1 ml inosithin (100 μg); 0.1 ml "accelerator" (containing 3.3 μl treated bovine serum); 0.1 ml buffer or 0.1 ml of 0.1 M EDTA;

0.1 ml of 0.025 M calcium chloride; and 0.1 ml of thrombokinase (0.003 μg). Under these conditions the accessory factors were necessary for rapid production of thrombin; and EDTA exerted a profound inhibition. After 10 minutes of activation, a sample of the control test produced a test clot in 25 seconds. A sample from the test with EDTA failed to clot fibrinogen in 1 hour.

On the other hand, when enough thrombokinase was used to convert prothrombin without the help of accessory factors, addition of EDTA did not suppress production of thrombin. However, when EDTA was 0.01 M in the activation mixture, it was still strong enough, after a sample was mixed with 3 parts of fibrinogen, to retard coagulation of the fibrinogen by thrombin. This effect has long been known (27). In the present control experiments, EDTA retarded the clotting of fibrinogen more than an equimolar concentration of oxalate and more than 10 times its molar concentration of added sodium chloride. Because of this retardation, a special empirical chart would have been required to convert clotting times to NIH units of thrombin.

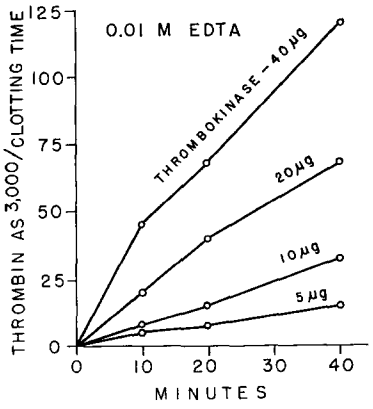

FIGURE 5. Activation of prothrombin by thrombokinase in the presence of 0.01 M EDTA. Relative thrombin values were obtained by dividing 3,000 by the clotting time, so that a 30 second clotting time would give a relative thrombin value of 100.

To avoid this, the results of Fig. 5 are presented in a form based directly on clotting time. Each test contained: 0.5 ml prothrombin (300 units); 0.3 ml buffer; 0.1 ml of 0.1 M EDTA; 0.1 ml of peak electrophoretic fraction of thrombokinase, (5 to 40 μg), diluted with buffer.

As seen in Fig. 5, thrombokinase activated prothrombin in the presence of 0.01 M EDTA; and the rate depended directly on the amount of thrombokinase. It is concluded that thrombokinase can function without the help of calcium ions, or of other ions which are tightly bound by EDTA.

DISCUSSION

The thrombokinase prepared from bovine plasma in 1951 (13) had been purified from a barium sulfate eluate; and almost all thrombin had been removed. This made it possible to outline basic relationships.

At each subsequent occasion, results have been in accord with the conclusions reached in 1951: that the action of thrombokinase was not entirely

dependent on ionic calcium; that platelets or brain lipid functioned as accessory factors in the production of thrombin; and that it was these accessory factors which depended on ionic calcium. Further studies (32, 33) made it necessary to take into account the *possibility* of an additional type of accessory factor, that represented by the accelerator of bovine barium carbonate–treated serum (34–39). Since then, a five reagent test system has been used:

Primary factors	*Accessory factors*
Prothrombin, thrombokinase	Ca^{++}, phospholipid, accelerator

With the other four reagents constant, the rate of thrombin production is determined by the amount of added thrombokinase; and this is the basis of the working assay. As previously considered (33), the accelerator reagent might function as an accessory for thrombokinase, or possibly as a potential source of more thrombokinase.

Recently, Marciniak and Seegers (40) described their autoprothrombin C; and they acknowledged that it may well be the same as the thrombokinase previously described at this laboratory. It is measured in a similar five reagent system and is reported to catalyze activation of prothrombin in the presence of citrate. However, they believe it is a derivative of prothrombin.

Esnouf and Williams (41) have reported that their activated factor X has an esterase activity which is reminiscent of thrombokinase as defined in this laboratory. They further comment on the similarity of purification procedures, behavior on DEAE-cellulose, and the observed autoactivation.

Macfarlane (42) has suggested that factor X can be activated by Russell's viper venom. His results indicate that the active product, with phospholipid and factor V, causes rapid prothrombin conversion. On the basis of studies with deficient human plasmas, Ratnoff (43) has tentatively considered a series of reactions which ends with the formation of "active plasma thromboplastin" by factor X, platelets, and factor V.

The possibility that factor X is identical with thrombokinase or its precursor requires serious consideration, and careful scrutiny. It may be recalled that factor V was once considered to resemble the precursor of thrombokinase (18), at the time when factor V was said to give rise to factor VI, which then activated prothrombin (35). It subsequently became clear, however, that this was not to be the defining property of factor V (44). In the case of SPCA (factor VII), there was opportunity for direct cross-testing of materials; and Alexander outlined the differences as well as the similarities found (45). Although thrombokinase is defined by its activation of prothrombin, it will

be desirable ultimately to find its equivalent in the clinically oriented system of numbered clotting factors.

Even when all the tests are performed in the same laboratory it is sometimes difficult to establish the identity of factors defined or measured in different ways. Microgram quantities of thrombokinase were used in this study to activate prothrombin in the presence of EDTA. But nanogram (10^{-9} gm) quantities were used in the potentially less specific five reagent system. Is it actually the same factor that is being measured in both systems?

In an earlier electrophoretic analysis of partially purified thrombokinase, each fraction was assayed for thrombokinase in the presence of oxalate and also in the five reagent system. The two assays gave closely parallel activity curves (46). The purest preparations have been active in both systems and to a parallel extent. Accumulating evidence indicates that a single factor, thrombokinase, has been isolated, and has been measured in different ways.

Much of the technical work was done by Harriet F. Brown, William S. Bartek, and Thomas R. Saxton.

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REFERENCES

1. MILSTONE, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1960, **103**, 361.
2. MILSTONE, J. H., *Nature*, 1960, **187**, 1127.
3. MILSTONE, J. H., *J. Gen. Physiol.*, 1962, **45**, No. 4, pt. 2, 103.
4. MILSTONE, J. H., *J. Gen. Physiol.*, 1959, **42**, 665.
5. WHITE, S. G., LAGEN, J. B., AGGELER, P. M., and GEYER, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1953, **83**, 384.
6. NEWLANDS, M. J., and WILD, F., *Nature*, 1955, **176**, 885.
7. SPAET, T. H., AND KROPATKIN, M., *Proc. Soc. Exp. Biol. and Med.*, 1957, **95**, 492.
8. SCHULMAN, I., CURRIMBHOY, Z., FORT, E., ERLANDSON, M. E., SCHORR, J. B., and SMITH, C. H., *Am. J. Dis. Child.*, 1957, **94**, 561.
9. CONNOR, W. E., and CARTER, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 38.
10. OWEN, C. A., JR., and THOMPSON, J. H., JR., *Am. J. Clin. Path.*, 1960, **33**, 197.
11. HYUN, B. H., DAWSON, E. A., BUTCHER, J., and CUSTER, R. P., *Am. J. Clin. Path.*, 1960, **33**, 209.
12. SHERRY, S., and TROLL, W., *J. Biol. Chem.*, 1954, **208**, 95.
13. MILSTONE, J. H., *J. Gen. Physiol.*, 1951, **35**, 67.
14. KUNITZ, M., *J. Gen. Physiol.*, 1947, **30**, 291.
15. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.

16. MORAWITZ, P., *Beitr. chem. Physiol. u. Path.*, 1904, **5**, 133.
17. EAGLE, H., and HARRIS, T. N., *J. Gen. Physiol.*, 1937, **20**, 543.
18. MILSTONE, J. H., *Science*, 1947, **106**, 546.
19. KUNITZ, M., AND NORTHROP, J. H., *J. Gen. Physiol.*, 1934, **17**, 591.
20. KUNITZ, M., *J. Gen. Physiol.*, 1940, **24**, 15.
21. ALDERTON, G., WARD, W. H., and FEVOLD, H. L., *J. Biol. Chem.*, 1945, **157**, 43.
22. CHRISTENSEN, L. R., and SMITH, D. H., JR., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74**, 840.
23. KLINE, D. L., *J. Biol. Chem.*, 1953, **204**, 949.
24. MILSTONE, J. H., *Yale J. Biol. and Med.*, 1952, **25**, 19.
25. SCHWARZENBACH, G., and ACKERMANN, H., *Helv. Chim. Acta*, 1948, **31**, 1029.
26. SCHWARZENBACH, G., and FREITAG, E., *Helv. Chim. Acta*, 1951, **34**, 1503.
27. DYCKERHOFF, H., MARX, R., AND LUDWIG, B., *Z. ges. exp. Med.*, 1942, **110**, 412.
28. PROESCHER, F., *Proc. Soc. Exp. Biol. and Med.*, 1951, **76**, 619.
29. HADLEY, G. G., and LARSON, N. L., *Am. J. Clin. Path.*, 1953, **23**, 613.
30. LEIKIN, S., and BESSMAN, S. P., *Blood*, 1956, **11**, 916.
31. DIDISHEIM, P., *Science*, 1959, **129**, 389.
32. MILSTONE, J. H., *Yale J. Biol. and Med.*, 1952, **25**, 173.
33. MILSTONE, J. H., *J. Gen. Physiol.*, 1955, **38**, 757.
34. OWREN, P. A., *Proc. Norweg. Acad. Sc.*, 1944, 21.
35. OWREN, P. A., *Acta Med. Scand.*, 1947, suppl. 194.
36. FANTL, P., and NANCE, M. H., *Nature*, 1946, **158**, 708.
37. FANTL, P., and NANCE, M. H., *Med. J. Australia*, 1948, **1**, 128.
38. MURPHY, R. C., WARE, A. G., and SEEGERS, W. H., *Am. J. Physiol.*, 1947, **151**, 338.
39. WARE, A. G., and SEEGERS, W. H., *Am. J. Clin. Path.*, 1949, **19**, 471.
40. MARCINIAK, E., and SEEGERS, W. H., *Canad. J. Biochem. and Physiol.*, 1962, **40**, 597.
41. ESNOUF, M. P., and WILLIAMS, W. J., *Thromb. Diath. Haemorrhag.*, 1961–62, **7**, suppl. 1, 213.
42. MACFARLANE, R. G., *Brit. J. Haematol.*, 1961, **7**, 496.
43. RATNOFF, O. D., and DAVIE, E. W., *Biochemistry*, 1962, **1**, 677.
44. OWREN, P. A., *Thromb. Diath. Haemorrhag.*, 1960–61, **6**, suppl. 1, 387.
45. ALEXANDER, B., *New England J. Med.*, 1955, **252**, 484.
46. MILSTONE, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1959, **101**, 660.