PREPARATION OF THROMBOKINASE FROM BOVINE PLASMA*

By J. H. MILSTONE, M.D.

(From the Department of Pathology, Yale University School of Medicine, New Haven)

(Received for publication, October 14, 1958)

ABSTRACT

Thrombokinase is prepared from bovine plasma by a procedure involving: treatment with diatomaceous silica, adsorption on barium sulfate, flowing elution with two successive phosphate buffers, ammonium sulfate fractionation, "spontaneous" activation in concentrated solution, and isoelectric precipitation. The yield of nitrogen is 0.002 per cent, corresponding to 1.2 mg. protein per liter of plasma.

When diluted back to the volume of parent plasma, and complemented by calcium plus cephalin, the product causes appreciable activation of prothrombin in 1 minute. Thus, the quantity of thrombokinase obtainable is compatible with a physiologic role. In the more complex system used for routine assay, thrombokinase can be supplied by crude plasma at a dilution of 1/500. In parallel tests, the product appears to be more active than its parent plasma, although it contains only 0.002 per cent of the nitrogen. However, the thrombokinase of the product has been activated, whereas the thrombokinase of the plasma is probably in an inactive precursor state.

When diluted back to the volume of parent plasma, to a concentration of 0.2 microgram nitrogen per ml., thrombokinase can slowly activate prothrombin in the presence of oxalate, and without the addition of accessory factors. Activation of prothrombin in the presence of oxalate is faster with higher concentrations of thrombokinase.

According to the view developed in this laboratory, thrombokinase is an enzyme which arises from a plasma precursor during the coagulation of blood. Thrombokinase converts prothrombin to thrombin; and the rate of thrombin production is accelerated by platelets plus calcium ions. Prior statements of these views from other laboratories have been acknowledged elsewhere (1, 2).

In the past, thrombokinase has been prepared from plasma as a crude material in an exploratory study (3), and later as a concentrated by-product obtained during the preparation of prothrombin (4). The amount of thromboki-

* This investigation was supported by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-87.

nase obtainable made it possible to outline basic relationships; but it was not enough for more extensive work. The present paper describes a method for preparing concentrated thrombokinase from 21-liter batches of plasma. The concentrate is one-thousandth the volume of the parent plasma and it contains 0.002 per cent of the nitrogen; yet its activity can be demonstrated in thousand- and millionfold dilutions.

Concentrated thrombokinase can activate prothrombin in the presence of excess oxalate (4). This is a property of considerable theoretical interest. It shows that thrombokinase can function without the aid of such accessory factors as platelets or cephalin, which require ionic calcium. Thus it helps to single out thrombokinase as the prime activator of prothrombin.

Despite its theoretical interest, this effect has not been widely observed in recent years. Therefore it has seemed worth while to outline stepwise the conditions necessary to elicit this property. These conditions are not quite as stringent as heretofore thought. If the thrombokinase is sufficiently active and sufficiently pure, it is not absolutely necessary that it be tested at high concentration. It is of particular interest that the present preparations are detectable in the presence of oxalate when they are diluted back to the volume of the parent plasma.

EXPERIMENTAL

Methods and Materials

Coagulation Tests.—Essentially as described (3). All tests are performed in a water bath at 28°C. Each batch of fibrinogen is tested with a working standard of glycerinized thrombin (5), which in turn has been standardized against dry thrombin received from the National Institutes of Health through the courtesy of Dr. W. G. Workman. The batch which was used for the tests herein illustrated clotted in 37 seconds after addition of 0.1 ml. thrombin, containing 0.5 NIH unit, to 0.3 ml. of the fibrinogen.[1] Thrombin unitage is estimated on this basis for Figs. 1 and 2.

Prothrombin.—Prepared by a modification of methods previously outlined (2, 4). For the assay of prothrombin, it is activated in dilute state in a five reagent system (2) and tested against standardized fibrinogen. One unit of prothrombin is taken as that amount which produces 1 NIH unit of thrombin.

Buffer for Tests.—Veronal-buffered saline, pH 7.4 (4).

Cephalin for Tests.—Prepared from bovine brain, by the method of Howell (6). 0.1 per cent suspension in buffer used for tests.

[1] The fibrinogen used in this laboratory has no added acceleratory agent such as acacia; and the end-point is the formation of a solid, invertible clot. Consequently the clotting time observed with a given amount of thrombin is considerably longer than it is when the fibrinogen has an added acceleratory agent and when the end-point is the first appearance of fibrin. The method by which the fibrinogen is prepared may also have an effect. For several reasons, including the subjective nature of some end-points, it is desirable to assay thrombin in terms of a stable standard.

"Accelerator."—Bovine serum, after removal of components adsorbed by 40 mg. barium carbonate per ml. 1/60 dilution in buffer used for tests.

Buffered Albumin.—Crystallized bovine plasma albumin, Armour and Co., Chicago. Dialyzed at least 3 days against veronal-buffered saline. 0.1 per cent solution used for tests.

Oxalated Bovine Plasma.—Obtained from Swift and Co., Chicago, through Sperry and Barnes, Co., New Haven. The writer is indebted to Dr. W. H. Seegers for advising him that this material is commercially available. Judged from a letter from Swift and Co., the anticoagulant is similar to that of Seegers, Loomis, and Vandenbelt (7); and the final concentration of oxalate is within the range 0.006 to 0.015 M. The plasma is received in 50 pound quantities, frozen in wooden kegs or metal cans.

Hyflo.—Hyflo super-cel, a grade of diatomaceous silica supplied by Johns-Manville Corporation, New York.

E and D Filter Paper.—The Eaton-Dikeman Company, Mt. Holly Springs, Pennsylvania.

Barium Sulfate.—U.S.P., "Suitable for x-ray diagnosis," Merck and Co., Rahway, New Jersey.

Phosphate-Buffered Sodium Chloride, pH 6.7.—

4 M NaCl	150 ml.
0.1 M KH_2PO_4	300 "
0.1 N NaOH	144 "
Distilled water	to 6 liters

0.1 M Phosphate, pH 6.6.—

Na_2HPO_4	34.1 gm.
KH_2PO_4	49.0 "
Distilled water	to 6.0 liters

0.4 M Phosphate, pH 6.6.—

Na_2HPO_4	170 gm.
KH_2PO_4	163 "
Distilled water	to 6 liters

0.4 SAS. Phosphate.—

0.4 M phosphate, pH 6.6	1 liter
$(NH_4)_2SO_4$	242 gm.

0.02 M Acetate, pH 5.2.—

$CH_3COONa \cdot 3H_2O$	12.520 gm.
4 M CH_3COOH	7.0 ml.
Distilled water	to 6.0 liters

Kjeldahl-Nitrogen (Non-Dialyzable).—Prior to analysis, a given volume of each fraction, or of diluted fraction, is dialyzed against cold 0.4 M phosphate, pH 6.6, and diluted to a suitable multiple of the given volume. A sample of appropriate size is digested and distilled by the micromethod of Hiller, Plazin, and Van Slyke (8). The distillate is collected and titrated according to Kunitz (9).

The Working Assay

Although thrombokinase, by itself, can activate prothrombin, it is potentiated by calcium and cephalin; and the effect is further augmented by an

"accelerator" reagent prepared from bovine serum. In this complex system, minute quantities of thrombokinase can be detected; and when the remainder of the system is constant, the rate of thrombin production is determined by the amount of thrombokinase (2).

For present purposes, the thrombin-producing system is composed as follows: 0.5 ml. prothrombin (34 units), 0.1 ml. cephalin, 0.2 ml. "accelerator," 0.1 ml. calcium chloride, and 0.1 ml. of the solution to be assayed for thrombokinase. Plasma or plasma derivatives are diluted with buffered albumin before they are tested as a source of thrombokinase. In contrast to the previously described

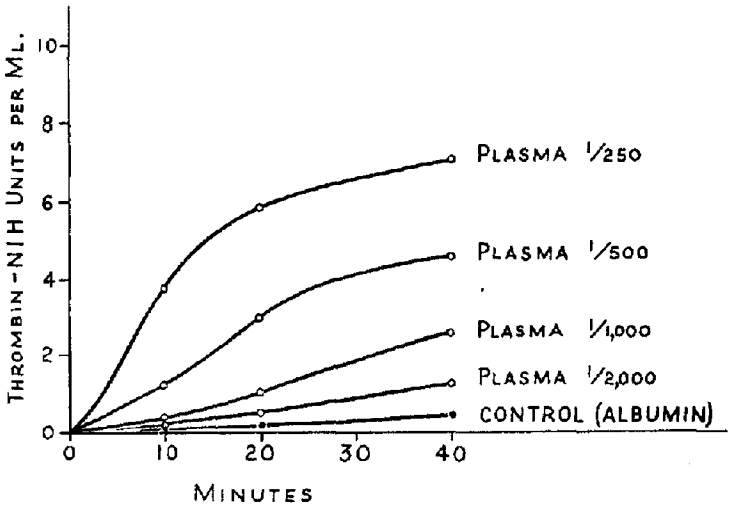

FIG. 1. Thrombin production in a system containing prothrombin, cephalin, "accelerator," and calcium, with varying dilutions of plasma as a source of thrombokinase.

"accelerator" reagent (2), the present one has not been passed through a column of diatomaceous earth; and the controls without separately added thrombokinase show some thrombin production, as illustrated in Fig. 1. Within limits, the production of thrombin becomes increasingly faster as increasing amounts of plasma are included. In Fig. 1, it is seen that the assay system can respond to plasma at a final dilution of 1/2,000.

The test system makes no special allowance for the strong probability that thrombokinase exists in plasma in the form of an inactive precursor. The activation of this precursor during the tests probably contributes to the S shape of the curves, which is perceptible in Fig. 1 and more pronounced in the plasma test of Fig. 2. Even with five reagents in its first stage, the system may not control all significant variables. Consequently, the test might well respond to factors other than thrombokinase or its precursor. A three stage test would remove some of these limitations, but not all.

The present system does have the advantage of assaying a thrombokinase preparation at such high dilution as to minimize the effect of contaminants in the preparation. And notwithstanding its limitations, the assay is a useful guide to purification. Moreover, it leads to concentrated thrombokinase preparations which can be identified in a more specific manner.

Preparation of Thrombokinase

1. Oxalated Plasma.—A coil of clean, bright copper tubing is arranged so that tap water runs into it at 37–39°C., and out of it *via* rubber tubing into the sink. The coil is placed on the surface of the frozen plasma and pressed down as thawing progresses. The 50 pound mass is completely thawed in 80 to 120 minutes; and then cold water is run through the coil until the plasma is brought to 28°C. The pH of the plasma is 7.0.

TABLE I

Kjeldahl-Nitrogen at Successive Steps of the Procedure

Product	Volume	Nitrogen	Total nitrogen	Yield of nitrogen
	ml.	*mg./ml.*	*mg.*	*per cent*
1. Oxalated plasma	21,168	10.78	228,191	100.0
2. Hyflo filtrate and washings	22,970	9.71	223,039	97.7
3. Second eluate from $BaSO_4$	8,064	0.023	185	0.081
4. 0.4 to 0.5 SAS fraction	305	0.230	70.2	0.031
5. Activated concentrate	22	2.76	60.7	0.027
6. Redissolved isoelectric precipitate	21.2	0.191	4.05	0.0018

SAS, saturated ammonium sulfate.

The figures for volume and nitrogen are averages, taken from determinations on at least two preparations.

2. Treatment with Hyflo.—This clarifies the plasma and probably adsorbs some protein. In contrast to results with hyflo under other conditions (4, 10) very little thrombokinase is removed. The plasma is stirred for 10 minutes with 2.2 kg. hyflo and filtered on 4 Buchner funnels. Each funnel is prepared with a No. 615 E and D filter paper, 23 cm. in diameter, precoated with 20 gm. hyflo. The filter cakes are packed; and each is washed by drawing 1.5 liters of phosphate-buffered sodium chloride, pH 6.7, through it. The filtrate and washings are combined and stirred with 1.36 kg. sodium chloride. The pH is 7.4.

3. Adsorption on Barium Sulfate and Fractional Elution.—The salted filtrate is stirred with 0.44 kg. barium sulfate, continuously for 10 minutes. Now 0.3 kg. hyflo is added and stirred 1 minute. The slurry is filtered with strong suction on 4 Buchner funnels prepared as for the preceding filtration. The filtrate is discarded.

The filter cakes are now treated as flat chromatographic columns. Through each cake, 3 L. 0.1 M phosphate, pH 6.6, is drawn in 15 to 20 minutes. The operation is repeated with another 3 liter portion of 0.1 M phosphate. (A small portion of phosphate is allowed to remain on the cakes, if the procedure is interrupted for 15 to 60 minutes at this point). This process washes out unadsorbed protein and elutes

much of the adsorbed prothrombin, along with some of the desired material. All this is discarded.

Through each cake, 2 liters 0.4 M phosphate, pH 6.6 is drawn in 20 to 30 minutes, to yield the second eluate. Its volume is usually 7.9 to 8.3 liters.

4. Ammonium Sulfate Fractionation.—2 kg. ammonium sulfate is dissolved in the second eluate to bring it to 0.4 saturation. After 15 minutes, 80 gm. hyflo is added. The well mixed suspension is filtered on a Buchner funnel, prepared with a 23 cm. E and D No. 612 filter paper, precoated with 20 gm. hyflo. The filter cake is washed with 300 ml. 0.4 SAS:phosphate and discarded.

The volume of the combined filtrate and washings is usually 8.9 to 9.3 liters, and the pH is 6.0. The solution is brought to 0.5 saturation by addition of 0.59 kg. ammonium sulfate, stirred well and stored overnight at 4°C. The cold suspension is stirred with 32 gm. hyflo and filtered on a small Buchner funnel, prepared with a 9 cm. E and D No. 612 paper, precoated with 8 gm. hyflo. Although this is performed at the usual ambient room temperature, the last portion of the filtrate is about 10°C. The pH is 5.9. The packed cake is extracted at room temperature by drawing 300 ml. 0.4 M phosphate, pH 6.6 through it in 30 minutes.

This extract is the 0.4 to 0.5 SAS fraction of Table I. Its pH is 6.2 and its volume about 305 ml. It is brought to a little more than 0.5 saturation by 99.5 gm. ammonium sulfate. After the well mixed suspension is allowed to stand for 15 to 30 minutes, the precipitate is collected by centrifugation.

5. Activation of the Concentrate.—The precipitate is dissolved by stages, in 5 ml. distilled water plus 5 ml. normal sodium hydroxide. The sodium hydroxide is cautiously added dropwise, 0.5 ml. at a step. At each step, care is taken to assure that enough protein is dissolved to prevent overalkalinization of the solution. After dilution to 22 ml. with distilled witer, the pH of the solution is 8.8. It is stored at 4°C. for 6 to 8 days.

In pilot studies, carried through from plasma to concentrate in 1 day, activity tests on the concentrates display S-shaped curves, suggesting that the thrombokinase is still in the precursor form. Such curves are not readily observable with a 2 day preparation; or with a 1 day preparation after aging. But, in all cases, the apparent thrombokinase activity, as estimated by the working assay, increases several fold in a few days at 4°C. The amount of thrombin also increases, and then diminishes toward the end of the week. The activated concentrate is stored at −23°C. At this temperature its activity is well preserved for months.

6. Isoelectric Precipitation.—The thawed concentrate is dialyzed in a rocking dialyzer (11) against distilled water at 3.5°C. for 4½ hours, and then diluted with 550 ml. cold 0.02 M acetate, pH 5.2. The very fine precipitate is collected by centrifugation at 4°C.

The supernate, with pH 5.2 and specific conductance 0.0014 to 0.0017 mho at 25°C., is discarded. The precipitate is dissolved in 20 ml. cold 0.05 M NaCl:0.02 M Na_2HPO_4, (pH 8.7–9.0) to give a solution at pH 7.8. The addition of 550 ml. cold 0.02 M acetate, pH 5.2, reprecipitates the protein, which is collected by centrifugation. The precipitate is dissolved in 0.05 M NaCl:0.02 M Na_2HPO_4 to give a final volume of 21.2 ml. A small amount of insoluble material is removed by centrifugation. The solution, at pH 7.7–7.8, is stored at −23°C.

The Activity of the Product in Relation to Its Physiologic Importance

It would be hard to consider that a plasma derivative was physiologically important as a clotting factor unless it could be shown that it was present in, or obtainable from, plasma in sufficiently large quantity to exercise its proposed function. The results of Fig. 1, with unfractionated plasma, suggest that the quantity of available thrombokinase is quite sufficient. But it is especially remarkable that the purified thrombokinase can exert an effect when diluted back to the original volume of plasma and a few thousand times further.

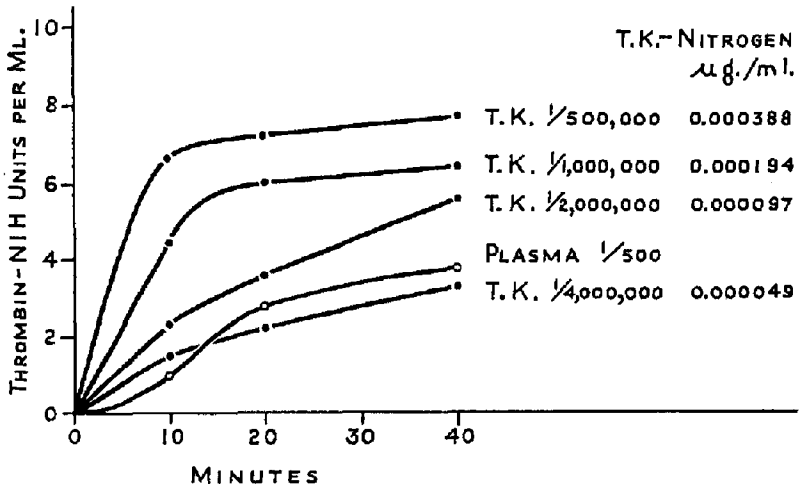

FIG. 2. In the working assay, the purified thrombokinase (*T.K.*), appears to be more active than its parent plasma. One ml. *T.K.* is derived from 1,000 ml. plasma.

Since product 6 of Table I occupies one-thousandth the original volume of plasma, it is back to plasma volume when diluted 1/1,000. Yet, as seen in Fig. 2, it exerts an effect in the working assay when tested in a final dilution of 1/4,000,000. Fig. 2 shows the effect of these dilutions when tested in comparison with their own parent plasma, a sample of which had been stored at −23°C. during the preparation. Although the oxalate of the plasma does not significantly interfere at 1/500 dilution, the dilutions of thrombokinase are made up to contain a little more added oxalate than the comparable dilutions of plasma, calculated on the basis of 0.02 M added oxalate in the 1/1,000 dilution of thrombokinase and 0.015 M oxalate in the undiluted plasma.

It is evident from Fig. 2 that a 1/500,000 dilution of thrombokinase is more active than a 1/500 dilution of its parent plasma. This would make the yield of activity appear to be more than 100 per cent, if the tests were strictly comparable. But they are not. Thrombokinase probably exists in the plasma in a precursor form, which needs to be activated during the course of the assay;

whereas product 6 contains thrombokinase which has already been activated. The difference is reflected in the different shapes of the curves in Fig. 2.

It is further remarkable that thrombokinase is detectable in minute amount, in terms of Kjeldahl-nitrogen. As seen in Fig. 2, product 6 displays conspicuous activity when its nitrogen concentration is in the range of 0.0001 microgram per ml. This extraordinary result provokes speculation, particularly since these ultraminute amounts are effective only in the presence of the "accelerator" reagent, and the contributions of the "accelerator" reagent are not well under-

TABLE II

Activity of Thrombokinase When Diluted back to Original Plasma Volume and Tested with 34 Units of Prothrombin per Ml.

Test No.	Mixtures						Period of Incubation						
	Prothrombin	Buffer	Cephalin	"Accelerator"	$CaCl_2$	Kinase	1 min.	2 min.	4 min.	8 min.	16 min.	32 min.	64 min.
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
1	0.5	—	0.1	0.2	0.1	0.1	30	21	19	19	17		
2	0.5	0.1	0.1	0.2	0.1	—			n.c.	n.c.	2,040	990	360
3	0.5	0.2	0.1	—	0.1	0.1	165	100	70	57	52		
4	0.5	0.3	0.1	—	0.1	—			n.c.	n.c.	n.c.	n.c.	n.c.
5	0.5	0.3	—	—	0.1	0.1			855	1,080	1,320	900	750
6	—	0.8	—	—	0.1	0.1			2,760	2,460	n.c.	3,120	n.c.

Prothrombin, stock solution containing 67.3 units per ml.

Kinase, product 6, (see Table I), diluted 1/100 in buffered albumin.

At the specified times, 0.1 ml. of mixture was added to 0.3 ml. oxalated fibrinogen. The resulting clotting times are given in the body of the table. A vacant place indicates that no sample was tested at the particular time.

n.c., no clot in 3600 seconds.

stood. It may further be recalled that thrombokinase can function autocatalytically; and thus it can be effective in minute amount in those systems which contain prothrombokinase (4).

Whatever the explanation might be, it is desirable to examine product 6 in further tests, some of which do not include the "accelerator" reagent. Table II shows what thrombokinase does when diluted 1/100, then ten times further, to original plasma volume. In these tests the thrombokinase-nitrogen is 0.194 microgram per ml. As seen in Test 1, it causes considerable activation of prothrombin within the 1st minute. In Test 2 thrombokinase has been omitted; and the production of thrombin is a few hundred times slower. That thrombin is produced at all in Test 2, indicates that some thrombokinase is available in the test system. This is no longer evident in Test 4, from which the "accelerator" reagent has been omitted. Nevertheless, Test 3 shows that thrombo-

kinase can still cause appreciable activation of prothrombin in the 1st minute, without the aid of the "accelerator" reagent.

When it is considered that much of the potential activity is discarded and otherwise lost during the preparation of thrombokinase from plasma, and when it is seen how rapidly the product can function when diluted back to plasma volume, there is little doubt that thrombokinase is obtainable from plasma in sufficient quantity to be of physiologic significance.

The Yield of Thrombokinase in Relation to Its Action in the Presence of Oxalate

Previous studies on this phenomenon have emphasized basic relationships (2, 4) and have shown that, if the thrombokinase were concentrated enough, it could activate prothrombin moderately fast in the presence of oxalate (12). The following experiments demonstrate this activity in terms of the amount obtainable from plasma. They further reveal that this effect may not be manifest if: (*a*) the thrombokinase is obscured by too much contaminating thrombin, (*b*) the incubation period is too short, (*c*) the concentration of substrate prothrombin is too low, (*d*) the concentration of thrombokinase is too low.

In the course of the preparation, the effect is not readily demonstrable with product 5, because there is too much thrombin. Most of that thrombin is removed in the next step, but enough remains in product 6 to confuse the issue, temporarily. With the system of Table II, the clue can easily be overlooked. Test 5 suggests that thrombokinase is activating the prothrombin and slowly outpacing a simultaneous loss of thrombin. But this is dubious, even though the amount of thrombin is large compared with that revealed by Test 6 to be present in the thrombokinase preparation.

In Table III, the kinase is again diluted back to plasma volume. But now the concentration of prothrombin is increased to 300 units per ml. Test 1 shows a slow, but now quite definite, production of thrombin. Test 2 shows a parallel production of thrombin when calcium is omitted; and Test 3 shows a comparable effect when oxalate is added. As previously demonstrated in similar experiments (12) the faster clotting times in Test 1 reflect the influence of calcium on the clotting of fibrinogen. They are not due to faster production of thrombin, in this particular system. (Calcium would cause a pronounced acceleration of thrombin production in a cephalin-containing system.) Test 4 again reveals the presence of thrombin in the kinase preparation. Test 5 is a control on the prothrombin reagent.

If now, the concentration of thrombokinase is increased tenfold, as in Table IV, the rate of thrombin production is correspondingly increased, in the presence of calcium, (Test 1), and in the presence of oxalate, (Test 3). Now, however, the thrombin which contaminates the kinase preparation becomes more conspicuous, as in Test 4; and another possibility presents itself. It might be supposed that it is not the kinase, but the contaminating thrombin, which

activates prothrombin in the presence of oxalate. It has previously been shown that this is not so (4), but it is desirable to repeat the demonstration with the new preparation of thrombokinase.

TABLE III

Activity of Thrombokinase When Diluted back to Original Plasma Volume and Tested with 300 Units of Prothrombin per Ml.

Test No.	Mixtures					Period of incubation				
	Pro-thrombin	Buffer	$CaCl_2$	Oxalate	Kinase	10 min.	20 min.	40 min.	80 min.	160 min.
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
1	0.5	0.3	0.1	—	0.1	770	640	430	225	120
2	0.5	0.4	—	—	0.1	2,100	1,740	1,000	560	250
3	0.5	0.3	—	0.1	0.1	2,640	1,980	1,120	680	280
4	—	0.8	0.1	—	0.1	3,240	2,940	3,240	3,600	n.c.
5	0.5	0.4	0.1	—	—	n.c.	n.c.	n.c.	n.c.	n.c.

Prothrombin, stock solution containing 600 units per ml.
Kinase, product 6, (see Table I), diluted 1/100 in buffered albumin.
$CaCl_2$, 0.025 M.
Oxalate, 0.1 M potassium oxalate.

TABLE IV

Activity of Thrombokinase When Diluted back to One-Tenth Original Plasma Volume

Test No.	Mixtures						Period of incubation			
	Pro-thrombin	Buffer	$CaCl_2$	Oxalate	Albumin	Kinase	10 min.	20 min.	40 min.	80 min.
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*sec.*	*sec.*	*sec.*	*sec.*
1	0.5	0.3	0.1	—	—	0.1	94	59	34	22
2	0.5	0.4	—	—	—	0.1	160	108	70	38
3	0.5	0.3	—	0.1	—	0.1	164	118	69	44
4	—	0.8	0.1	—	—	0.1	195	195	240	258
5	0.5	0.3	0.1	—	0.1	—	n.c.	n.c.	n.c.	n.c.

Prothrombin, stock solution containing 600 units per ml.
Kinase, product 6, (see Table I), diluted 1/10 in buffered albumin.
Oxalate, 0.1 M potassium oxalate.

To 18.8 ml. of product 6, 28.2 ml. saturated ammonium sulfate is added to 0.6 saturation. The precipitate is collected by centrifugation and dissolved in 20 ml. phosphate-buffered sodium chloride, pH 6.7. Insoluble material is removed by centrifugation, and 16.4 ml. saturated ammonium sulfate is added to 0.45 saturation. The precipitate is collected by centrifugation and dissolved in 20 ml. of phosphate-buffered sodium chloride solution. Precipitation at 0.45 saturation with ammonium sulfate is repeated six times. The seventh precipitate is dissolved in 5 ml. phosphate-buffered sodium chloride solution and dialyzed against veronal-buffered saline.

Before testing, it is diluted so that 1 ml. corresponds to 1 ml. of product 6. Much thrombokinase is discarded, or otherwise lost during this procedure.

In Test 1 of Table V, the reprecipitated thrombokinase is seen to activate prothrombin in the presence of oxalate at a faster rate than that of Test 3 in Table IV. Contrariwise, the thrombokinase of Table IV contains at least 15 times as much thrombin (Test 4) as that of Table V (Test 2). As seen in Test 2 of Table V, the thrombin has been reduced below the limits of the test.

In summary, the present preparation of thrombokinase can be diluted back to original plasma volume and still activate prothrombin in the presence of oxalate. Activation is faster with higher concentrations of thrombokinase, and does not depend on the presence of thrombin as a contaminant.

TABLE V

Activity of Thrombokinase after Further Removal of Thrombin

Test No.	Mixtures				Period of incubation			
	Prothrombin	Buffer	Oxalate	Kinase	5 min.	10 min.	20 min.	40 min.
	ml.	*ml.*	*ml.*	*ml.*	*sec.*	*sec.*	*sec.*	*sec.*
1	0.5	0.3	0.1	0.1	230	110	55	32
2	—	0.8	0.1	0.1	n.c.	n.c.	n.c.	n.c.

Prothrombin, stock solution containing 600 units per ml.

Oxalate, 0.1 M potassium oxalate.

Kinase, product 6 was precipitated once at 0.6 and seven times at 0.45 saturation with ammonium sulfate.

n.c., no clot in 3600 seconds.

DISCUSSION

The main purpose of the present work was to develop a method which would yield enough purified and concentrated thrombokinase for further study. The present product is neither final nor fully characterized. It is known to contain thrombin and may contain other factors. There is some evidence (3, 4) that thrombokinase is produced autocatalytically from a precursor. It is also advisable to contemplate a thrombokinase-inhibitor complex similar to that formed by trypsin and trypsin-inhibitor (13). How much of such inactive forms might occur in the present product is a matter for future inquiry.

The accumulating results have a pertinent relation to the "spontaneous" activation of prothrombin preparations. This phenomenon was observed, under various conditions, by Cekada in 1926 (14), by Mellanby in 1933 (15), by Mellanby and Pratt in 1940 (16), by the writer in 1942 (5), and by Ware and Seegers in 1948 (17). In the last three instances, it was reported that the "spontaneous" activation occurred in the presence of calcium-binding agents such as oxalate or citrate. It follows that, if such activation were not truly

spontaneous, but induced by a contaminating thrombokinase, then the thrombokinase must be capable of acting in the presence of oxalate or citrate. It further follows that if such a thrombokinase were to be found, a likely place would be those preparations in which "spontaneous" activation of prothrombin had occurred. Such preparations would, of course, also contain thrombin.

The foregoing theoretical consequences have been tested and found to be true. In 1949 (18), a "spontaneously" activated fraction was split into a "converter" subfraction and a thrombin subfraction. In 1951 (4), the "converter" was identified as thrombokinase and shown to activate prothrombin in the presence of oxalate. At step 5 of the present method, there is again a mixture of thrombokinase and thrombin, resulting from "spontaneous" activation. At the next step, most of the thrombin remains in solution when the thrombokinase is subjected to isoelectric precipitation at pH 5.2. The ratio of thrombin to thrombokinase can further be reduced by repeated precipitation of the kinase by 0.45 saturated ammonium sulfate.

Activation of prothrombin in the presence of oxalate can be induced by the present thrombokinase at a concentration of 0.194 microgram nitrogen per ml., corresponding to about 1.2 microgram protein per ml. From this it may be estimated that a prothrombin solution containing more than 120 micrograms protein per ml. would need to have less than 1 per cent of its protein in the form of contaminating thrombokinase, in order to become activated "spontaneously" in the presence of oxalate. The required amount of contamination would undoubtedly vary with the conditions.

REFERENCES

1. Milstone, J. H., *Medicine*, 1952, **31**, 411.
2. Milstone, J. H., *J. Gen. Physiol.*, 1955, **38**, 757.
3. Milstone, J. H., *J. Gen. Physiol.*, 1948, **31**, 301.
4. Milstone, J. H., *J. Gen. Physiol.*, 1951, **35**, 67.
5. Milstone, J. H., *J. Gen. Physiol.*, 1942, **25**, 679.
6. Howell, W. H., *Am. J. Physiol.*, 1912, **31**, 1.
7. Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, **6**, 85.
8. Hiller, A., Plazin, J., and Van Slyke, D. D., *J. Biol. Chem.*, 1948, **176**, 1401.
9. Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291.
10. Milstone, J. H., *J. Gen. Physiol.*, 1955, **38**, 743.
11. Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1927, **11**, 641.
12. Milstone, J. H., *Yale J. Biol. and Med.*, 1952, **25**, 19.
13. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.
14. Cekada, E. B., *Am. J. Physiol.*, 1926, **78**, 512.
15. Mellanby, J., *Proc. Roy. Soc. London, Series B*, 1933, **113**, 93.
16. Mellanby, J., and Pratt, C. L. G., *Proc. Roy. Soc. London, Series B*, 1940, **128**, 201.
17. Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **174**, 565.
18. Milstone, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 315.