FRACTIONATION OF PLASMA GLOBULIN FOR PROTHROMBIN, THROMBOKINASE, AND ACCESSORY THROMBOPLASTIN*

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Although the blood-clotting system presents increasing complexities, it is still to be hoped that there is a simple basic mechanism at its core. If this basic mechanism were first apprehended, one could then begin to superimpose the complexities.

The facts at hand are consistent with the following concept of the basic mechanism:

$$\text{1. Prokinase} \xrightarrow{\text{Kinase}} \text{Kinase}$$

$$\text{2. Prothrombin} \xrightarrow{\text{Kinase}} \text{Thrombin}$$

$$\text{3. Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

in which all three precursors are substances closely associated with the plasma globulins, and all three reactions are enzymatic.

Kinase has been concentrated from a globulin derivative of plasma; and it has been shown to activate prothrombin, even in the presence of excess oxalate. Moreover, such kinase preparations, used at high dilution, have been shown to accelerate the activation of crude prokinase. This is in accord with the view that kinase activates prokinase.

An additional derivative of plasma globulin has been found to have an accessory thromboplastic effect similar to that of platelets, or of brain "cephalin." These accessory thromboplastins do accelerate production of thrombin in crude systems. But they differ from kinase in that they are not direct activators of purified prothrombin. They appear to be complicating factors superimposed on the basic mechanism. Further particulars are now given by the accompanying experiments.

"Kinase" has often been used as a short form of thrombokinase. Morawitz likened the action of thrombokinase on prothrombin to that of enterokinase

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on trypsinogen, and apparently considered it a direct activator (1). These working concepts are herein preserved. Howell stated that thromboplastin was contained in platelets and in brain "cephalin" fractions; and he did not consider his thromboplastin to be a direct activator (2, 3). To that extent, Howell's usage is preserved for the present accessory thromboplastins.

As a prologue to further purification, a method has been developed for separating clotting factors from large quantities of blood. The present account describes in detail a procedure previously outlined in brief (4).

The Fractionation Procedure

A flow-sheet is presented in Fig. 1 and the technical details are given in the section on materials and methods. The starting material is frozen euglobulin from slaughter-house plasma. The use of this material entails certain dis-

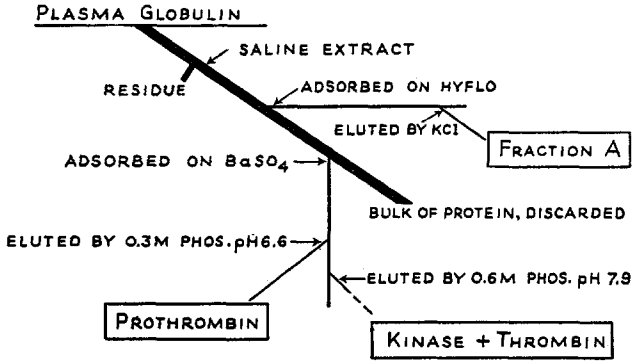

FIG. 1. Flow-sheet for fractionation procedure.

advantages which, for the present, are outweighed by the conveniences it offers in dealing with large quantities. Frozen euglobulin, representing more than 1,000 liters of plasma, has been used in developing this procedure. Globulin from more than 500 liters of plasma has been subjected to the consecutive adsorptions shown in Fig. 1.

When the frozen globulin is extracted with oxalated saline at pH 7.4 a large residue, apparently fibrin, remains undissolved. If left at room temperature a few hours, this residue becomes semifluid, and capable of liquefying fresh fibrin. A certain amount of fibrinolytic enzyme, or its precursor, is probably eliminated with the fibrin at this point.

The saline extract is then subjected to two successive adsorptions, the first by hyflo, the second by barium sulfate. The bulk of protein, including much fibrinogen, remains unadsorbed and is discarded. The adsorbent in each case, is collected by filtration, washed, and treated with eluent without disturbing

the filter-cake, thereby saving much time and motion. Potentially, these operations could be performed on much larger batches, and possibly could be adapted for use directly on oxalated plasma. They were evolved through unsuccessful attempts to achieve a true chromatographic fractionation on adsorption columns. The yields obtained by flowing elution have been as good as those obtained by stirring the resuspended adsorbent with the eluent. As indicated on the flow-sheet, two fractional elutions are performed on the barium sulfate.

TABLE I

Kjeldahl-Nitrogen in the Fractions Derived from 10 Liters of Plasma

Fraction	Volume	Nitrogen	Total nitrogen
	ml.	*mg./ml.*	*mg.*
Saline extract	1970	2.81	5,540
Fraction A	1000	0.203	203
Prothrombin	900	0.071	64
Kinase and thrombin	905	0.024	22

TABLE II

Activation of Prothrombin by Fraction A

Buffer	Pro-thrombin	Fraction A	$CaCl_2$	Period of activation					
				1 min.	2 min.	4 min.	8 min.	12 min.	16 min.
ml.	*ml.*	*ml.*	*ml.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*	*sec.*
0.6	0.3	0.1	0.1	720	315	83	35	27	26

Prothrombin preparation I was used for this test. The figures given in seconds report the time required for a 0.1 ml. sample to produce a clot, after being mixed with 0.3 ml. oxalated fibrinogen. For results without fraction A, see Table IV.

The distribution of Kjeldahl-nitrogen in the major fractions of a representative run is shown in Table I. Of the total Kjeldahl-nitrogen in the saline extract, about 5 per cent appears in the three major eluates, taken all together.

Fraction A

As is evident from Table I, fraction A was one-tenth the volume of the parent plasma. When used at 1/11 dilution in a prothrombin-calcium mixture fresh fraction A caused fairly rapid activation, as illustrated in Table II.

When fraction A was diluted to the point where its over-all thrombokinase activity was slight, it still showed a complementary thromboplastic effect when used together with crystallized trypsin, or with various globulin preparations derived from plasma. This property of fraction A fits into an experimental

pattern which is now becoming quite familiar. Recurrent demonstrations of this pattern, as observed in this laboratory, are summarized in Table III.

It was first found that washed platelets, plus a globulin preparation from slaughter-house plasma, would together produce thrombin from prothrombin, although neither was effective alone (5). The effect was clearly a complementary one, and could not be explained as the simple addition of two qualitatively similar actions.

Meanwhile, it was found that a minute quantity of crystallized trypsin could replace the crude globulin in this system (6). Larger quantities of trypsin activated prothrombin without the help of platelets. Indeed, trypsin could activate those prothrombins which had been purified to the extent that they were no longer readily activated by platelets plus calcium, or by brain lipid plus calcium. These results go far to substantiate the view of Eagle and Harris that trypsin activates prothrombin (7). There is now, however, this difference of interpretation: calcium plus platelets are not the equivalent of trypsin, but rather, they complement trypsin.

TABLE III

Functional Groups of Thromboplastic Materials

Direct activators of prothrombin	Accessory thromboplastins
Crude globulin (blood)	Platelets
Trypsin (pancreas)	Brain lipid*
Thrombokinase (blood)‡	Dilute, aged fraction A (blood)

* Crude *saline* extract of brain contains a direct activator, which is almost entirely absent from the lipid extract.

‡ In an earlier paper, a concentrate of this factor was temporarily designated "converter," with the preliminary statement that it might represent a thrombokinase (4).

It was next found that crude brain cephalin could substitute for platelets (6, 8). This strengthened the impression that it was the trypsin, and not the platelets or the brain lipid, which represented the primary activator of prothrombin. Prothrombin is considered to be a proenzyme. Trypsin activates some proenzymes. No lipid is known to do so.

Since the time of Morawitz (1), there has been a tendency to recognize two distinct groups of thromboplastic materials; and since the time of Howell (2) there has been an understandable desire to simplify the problem by assuming that they are all basically alike. However, Rumpf in 1913 (9) and later, Leathes and Mellanby (10) emphasized that certain lipids functioned in a different way than thrombokinases. Lenggenhager stated that his thrombokinin could be derived from plasma, but was not carried by platelets (11).

More recently, the contributions of Ferguson and Erickson (12), Quick (13), Owren (14), Zondek and Finkelstein (15) Fantl and Nance (16), Honorato (17), Ware Guest, and Seegers (18), Mann, Hurn, and Magath (19), Quick and Stefanini (20), Alexander, deVries, Goldstein, and Landwehr (21), and Jacox (22) have suggested in a variety of ways that at least two classes of biologic factors, beside the clas-

sical prothrombin, enhance the rate of over-all thrombin production. It would be rash to conclude arbitrarily that there are only two functional groups of thromboplastic materials, or only one type of thromboplastic lipid.

It is sufficient to note that experimental methods are now better able to distinguish between the two classes of thromboplastic materials listed in Table III and that the factor which is conspicuous in the *diluted* fraction A belongs to the group of accessory thromboplastins. Since the activation tests followed the pattern previously demonstrated (5, 6), they will not be reported in detail, except for the brief example in Table IV. There, it is seen that dilute fraction A plus kinase caused thrombin to be produced many times as fast as with fraction A or kinase alone. Other, corroborating, experiments showed that dilute fraction A and kinase together caused more rapid production of

TABLE IV

Complementary Effect of Dilute Fraction A and Dilute Kinase

Buffer	Prothrombin	Fraction A 1/10	Kinase 1/160	$CaCl_2$	Period of activation	
					10 min.	20 min.
ml.	*ml.*	*ml.*	*ml.*	*ml.*	*sec.*	*sec.*
0.7	0.2	0.1	None	0.1	>1800	>1800
0.6	0.2	0.1	0.1	0.1	60	42
0.7	0.2	None	0.1	0.1	>1800	>1800
0.8	0.2	None	None	0.1	>1800	>1800

Prothrombin preparation I was used for these tests. Fraction A had been stored at 5°C. for 28 days, and diluted just before the experiment. (The aging was not necessary.) The kinase was prepared from the kinase + thrombin fraction (*cf.* Fig. 1), as described under Methods. Figures in the body of the table give clotting times of 0.1 ml. samples mixed with 0.3 ml. oxalated fibrinogen.

thrombin than four times as much of either reagent without the other. Thus, the two reagents complemented each other in a way that could not be the simple addition of qualitatively similar effects.

Preparations were assayed for accessory thromboplastin on a comparative basis, by testing dilutions in prothrombin conversion mixtures which needed accessory thromboplastin, and comparing thromboplastic effects. For example, dilutions of various fractions could be substituted for dilute fraction A in the system of Table IV. In general, about half of such activity as found in the saline extract, was accounted for in fraction A; and perhaps as much as one-fifth of it remained with the bulk of protein, unadsorbed by the hyflo. This factor was most stable when the pH of fraction A was kept between 5.4 and 7.3; and there was an optimum near the middle of this range. The factor deteriorated more rapidly in 0.1 M NaCl than in 1.4 M KCl.

Attempts to separate the two complementary components of fraction A were only partly successful, and there were large apparent losses of activity. A large amount of accessory thromboplastin with relatively little kinase could be precipitated from fraction A at pH 6.4 in the presence of 0.3 saturated ammonium sulfate plus the fraction's original potassium chloride. From the resulting filtrate, thrombokinase could be precipitated by adding ammonium sulfate to 0.6 saturation. Studies of this kinase have not disclosed any essential difference from the kinase to be described below; and it is not necessary, as yet, to consider them as two different kinases. Of the total potential kinase available in the crude saline extract, a large part was removed with fraction A.

It is clear, however, that fraction A contains both kinase and accessory thromboplastin, and that it is the accessory thromboplastin which predominates when fraction A is diluted.

Prothrombin

Although a small amount of prothrombin could be detected in fraction A, more than 90 per cent of it went through the hyflo step along with the bulk of the proteins. In this material, the prothrombin was readily activated by adding calcium and fraction A. When brain lipid (which has extremely little kinase) was substituted for fraction A, there was a well marked latent period followed by a sharply accelerated production of thrombin. The interpretation offered is that prothrombokinase still accompanied the crude prothrombin at this stage of the procedure. Following the addition of calcium and brain lipid, the prokinase was activated autocatalytically, thereby furnishing the necessary kinase for the conversion of prothrombin.

The quantity of barium sulfate used to adsorb the prothrombin was chosen for optimal specific activity rather than maximal yield; and from 20 to 45 per cent of the prothrombin was left unadsorbed. The use of larger amounts of barium sulfate resulted in eluates with lower specific activities. Most of the adsorbed prothrombin could be eluted by 0.3 M phosphate, pH 6.6. Such prothrombin, although of a rather high specific activity, was still far from pure; and interesting differences have been noted in various subfractions. When the eluates were treated with filter-cel, precipitated with ammonium sulfate, and subjected to isoelectric fractionation, small fractions were obtained, in which only slight contamination with kinase or other significant impurity was recognized (see Methods).

Thrombokinase and Thrombin

Prothrombin eluates obtained with 0.3 M phosphate were not entirely stable. Eluates obtained with 0.1 M phosphate, pH 6.6, had less prothrombin, but also less lability. When parallel batches were eluted by different phosphate solutions, the prothrombin eluates obtained with the more concentrated or more

alkaline phosphate solutions showed greater lability. These differences in lability were large, and were still evident when the fresh eluates were promptly dialyzed and aged in the same medium.

Such differences also appeared in successive eluates. After the elution with 0.3 M phosphate, pH 6.6, a second elution was performed with 0.6 M phosphate,

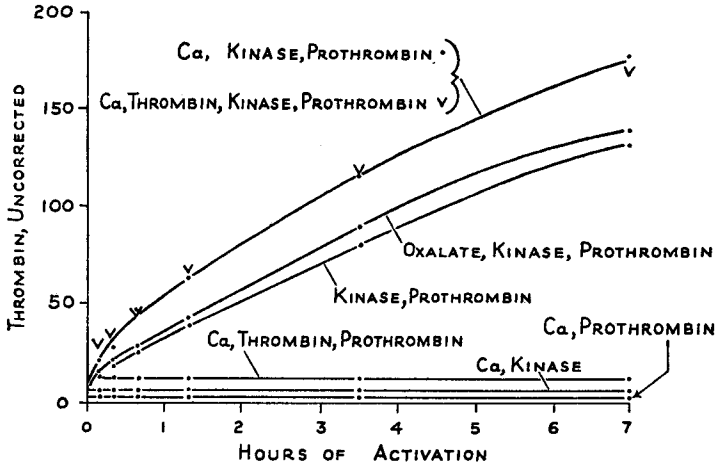

FIG. 2. Activation of prothrombin by kinase. All activation mixtures contained 0.4 ml. of prothrombin preparation II, except the "Ca, kinase" test, in which 0.4 ml. distilled water was substituted. Ca, kinase, thrombin, and oxalate were added where indicated, in the amount of 0.1 ml. The volume was made up to 1.1 ml. with veronal-buffered saline, and all mixtures were pH 7.3 by phenol red. The uncorrected thrombin values were derived by dividing 3,000 by the test clotting times. Minor, but very interesting, corrections which might have been made, are not discussed here because they do not affect the validity of the conclusions drawn in the text. The stock potassium oxalate was 0.1272 M, making the activation mixture more than 0.01 M with respect to oxalate. At the end of the experiment, the clear oxalated mixture was mixed with $CaCl_2$; and it gave a heavy precipitate. A further experiment showed that the activation rate was no slower with 3 times as much oxalate. In both cases oxalate was added before kinase. A later experiment showed that the same prothrombin reagent was not activated by oxalate plus brain lipid. The last 2 experiments are not illustrated in the figure.

pH 7.9. The protein concentrated from the second eluate contained not prothrombin, but thrombin. Since the evidence on this point is not yet complete, the following is offered only as a tentative explanation. An inactive form of kinase, possibly prokinase, was adsorbed with the prothrombin. During the subsequent steps, active kinase was liberated, and proceeded to activate the prothrombin, in the absence of ionic calcium. If this explanation were correct,

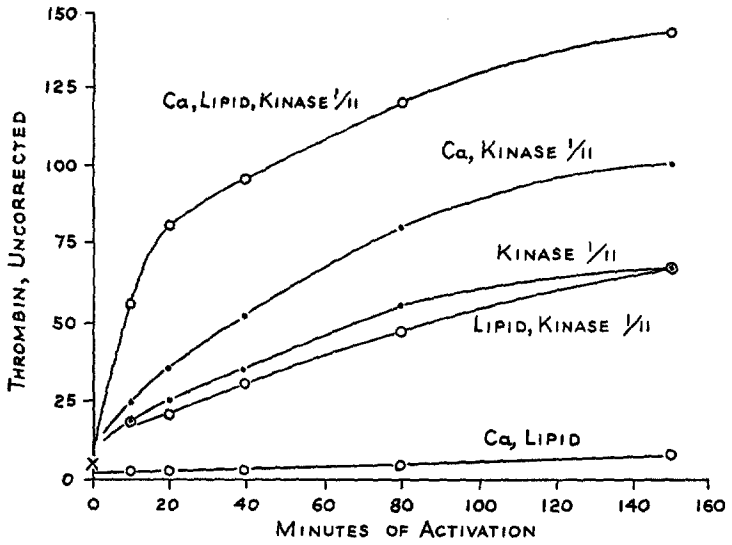

FIG. 3 *a*

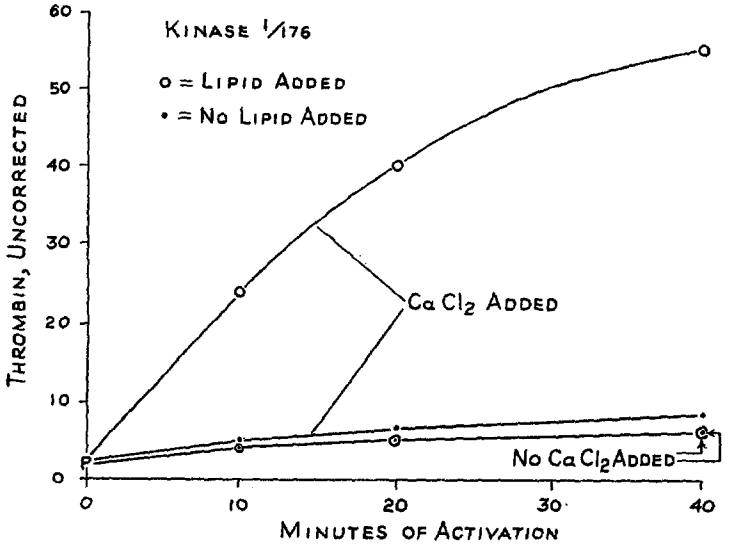

FIG. 3 *b*

FIGS. 3 *a* and 3 *b*. Fig. 3 *a* with kinase 1/11 and Fig. 3 *b* with kinase 1/176. The importance of calcium for the accessory effect of brain lipid. Prothrombin preparation II was present in all tests, which were performed like those of Fig. 2. The brain lipid was added as 0.1 ml. of a 1/100 dilution of a 10 per cent stock suspension. The dilutions of kinase are final dilutions in the activation mixtures. The X on the vertical axis of Fig. 3 *a* shows the amount of thrombin contaminant contributed by the prothrombin and kinase reagents at the start. The P on the vertical axis of Fig. 3 *b* shows the amount of thrombin contaminating the prothrombin. There has been much less of this annoyance with prothrombin preparation I (*cf.* Table IV).

then the concentrated protein should contain not only thrombin, but also kinase.

It did. By repeatedly selecting those proteins soluble in 0.35 but precipitated by 0.45 saturated ammonium sulfate, kinase subfractions were obtained which showed only slight contamination with thrombin. Proteins soluble in 0.45, but precipitated by 0.6 saturated ammonium sulfate, were selected to yield the thrombin subfractions.

Fig. 2 shows that the kinase could activate prothrombin with or without the addition of calcium chloride, and even in the presence of excess oxalate. The two lowermost curves reveal that both the prothrombin and the kinase reagents contained small amounts of thrombin, which did not increase during the test. The next higher line shows that a larger amount of thrombin, furnished by the thrombin subfraction, failed to activate prothrombin. The V-shaped symbols show that the same amount of thrombin subfraction had no effect on the activation of prothrombin by kinase.

Fig. 3 *a* reiterates that kinase can activate prothrombin without the addition of calcium. Without calcium, the further addition of brain lipid did not hasten production of thrombin. However, when kinase activated prothrombin in the presence of calcium, then the brain lipid was able to exert its accessory effect, as demonstrated by the contrast between the two uppermost curves. The effect of the lipid was more conspicuous in the early stages of activation (Fig. 3 *a*), and when the kinase was dilute (Fig. 3 *b*). Control experiments showed that lipid, Ca, and kinase did not produce thrombin unless prothrombin was included. Other controls showed that the principal effect of the lipid could not be attributed to a prompt hastening of the thrombin-fibrinogen reaction, with spurious increase in thrombin values.

Greater dependence on calcium furnishes another characteristic distinguishing the accessory thromboplastins from the primary activators of prothrombin. This applies alike to the different accessory thromboplastins listed in Table III. In experiments such as those of Fig. 3 *b*, either platelets or dilute fraction A could be substituted for brain lipid. But neither platelets nor dilute fraction A exerted much effect unless calcium chloride was also included.

As already indicated, fraction A contained a relatively large amount of accessory thromboplastin, along with some kinase. In the presence of calcium, and in the amount used for the experiment of Table II, it caused more rapid production of thrombin than the amount of kinase used for the experiments of Figs. 2 and 3 *a*. However, without the addition of calcium, the same amount of fraction A was practically inert. To this extent, the behavior of fraction A was like that of the artificial mixture of dilute kinase and accessory thromboplastin, illustrated in Fig. 3 *b*, but even less active without calcium.

Contrariwise, the behavior of concentrated kinase did not suggest such a mixture. If it is suspected that the activity of concentrated kinase depends on

its containing two biologic factors, it is difficult to suppose that one of them is accessory thromboplastin; because the latter is not effective in the presence of oxalate, whereas concentrated kinase is.

Previously (23), when crude kinase was obtained by conversion of crude prokinase, it activated prothrombin only very slowly, if the concentration of calcium was reduced. For this and other reasons, it is probable that such crude kinase contained accessory thromboplastin. Indeed, it was indicated at that time that the crude prokinase might contain an activator complex with more than one significant component.

Comparison of Fig. 3 *b* with Fig. 3 *a* demonstrates that smaller quantities of kinase could be detected if calcium and accessory thromboplastin were included in the system. With this more sensitive test, it was found that there was still a considerable amount of kinase in the thrombin subfraction. But the concentration of kinase, as so estimated, did not parallel thrombin. The ratio of kinase to thrombin was 100 times as great in the kinase subfraction as in the thrombin subfraction.

This degree of separation was achieved by rejecting a large part of the total activity in order to concentrate a small sample of kinase with little contaminating thrombin. Only 10 ml. of concentrate was derived from 55 to 60 liters of plasma. Because of the losses by rejection and other large losses, the concentrate does not afford a full measure of the potential activity of the parent plasma. As far as kinase and prokinase are concerned, the present methods are exploratory.

Table IV illustrates the complementary effect of dilute fraction A and dilute kinase. The amount of fraction A in the activation mixtures represents the equivalent of diluting fraction A to 11 times the volume of the parent plasma. The dilution of kinase in the mixtures corresponds to diluting 10 ml. of concentrate to 17.6 liters. In other experiments the kinase was detectable in the proportion of 10 ml. of concentrate diluted to the original 55 liters.

These quantitative relations offer no reason for doubting the physiologic significance of the kinase. However, other quantitative considerations cast much doubt on its purity. In some runs, there was almost as much kinase in the thrombin, as in the kinase subfraction, the principal difference being that the kinase subfraction had only 1/100 as much thrombin. It is quite possible that kinase represented only a small proportion of the total protein of these preparations. Moreover, 1 microgram of crystallized trypsin would replace 100 micrograms or more of kinase subfraction, in the activation of prothrombin. Like kinase, trypsin could activate prothrombin in the presence of oxalate.

The Activator of Prothrombokinase

The latent period in the activation of fresh, crude prothrombin is concerned with the activation of the prokinase which contaminates the prothrombin (23).

Various fractions described here contained a factor which could shorten the latent period. According to this presumptive two-stage test, the kinase subfraction was 100 times more concentrated in this factor, than was fraction A. This tended to associate the activator of prokinase with kinase, rather than with accessory thromboplastin.

With the more explicit three-stage analysis, it was found that the kinase subfraction, at high dilution, accelerated the activation of crude prokinase, as shown in Fig. 4 *a*. However, the thrombin subfraction was almost as capable in this respect, as shown in Fig. 4 *b*. These two subfractions had shown similar concentration of kinase, but a 100-fold difference in thrombin. In these two preparations, as well as in others, the capacity to accelerate the activation of prokinase was proportional to the concentration of kinase, not to the concentration of thrombin.

In the three-stage analysis, seed material diluted 45,056-fold in the prokinase mixture was later diluted 10-fold more in the prothrombin mixtures, (compare 1/450,560 with the dilution of $1/160 \times 1/11 = 1/1{,}760$ in Table IV), and finally 4-fold more in the fibrinogen mixtures. Under these conditions, the present seed materials could offer no interference in the second and third stages. The observed effect depended entirely on their acceleration of prokinase conversion. The amount of kinase added as seed was very small compared to that developed from the prokinase. This was further verified by a seeded three-stage test which followed the kinase activity during the first 2 minutes, before the rapid rise.

Materials and Methods

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

Filter-Cel.—Another grade of diatomaceous silica.

E & D Filter Paper.—The Eaton-Dikeman Co., Mt. Holly Springs, Pennsylvania.

$BaSO_4$.—U.S.P., "suitable for x-ray diagnosis," supplied by Merck and Co., Rahway, New Jersey. Some stocks of analytical reagent are not satisfactory for the present procedure. $BaSO_4$ was used for absorption of "serozyme" by Bordet and Delange (24), and of prothrombin by Dale and Walpole (25), and recently by others (21, 26, 27).

Oxalated Salt Solution.—	NaCl	36 gm.
	$K_2C_2O_4 + H_2O$	20 gm.
	Distilled water to	2 liters
4 M NaCl.—	NaCl	1404 gm.
	Distilled water to	6 liters
1.4 M KCl, pH 6.7.—	KCl	626 gm.
	0.1 M KH_2PO_4	300 ml.
	0.1 N NaOH	210 ml.
	Distilled water to	6 liters

The pH values for this and the following solutions were read with a glass electrode.

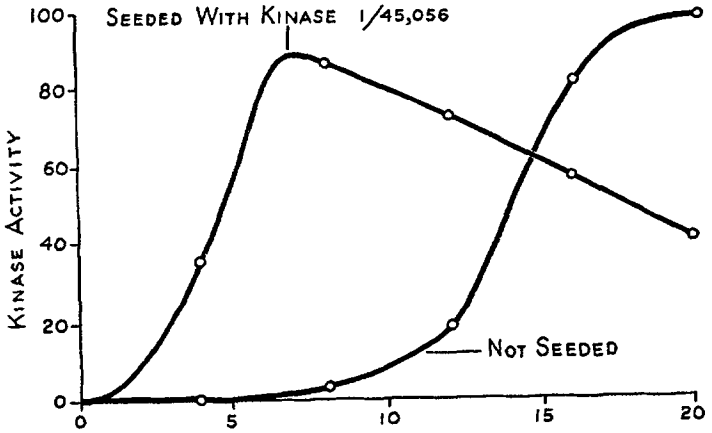

FIG. 4 *a*

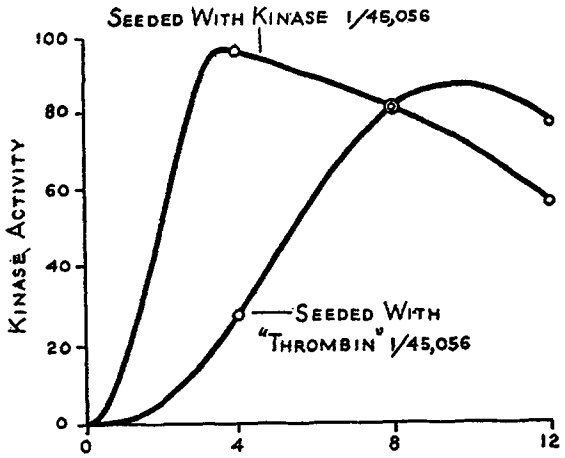

FIG. 4 *b*

FIGS. 4 *a* and 4 *b*. Fig. 4 *a* with unseeded control and Fig. 4 *b* with comparison of kinase and thrombin. Three-stage analysis showing that the kinase subfraction accelerated activation of prokinase more than did the "thrombin" subfraction. The kinase subfraction had a little more kinase, about 1/3 as much protein, and 1/100 as much thrombin, as compared with the "thrombin" subfraction. The dilutions given are final dilutions in the prokinase activation mixtures. The undiluted kinase had 0.35 mg. protein N per ml. by turbidity, and caused acceleration in dilutions beyond 1/45,056. 0.02 μg. protein N per ml. represents a conservatively high estimate of the amount required to cause detectable acceleration. For each test, 0.1 ml. of seed material (or buffer control) was added to 0.9 ml. crude prokinase, and 0.1 ml. $CaCl_2$ was added 15 seconds later. At 4 minute intervals, timed from the addition of $CaCl_2$, samples were assayed for kinase activity by determining how much prothrombin they would activate in 2 minutes.

The readings did not vary more than 0.1 unit from the values given.

Wash, pH 6.7.— 4 M NaCl 125 ml.
0.1 M KH_2PO_4 250 ml.
0.1 N NaOH 120 ml.
Distilled water to 5 liters

0.3 M phosphate, pH 6.6.—KH_2PO_4 81 gm.
1 N NaOH 275 ml.
Distilled water to 2 liters

Phosphate solutions were used by Munro and Munro (28) to elute prothrombin from $Al(OH)_3$, and by Fantl and Nance (29) to elute prothrombin from $BaSO_4$.

0.6 M phosphate, pH 7.9.—KH_2PO_4 163 gm.
1 N NaOH 1110 ml.
Distilled water to 2 liters

0.005 M acetate, pH 5.3.— 4 M CH_3 COONa 4.0 ml.
4 M CH_3 COOH 1.0 ml.
Distilled water to 4 liters

SAS.—Saturated ammonium sulfate.

0.1 SAS: 0.1 M acetate, pH 5.25.— SAS 100 ml.
4 M CH_3COONa 21 ml.
4 M CH_3COOH 4 ml.
Distilled water to 1 liter

0.35 SAS: 0.1 M acetate, pH 5.25.—SAS 350 ml
4 M CH_3COONa 21 ml.
4 M CH_3COOH 4 ml.
Distilled water to 1 liter

0.45 SAS: 0.1 M acetate, pH 5.25.—SAS 450 ml.
4 M CH_3COONa 21 ml.
4 M CH_3COOH 4 ml.
Distilled water to 1 liter

Veronal-Buffered Saline.—0.9 per cent NaCl: 0.02 M veronal, pH 7.4 (5).

Platelets.—Twice washed rabbit platelets (5).

Lipoid Thromboplastin.—That fraction of bovine brain, soluble in ether, but not in acetone (6).

Crystallized Trypsin.—Stock solution prepared as described (6).

Prothrombin Preparation I.—by Mg $(OH)_2$, as described (23).

Prothrombin Preparation II.—by $BaSO_4$, as described below.

Preparation of Major Fractions.—Frozen euglobulin was obtained from Armour and Co., of Chicago, through the cooperation of Dr. J. B. Lesh. Citrated bovine plasma was diluted with 10 volumes of cold tap water and the pH brought to 5.1 by addition of 1 per cent acetic acid. After settling overnight in the cold, the supernatant was discarded and the precipitate was shipped by air express, packed in dry ice. At the laboratory, this material was stored at −17°C. The weight of the frozen precipitate from 100 liters of plasma was usually between 4 and 6 kg. To avoid continuous changes in procedure, 540 gm. was arbitrarily taken as roughly equivalent to 10 liters of plasma.

540 gm. of precipitate was broken up with a cold chisel, passed through a meat grinder, and mixed with 900 ml. distilled water plus 1 liter of cold oxalated salt solution. This mixture was stirred electrically with a glass rod bent at the bottom into a wide triangle, thereby moving the entire suspension without causing much foaming. During the stirring, about 170 ml. 0.1 N NaOH was added dropwise until a phenol red spot test matched that of a pH 7.4 buffer. The mixture was centrifuged and the residue set aside. To the supernatant was added 400 gm. hyflo, which was kept suspended by occasional stirring for 20 minutes. A suspension of 100 gm. hyflo in 500 ml. wash was used to precoat an E & D No. 615 filter paper on a Buchner funnel 24 cm. in diameter. Finer filter paper was not satisfactory, leading to excessive slowing of filtration.

The filters were handled as if they were flat chromatographic columns.[1] Fluids were poured on in batches; and these were kept reasonably separate by waiting until only a thin layer of preceding fluid remained over the filter-cake, before the next was poured on.

The hyflo-globulin mixture was poured on the prepared filter and filtration with suction was performed slowly until the amber globulin just began to emerge. At this point filtration was stopped, and about 470 ml. filtrate was discarded. Filtration was then completed within 10 minutes. Now, 500 ml. wash was drawn into the filter-cake, which was then packed by pressing with a spatula. Next 1,000 ml. wash was drawn into the cake; and about 2450 ml. of amber filtrate was removed from the flask, and stirred with 800 ml. cold 4 M NaCl. The salted globulin could be processed at once or stored overnight at 4°C. Another 1,000 ml. of wash was drawn into the cake; the filtrate was discarded and the flask rinsed with distilled water. 800 ml. 1.4 M KCl was poured on the filter; and the suction was adjusted so that 20 minutes were required to draw the KCl into the cake. The filtrate was discarded. Another 800 ml. 1.4 M KCl was poured on the filter and filtration was completed slowly. The 800 to 1,000 ml. of opalescent filtrate was fraction A. About 140 minutes were required for the adsorption, filtration, washing, and elution. As routine, two batches were run, from the frozen globulin to fraction A in 5 hours.

Initially, difficulty was met in performing two successive adsorptions with globulin solutions which contained so much fibrinogen. Apparently this was largely due to the tendency of the fibrinogen to clot during the process. This difficulty was avoided by salting the globulin after the first adsorption and by selecting the proper stock of $BaSO_4$, for different stocks varied in their suitability for this procedure. If the salted globulin had been stored at 4°C., it was warmed for 15 minutes in a water bath at 26–30°C. Meanwhile a suspension of 10 gm. hyflo in 300 ml. 1.4 M KCl was used to precoat a No. 612 E & D filter paper on a Buchner funnel 24 cm. in diameter. 150 gm. $BaSO_4$ was added to the globulin and stirred continuously for 10 minutes. Then 60 gm. hyflo was added and stirred well for 2 to 3 minutes. Filtration was begun at moderate speed, the mixture being poured with care not to disturb much of the precoat. After the first few seconds, the suction was turned on full; and filtration was completed in 15 to 30 minutes. The $BaSO_4$–globulin suspension was followed by 500

[1] The writer is indebted to Dr. Harold G. Cassidy of Yale University, for an introductory discussion of chromatographic methods.

ml. 1.4 M KCl and the filter-flask was emptied. Then 1500 ml. wash was passed through; and the flask was emptied and rinsed with distilled water. The first elution was performed by drawing 900 ml. 0.3 M phosphate, pH 6.6, through the cake in 30 to 40 minutes. The second eluate was obtained in a similar manner with 900 ml. 0.6 M phosphate, pH 7.9. Sometimes twice as much hyflo was needed for rapid filtration.

Sometimes the procedure was interrupted just before the first elution, and the washed $BaSO_4$ was stored at −17°C. Often, the $BaSO_4$ was stored in the freezer after the first elution; and the second eluates were prepared from the stored cakes, as needed.

As an extension of this major fractionation procedure, the following exploratory fractionations have been done.

Kinase and Thrombin Subfractions.—The second eluates from 6 $BaSO_4$ cakes were pooled. For each liter, 474 gm. solid ammonium sulfate was added, and stirred well. The precipitate was collected on a Buchner filter with 33 gm. hyflo as filter-aid. The moist, undisturbed filter-cake was kept in the refrigerator 23 hours and then at room temperature for ½ hour. For the next ½ hour, 600 ml. wash was drawn slowly through the cake, which was finally pressed with a spatula. To 650 ml. of amber filtrate, there was added 351 ml. SAS. After 2 hours, the precipitate was removed by centrifugation. To 980 ml. of supernatant was added 435 ml. SAS. The precipitate was collected by centrifugation, dissolved in 40 ml. wash, kept at 5°C. for 1 week, and mixed with 33 ml. SAS, to give a kinase precipitate and a thrombin supernatant. All fractionation was done at room temperature, but the fractions were stored at 5°C. at the end of each day.

The kinase precipitate was extracted with 20 ml. 0.35 SAS : 0.1 M acetate, and the residue discarded. The extract was mixed with 3.9 ml. SAS; and the resulting precipitate was extracted with 15 ml. 0.35 SAS : 0.1 M acetate. The extract was recentrifuged the next day to remove a precipitate which had formed overnight in the cold; and the extract was then mixed with 2.7 ml. SAS. The resulting precipitate was extracted with 5 ml. 0.35 SAS : 0.1 M acetate; and the extract was mixed with 0.9 ml. SAS. The resulting precipitate was dissolved in 15 ml. wash, and reprecipitated with 12.3 ml. SAS. The precipitate was dissolved in 20 ml. wash and reprecipitated with 16.4 ml. SAS. This last pair of operations, (20 ml. wash, then 16.4 ml. SAS) was repeated to make a total of 12 such precipitations. The final precipitate was dissolved in 10 ml. wash and dialyzed against several changes of cold veronal-buffered saline, over a period of 4 days.

76 ml. of the thrombin supernatant was mixed with 28.5 ml. SAS. The resulting precipitate was extracted with 20 ml. 0.45 SAS : 0.1 M acetate. 22 ml. extract plus 8.3 ml. SAS gave a precipitate which was extracted with 10 ml. 0.45 SAS : 0.1 M acetate. This extract plus 3.8 ml. SAS gave a precipitate, which was extracted with 5 ml. 0.45 SAS : 0.1 M acetate. After removal of a precipitate which formed overnight in the cold, the extract was diluted with 5 ml. wash, and dialyzed in the same beaker with the kinase subfraction.

The principle involved in this separation has been used repeatedly, with many variations in detail.

Prothrombin Preparation II.—900 ml. fresh prothrombin eluate was stirred 10 minutes with 13.5 gm. filter-cel, and filtered through No. 612 E & D paper on a Buch-

ner funnel 9 cm. in diameter. Two such batches were combined and stirred with 352 gm. ammonium sulfate per liter of filtrate. After ½ to 1 hour, 10 gm. hyflo was stirred in, and the suspension filtered on a 9 cm. E & D No. 612 paper, precoated with 1 gm. hyflo (suspended in 0.65 SAS). The filter-cake was washed with 100 ml. of 0.45 SAS : 0.1 M acetate. Then 2 successive 100 ml. portions of 0.1 SAS : 0.1 M acetate were slowly drawn through the filter over a period of 30 to 45 minutes, to yield about 200 ml. of prothrombin solution. This was dialyzed in the cold against several changes of 0.005 M acetate, pH 5.3, and the resulting precipitate removed by centrifugation in the cold. To the supernatant, cold 0.1 N HCl was cautiously added until a spot test with brom cresol green matched that of a pH 4.4 buffer. The precipitate was removed by centrifugation in the cold. After the supernatant had been at 5°C. for a day or two more precipitate formed; and this was removed. The supernatant contained prothrombin, with only traces of kinase.

Several variations of this method have been used, all of them having in common the selection of prothrombin remaining in solution after isoelectric precipitation at pH 4.4 to 4.8. At this point most of the prothrombin is lost in the precipitate, along with kinase or its precursor. However, the supernatant is almost as low in kinase as is prothrombin preparation I; and it differs from preparation I in an interesting way, which will be described elsewhere (8).

Three-Stage Analysis and Other Coagulation Tests.—As described (23).

Protein-N.—Turbidity method of Northrop (30), standardized by Kjeldahl (31) on crude globulin.

Kjeldahl-N.—Microanalysis, using digestion and distillation procedure of Hiller, Plazin, and Van Slyke (32). The distillate was collected and titrated according to Kunitz (31).

DISCUSSION

The understanding of the clotting system will depend ultimately on the isolation of its components. To do this, it is desirable to know what are the components to be isolated, and to have some idea of what they do and how they can be measured. Thus, even a purification program needs a working hypothesis. At the present stage of purification, it is profitable to check the new facts against the basic hypothesis:

$$\text{1. Prokinase} \xrightarrow{\text{Kinase}} \text{Kinase}$$

$$\text{2. Prothrombin} \xrightarrow{\text{Kinase}} \text{Thrombin}$$

$$\text{3. Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

The crucial questions concern kinase, which is defined as a direct activator of prothrombin. It is concluded that the concentrated plasma derivatives herein called kinase do contain kinase; for they have never failed to activate a prothrombin preparation, no matter how purified.

Platelets, brain lipid, and *dilute* fraction A are considered not to be primary

activators; for they cause little or no activation of purified prothrombin, even in the presence of calcium. However, they do accelerate production of thrombin, provided that both kinase and calcium are included with the prothrombin. Therefore, they are classed as accessory thromboplastins. The rate of over-all thrombin production depends on the rates of prothrombin activation, of side reactions, and of thrombin inactivation. This leaves open several conceivable modes of action for these thromboplastins.

Concentrated kinase activates prothrombin in the presence of oxalate. Accessory thromboplastins are effective only when calcium is added, and are practically inert in the presence of oxalate. Hence, they do not significantly contribute to the effect of kinase in the presence of oxalate, even if they happen to contaminate the system. It is concluded that kinase can activate prothrombin, unaided either by ionic calcium or by accessory thromboplastin of the type studied.

Heretofore, concentrated kinase preparations, as here described, have not been available for continuous and repeated study. When kinase is dilute and the test system crude, the addition of accessory thromboplastin plus calcium makes an enormous difference in the rate of thrombin production. Under these conditions, calcium and accessory thromboplastin (*e.g.* platelets, "cephalin") can easily be mistaken for direct activators. They often have been. It is now seen that ionic calcium and accessory thromboplastin are neither absolutely necessary, nor together sufficient, for the activation of prothrombin.

Under proper conditions, highly purified thrombin preparations will show kinase activity. But the ratio of kinase to thrombin varies, and is 100 times as great in the present kinase fraction as in the thrombin fraction. This strongly favors the view that kinase and thrombin are distinct entities, and that kinase commonly contaminates thrombin.

The kinase preparations sharply accelerate the conversion of crude prokinase. Other fractions behave similarly. But the extent to which the fraction can be diluted and still show this effect, is roughly proportional to the amount of kinase, and far out of proportion to thrombin or to accessory thromboplastin. Although more complicated interpretations must be kept in mind, the simplest explanation is that kinase activates prokinase. It has already been shown that the conversion of crude prokinase follows the course of an autocatalytic reaction (23).

Several years ago, it was reported that concentrated crude prothrombin in ammonium sulfate solution, changed to thrombin without the addition of extraneous activators and in the absence of ionic calcium. The essential condition for "spontaneous" activation seemed to be the concentration of the biologic material, which might have been either the prothrombin itself, or some accompanying factor (33). It has now been shown that *dilute* prothrombin in *dilute* salt solution can be activated in the presence of oxalate by *concentrated* kinase. Moreover, it has been possible to find

kinase in thrombins resulting from "spontaneous" activation, and to separate some of the kinase from the thrombin in a moderately effective manner.

During the present work, it was found extremely difficult to remove the last traces of kinase and its precursor from prothrombin subfractions. These traces, although of little consequence in dilute solution would become more effective when the protein was concentrated. Until there is some valid criterion for distinguishing a truly spontaneous conversion of prothrombin, it is well to suspect that those prothrombin concentrates which become active "spontaneously" are contaminated with prokinase or kinase.

"Spontaneous" activation of prothrombin was encountered by Cekada (34) and Mellanby (35), and probably also by Parfentjev (36). Quite recently, Seegers, McClaughry, and Fahey reported the autocatalytic kinetics observed for the activation of concentrated prothrombin in sodium citrate (37). They did not discuss the possibility that their prothrombin might be contaminated with prokinase, and their thrombin with kinase. A plasma derivative that activated prothrombin in the presence of oxalate was described by Tagnon (38) who compared his factor to the thrombozyme of Nolf (39).

Although ionic calcium is ordinarily very important for the conversion of prothrombin, there is now ample evidence that it is not indispensable. Calcium is also important for the activation of prokinase (23). That calcium may not be absolutely necessary for this reaction either, has been suggested by incidental observations which require further study.

It has usually been possible to substitute crystallized trypsin for thrombokinase. However, the two do not appear to be the same. Certain preparations of serum protein have here been found to depress the activation of prothrombin by trypsin, whereas they exerted little influence on the activation of prothrombin by kinase (*cf.* Travis and Ferguson (40)). It is also probable that thrombokinase is distinct from that particular fibrinolytic enzyme called plasmin (41). This does not mean that thrombokinase is not an enzyme, nor does it even exclude the possibility that the kinase, under proper conditions, might show some proteolytic or fibrinolytic activity.

Although accessory thromboplastin was recovered in good yield from the plasma globulin, it is not certain to what extent this material is distinct from the functionally similar factor in platelets. The globulin precipitate from slaughter-house plasma may well have included platelet material. Further, there is some doubt that plasma, as it circulates during life, is ever entirely free of platelet material. On the other hand, the possibility must be considered that *functionally* similar material might originate from sources other than platelets, and also be found in the plasma globulins. Regardless of source, it is advantageous to group all such functionally similar materials together as accessory thromboplastins. This preserves for platelets and for brain "cephalin" the designation "thromboplastin," applied to them by Howell (2, 3). They are now further qualified as accessory thromboplastins in order to distinguish them more emphatically from the primary activator, thrombokinase. It is quite probable that accessory thromboplastic action is of the utmost importance physiologically.

The effect of accessory thromboplastin on the production of thrombin was found to be more conspicuous when the kinase was diluted. In fact, it was often difficult to detect dilute kinase without adding accessory thromboplastin. There are many conceivable explanations, some of which have been discussed elsewhere (6). One

possibility which must be admitted here is that various subfractions of the plasma globulins might contain anticoagulant material which tends to obscure the kinase. Howell repeatedly portrayed the function of his thromboplastin as an opposition to some anticoagulant (2, 3).

It is unnecessary to speculate further on the surprises this topic has in store. The chief present purpose is to group these accessory thromboplastins together on a functional basis and to set them aside, so that they no longer obscure the basic mechanism. With regard to the basic mechanism, the hypothesis fits the new facts. Many reports from other laboratories can be brought into harmony with this hypothesis if it is clearly appreciated that: First, kinase and accessory thromboplastin are different factors, often mistaken one for the other, and often coexisting in plasma globulin derivatives; second, kinase is a frequent and hitherto unrecognized contaminant of highly purified thrombin preparations.

SUMMARY

1. Crude globulin from more than 1,000 liters of citrated bovine plasma has been used in developing a procedure for moderately large scale separation of clotting factors. Fraction A, prothrombin, kinase, and thrombin fractions were prepared. Fraction A contained both kinase and accessory thromboplastin, the latter predominating when fraction A was diluted.

2. When prothrombin was activated by kinase, the rate of thrombin production was enhanced by the addition of platelets, or brain lipid, or dilute fraction A. These accessory thromboplastins caused this acceleration only when calcium chloride was added. Even with calcium, they were not effective unless kinase was present.

3. In contrast, the action of kinase was not entirely dependent on either ionic calcium or accessory thromboplastin. The concentrated kinase fraction activated prothrombin in the presence of excess oxalate. Although kinase often contaminates highly purified thrombins, it is probably distinct from thrombin. The ratio of kinase to thrombin was 100 times as great in the kinase fraction as in the thrombin fraction.

4. The kinase fraction, diluted 45,000-fold, to protein-nitrogen concentrations as low as 0.02 microgram per ml., accelerated the conversion of crude prokinase in three-stage tests.

5. The findings are consistent with the following concept of the basic enzymatic mechanism:

$$\text{1. Prokinase} \xrightarrow{\text{Kinase}} \text{Kinase}$$

$$\text{2. Prothrombin} \xrightarrow{\text{Kinase}} \text{Thrombin}$$

$$\text{3. Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

It is now added that calcium and accessory thromboplastin exert their effects by impinging on the basic mechanism, in a chemically secondary or indirect manner.

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