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Jessica H. Lewis, John H. Ferguson

*J Clin Invest.* 1953;32(10):915-921. <https://doi.org/10.1172/JCI102816>.

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# PARTIAL PURIFICATION OF HUMAN PROTHROMBIN AND PRO- CONVERTIN: THEIR CHARACTERISTICS AND INTERACTION<sup>1</sup>

BY JESSICA H. LEWIS AND JOHN H. FERGUSON

(From the Department of Physiology, University of North Carolina, Chapel Hill, N. C.)

(Submitted for publication April 6, 1953; accepted May 22, 1953)

Independently and almost simultaneously a number of investigators recognized the fact that at least two plasma factors are important in the conversion of prothrombin to thrombin in the presence of *tissue* thromboplastin and calcium. Various names have been applied to these factors and, at a recent Macy Conference on blood clotting (1), most of the investigators agreed that they could be grouped into two entities as follows:

lability, it must be prepared specially for each individual experiment, and because we wished to study the reactions of proconvertin and prothrombin in the presence of accelerin rather than proaccelerin.

*Thromboplastin*: Soluplastin, obtained through the courtesy of Dr. E. W. Blanchard, Schieffelin and Company. Dog lung thromboplastin, freshly prepared fraction IV + VI of perfused dog lung, previously shown to have high thromboplastic potency as well as fibrinolysokinase activity (30).

Relatively labile factor	Relatively stable factor
Labile factor (Quick [2, 3])	SPCA precursor (Alexander et al. [1, 13-20])
Factor V, Proaccelerin (Owren [4, 5])	Co-factor V, Proconvertin (Owren [1, 21, 22])
Plasma Ac globulin (Ware, Guest, and Seegers [6, 7])	Factor VII (Koller, Loeliger, and Duckert [23])
Prothrombin accelerator (Fantl and Nance [8, 9])	Prothrombin accelerator (MacMillan [24])
Plasmatic co-factor of thromboplastin (Honorato, Rojas, and Ivanovic [10, 11])	Co-thromboplastin (Mann and Hurn [25, 26])
Plasma prothrombin conversion factor (Stefanini [12])	Stable prothrombin conversion factor (Owen, Bollman, and Magath [27, 28])

Without prejudice and for the sake of simplicity, we have chosen to use the terms proposed by Owren, namely, proaccelerin and proconvertin for the plasma factors, and accelerin and convertin for the active (serum) factors.

The investigations presented in this paper deal with a method for the partial separation of prothrombin and proconvertin from human plasma and the study of their interactions.

## MATERIALS

*Imidazole buffer*: pH 7.3 (26).

*Accelerin* (Accelerator Globulin, AcG): Barium carbonate treated beef serum prepared according to the method of Ware and Seegers (29). For the purposes of the present study this crude material served as a satisfactory source of accelerin, free from prothrombin and proconvertin, and retained full potency on storage at -20° C. for one year or longer. In additional experiments, which will be reported separately, we have employed highly purified human proaccelerin. This material was not used in the present experiments as, due to its marked storage

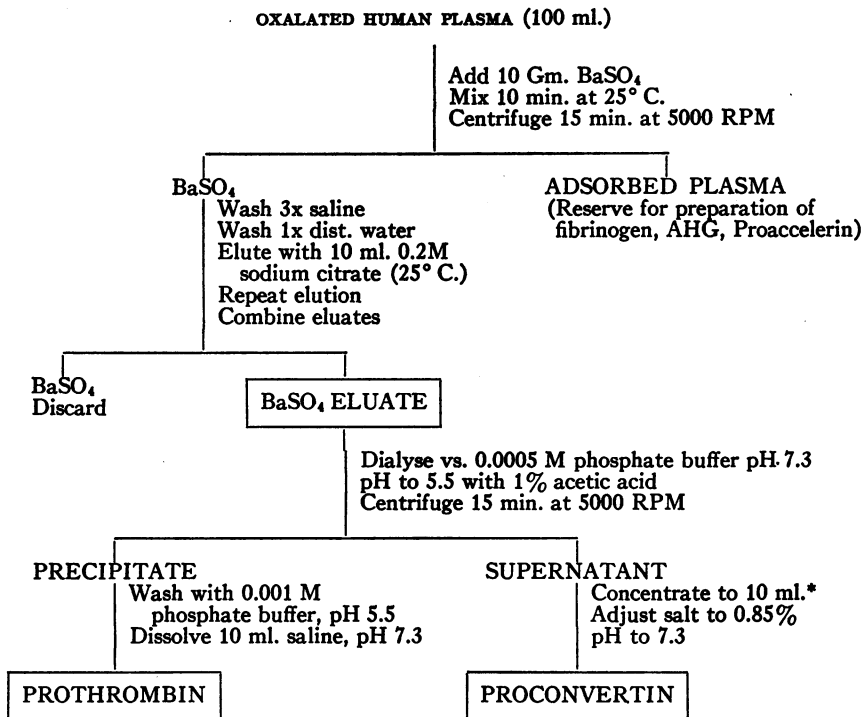
*Fibrinogen*: Human plasma fraction I (Cohn) prepared as a 0.7 per cent solution in 0.85 per cent sodium chloride (pH 7.3) and adsorbed with 50 mg. barium sulfate per ml. Although this material contains a high concentration of citrate, treatment with barium sulfate apparently did improve its stability. The fraction I was supplied through the courtesy of the American National Red Cross and Squibb and Company.

## METHODS

*Plasma fractionation*: Many fractionation procedures were attempted in the separation of prothrombin and proconvertin. Although the method outlined below does not supply absolutely pure fractions, it does yield sufficient separation of prothrombin and proconvertin to allow study of the properties of these materials. All procedures were carried out at 4° C. unless otherwise stated. Various attempts to further purify the two fractions were made, but repeated reprecipitation and readsorption and elution from barium sulfate did not lead to preparations of significantly greater purity.

*Thrombin activation mixtures*: All activation mixtures (except controls in Table I) contained 0.5 ml. imidazole buffer, 0.5 ml. thromboplastin (diluted 1 to 5 with saline), 0.2 ml. accelerin (diluted 1 to 16 with saline) and 0.5 ml. 0.15 M CaCl<sub>2</sub>. Prothrombin and proconvertin were added in 0.1 ml. amounts unless otherwise stated, and in all cases, sufficient 0.85 per cent sodium chloride was added to

<sup>1</sup> This investigation was supported in part by a research grant from the Division of Research Grants of the National Institutes of Health, U. S. Public Health Service.



\* Concentration may be accomplished by evaporation from a dialysis bag, evaporation in vacuo, dialysis against concentrated PVP, or lyophilization. The first method was usually employed.

bring the total volume to 5 ml. Employing the BaSO<sub>4</sub> Eluate (previously dialyzed to remove sodium citrate), which contains both prothrombin and proconvertin, the above concentrations of CaCl<sub>2</sub>, thromboplastin, and ac-celerin were found to be optimal.

Activation and clotting were carried out at 25° C. Experiments showed that a greater yield of thrombin and more reproducible results were obtained when activation was carried out in siliconed tubes, which prevent the adsorption of the formed thrombin onto glass. At intervals, 0.2 ml. samples of the activation mixtures, in siliconed tubes, were removed with siliconed pipettes and added to 0.2 ml. fibrinogen, in glass tubes, and the clotting times obtained. The concentration of each lot of prothrombin was adjusted so that approximately a 10 second clotting time was obtained after complete activation under optimal conditions.

*Thrombin "unitage"*: For simplification in interpretation of the results we have converted clotting times to "units." The most active clotting mixture, after full activation, was arbitrarily chosen as containing 100 thrombin "units" (per 0.2 ml.). Precise dilutions of this mixture were prepared in siliconed tubes, employing, as diluting fluid, the same materials as in the activation mixture but without the prothrombin or proconvertin. Clotting times for each dilution were obtained in triplicate, and a curve, relating concentration of the thrombin mixture and clotting time, constructed. By reference to this curve, clotting times could then be converted to these arbitrary "units."

The results presented below are those of individual experiments, performed in duplicate, each of which is illustrative of a number of similar experiments carried out on different lots of prothrombin and proconvertin.

TABLE I  
*Controls (run 4)\**

Mixtures	Soluplastin ( <i>dil. 1-5</i> )	AcG ( <i>dil. 1-10</i> )	Prothrombin	Proconvertin	Thrombin yield "units"	Incubation time (25° C.) minutes
A	0.5	0.2	0.1	0.3	100.0	3
B	0.5	0.2	0.1		95.0	10
C	0.5	0.2		0.3	4.0	3
D	0.5		0.1	0.3	5.5	60
E		0.2	0.1	0.3	1.0	60

\* All activation mixtures contained 0.5 ml. 0.15 M CaCl<sub>2</sub>, 0.5 ml. imidazole buffer, and the reagents listed above in a total volume of 5 ml.

## RESULTS

*Identification and purity of reagents:* In order to establish the purity of our reagents a number of control tests were carried out.

Five ml. mixtures containing 0.5 ml. imidazole and the other ingredients (calcium, thromboplastin, accelerin, prothrombin, proconvertin) either singly or in any of the possible combinations were tested for thrombin formation at intervals during a one hour incubation period. Appreciable thrombin was formed only in those mixtures shown in Table I, which is illustrative of a typical run. The complete mixture (A) activated rapidly, and this mixture was designated as yielding 100 "units" of thrombin. In the absence of proconvertin (B), 95 "units" of thrombin were formed, but the time of complete activation was considerably slower. When proconvertin was activated in the absence of added prothrombin (C), 4.0 "units" of thrombin were formed. If accelerin or thromboplastin were omitted (D,E) there was only slight activation during a one hour period.

It seems probable that a pure prothrombin preparation would *not* activate in the absence of proconvertin. In one experiment, the possibility that proconvertin might be present as a contaminant of thromboplastin was explored. Various thromboplastin preparations (rabbit brain, dog brain, beef lung, dog lung, cephalin) were substituted for soluplastin, but in each case some activation of the prothrombin occurred.

All of our prothrombin preparations have activated in the absence of added proconvertin, yield-

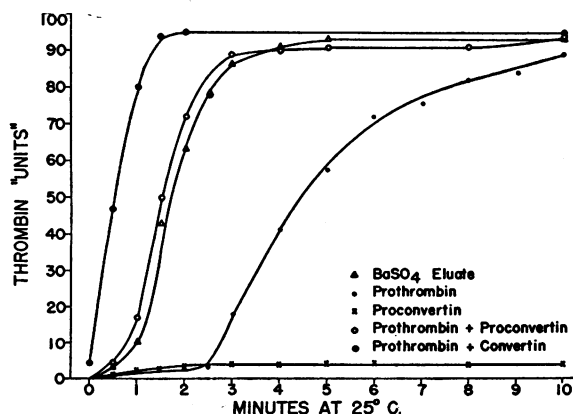


FIG. 1. THROMBIN FORMATION FROM HUMAN BaSO<sub>4</sub> ELUATE, PROTHROMBIN, AND PROCONVERTIN

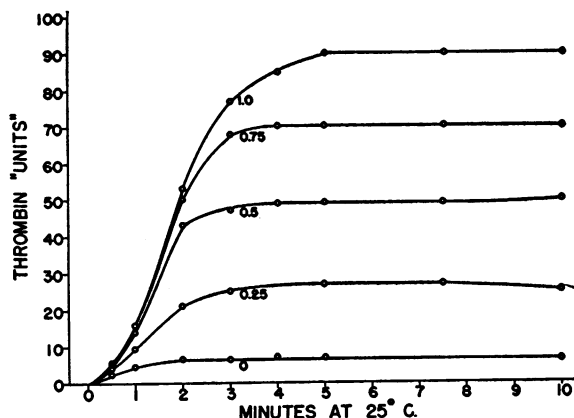


FIG. 2. EFFECT OF VARYING PROTHROMBIN CONCENTRATION

The prothrombin solution was diluted 1 to 10 and employed in 1.0, 0.75, 0.5 and 0.25 ml. amounts. All tests contained 0.2 ml. proconvertin.

ing from 70 to 100 per cent of the expected quantity of thrombin. We have roughly estimated the quantity of contaminating proconvertin from the incubation time necessary to reach maximal activation. This has varied from 10 to 30 minutes with different lots of prothrombin. The quantity of prothrombin contaminating a proconvertin preparation can be readily assayed, as it is rapidly converted to thrombin. In different fractionation runs the proconvertin preparations have formed from 0.5 to 6 "units" of thrombin.

In Figure 1, the results of activation of the BaSO<sub>4</sub> Eluate and its two fractions, "prothrombin" and "proconvertin," are compared. The BaSO<sub>4</sub> Eluate activated rapidly, while the prothrombin activated much more slowly, but yielded almost as much thrombin. The proconvertin also activated rapidly, but the thrombin yield was low. When the prothrombin and proconvertin fractions were mixed, the activation curve closely resembled that of the initial BaSO<sub>4</sub> Eluate. The most rapid activation was observed in a test in which convertin, formed by pre-incubation of the proconvertin with calcium and thromboplastin, was added to the prothrombin (see below).

*Effects of varying prothrombin concentration:* In this experiment (Figure 2) prothrombin in varying amounts was activated in the presence of a standard concentration of proconvertin. The thrombin yields, allowing for that formed from the proconvertin, were found to be proportional to the

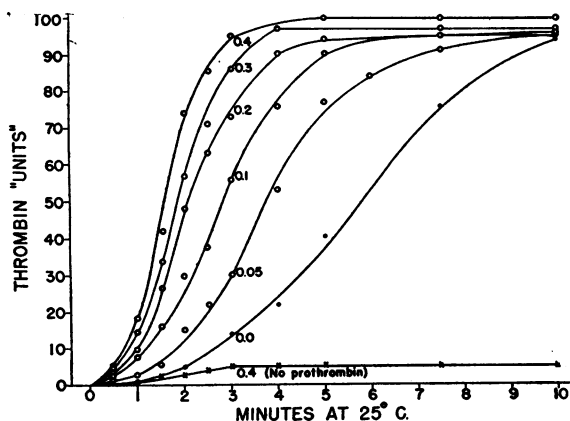


FIG. 3. EFFECTS OF VARYING PROCONVERTIN CONCENTRATION

added prothrombin concentrations. The proconvertin alone (curve 0) yielded 6 "units" of thrombin. If these 6 "units" were subtracted from the observed thrombin "units," they became proportional to the initial prothrombin concentrations: 84, 64, 44, 21 : : 1.0, 0.75, 0.5, 0.25.

The same concentrations of prothrombin were activated in the absence of added proconvertin (not illustrated). The activation rates were very slow, the maximal thrombin yield (between 70 to 80 per cent of that expected) being obtained after 15 to 30 minutes incubation.

**Effects of varying proconvertin concentration:** In this experiment the prothrombin concentration was maintained constant, and the proconvertin concentration varied. The results (Figure 3) clearly show that the rate of activation of prothrombin (in the presence of standard concentrations of calcium, tissue thromboplastin, and accelerin) is dependent upon the concentration of proconvertin. The small differences in final thrombin yields may be accounted for by the varying amounts of prothrombin present as a contaminant of the proconvertin.

**Activation of proconvertin:** In even the highest concentration of proconvertin employed in the experiment illustrated in Figure 3, there was an initial lag in thrombin formation during the first minute of incubation. Pre-incubation of the proconvertin with thromboplastin and calcium, as first suggested by Mann (25) in his studies on co-thromboplastin, eliminates this lag (see Figure 1). Experiments not illustrated showed that the addition of accelerin during the pre-incubation was

not essential to this conversion of proconvertin to convertin. Pre-incubation of the proconvertin with either calcium or thromboplastin alone did not cause the formation of convertin. Additional experiments also showed that the addition of thrombin to the usual proconvertin-prothrombin mixture did not eliminate the initial lag in thrombin formation.

**Rate of activation of proconvertin:** In a series of identical mixtures, the proconvertin was incubated with calcium and thromboplastin for varying lengths of time before addition to the prothrombin and accelerin. The results are illustrated in Figure 4. After 10 minutes pre-incubation of proconvertin, the initial lag in thrombin formation had been eliminated. Longer pre-incubation did not increase the convertin activity.

**Effects of varying convertin concentration:** In this experiment, various amounts of proconvertin were pre-incubated for 10 minutes with calcium and thromboplastin before addition to prothrombin and accelerin. The results, illustrated in Figure 5, show that the rapidity of activation of prothrombin was dependent upon the convertin concentration.

**Action of thromboplastin:** The experiments presented above showed that calcium and thromboplastin convert proconvertin to convertin. We attempted to determine whether calcium and thromboplastin were also important in the conversion of prothrombin to thrombin in the presence of *convertin* and accelerin. In order to do this, proconvertin was incubated with calcium and thromboplastin and then attempts were made to remove these activators. Although a number of

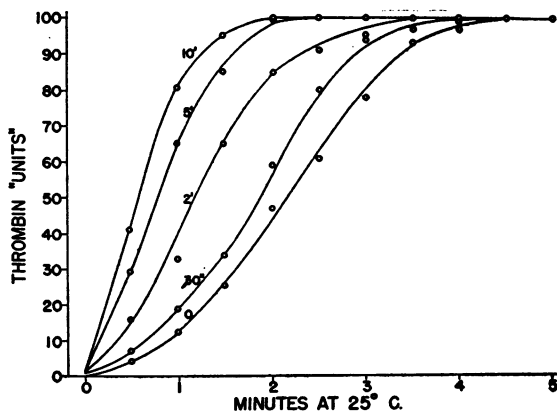


FIG. 4. ACTIVATION OF PROCONVERTIN

procedures for the removal of calcium (passage through the ion-exchange resin, Dowex 50, oxalation, citration, and dialysis) were tried, no convertin activity could be demonstrated after removal of the calcium.

On the other hand, substituting freshly prepared dog lung thromboplastin for soluplastin, it was possible to show full convertin activity after removal of most of the thromboplastin by high speed centrifugation. The results are shown in Figure 6. In the first test (A), calcium, dog lung thromboplastin, and proconvertin were incubated for 10 minutes and then added to prothrombin and accelerin. In the next test (B) proconvertin was again incubated with thromboplastin and calcium, and the mixture was centrifuged at 20,000 RPM for 20 minutes before adding to the prothrombin and accelerin. In test (C) the same procedure of incubation and centrifugation was followed as for test (B), but fresh thromboplastin was added along with the prothrombin and accelerin in the final activation mixture. In the last test (D), the calcium and thromboplastin were incubated, without proconvertin, and centrifuged before adding to the prothrombin, proconvertin, and accelerin. This test shows that a trace of thromboplastin remained after the centrifugation, as some thrombin was formed. In the absence of thromboplastin, no activation was observed. The similarity between curves (A) and (C) and their striking difference from curve (B) suggest that convertin is present after the removal of thromboplastin, but that it can fully activate the prothrombin only in the presence

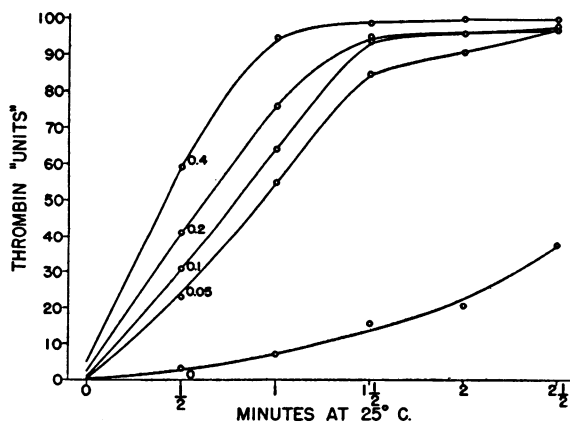


FIG. 5. EFFECTS OF VARYING CONVERTIN CONCENTRATION

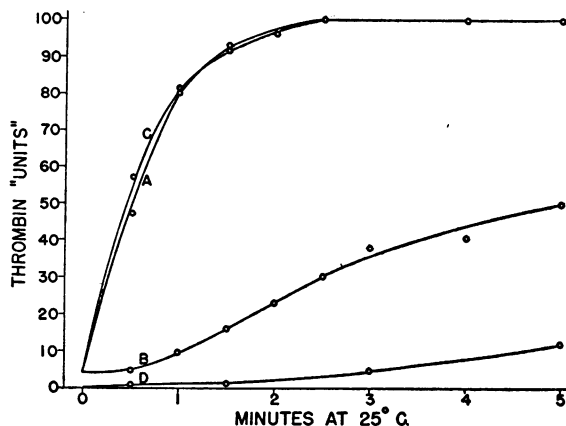


FIG. 6. ACTION OF THROMBOPLASTIN (SEE TEXT)

of adequate thromboplastin, as well as accelerin and calcium.

*Stability of prothrombin and proconvertin:* In order to test the stability of prothrombin and proconvertin, samples were heated to various temperatures, while control samples were kept at 4° C. In Table II the residual activity, after treatment, is recorded as percentage of that in the control samples. Proconvertin activity was estimated by comparison to a series of activation curves in which the concentration of the control proconvertin was varied. The results suggest that both fractions are relatively heat labile.

#### DISCUSSION

These investigations show that human prothrombin and proconvertin can be partially separated from oxalated blood plasma by a simple fractionation procedure. Koller, Loeliger, and Duckert (23) and Owren (1, 22) have accomplished a similar separation of these factors from bovine blood by using a differential adsorption method. However, they state that this technic does not work on human blood. The excellent studies of Alexander and his co-workers (1, 13-20) have shown that human serum contains, in addition to accelerin, another factor, which they have called SPCA. Apparently SPCA is very similar to proconvertin and may prove to be a mixture of proconvertin and convertin. Our studies confirm many of the findings of these workers.

Activation of our human plasma fractions in the presence of standard concentrations of calcium,

TABLE II  
Stability of prothrombin and proconvertin

Temperature	Residual activity	
	Prothrombin	Proconvertin
37° C. for 4 hours	82	100
56° C. for 5 mins.	30	34
65° C. for 3 mins.	14	28

thromboplastin, and accelerin gave final thrombin yields which were quantitatively dependent upon the initial concentration of prothrombin, but not of proconvertin. On the other hand, rate of thrombin formation was dependent upon proconvertin concentration and, in the presence of a fixed amount of prothrombin, the quantity of thrombin measured in early stages of activation was almost directly proportional to the proconvertin concentration. This strongly suggests that the ordinary one-stage prothrombin time (Quick test), in the presence of normal proaccelerin, largely reflects proconvertin, rather than prothrombin concentration.

Our findings that calcium and thromboplastin (but not accelerin) activate proconvertin to convertin extend the data of Mann and Hurn (25, 26), Landwehr and Alexander (18), and Owren (1), which previously pointed to some non-prothrombin plasma factor being activated by calcium and thromboplastin. In our cited isolated systems, complete activation of proconvertin by calcium and thromboplastin required about ten minutes, but other experiments, not illustrated, indicate that the rate of convertin formation is very dependent upon the potency of the thromboplastin. This observation might explain the fact that fractions, which we have prepared from spontaneously clotted serum, have contained primarily proconvertin.

During spontaneous plasma coagulation, thrombin formation is very slow during the first few minutes. This "lag phase" can be partially eliminated by introducing tissue thromboplastin, which by-passes the physiological action of a number of plasma substances and platelets. In our isolated system, we are able to reduce still further this "lag phase" by employing accelerin instead of proaccelerin. Complete elimination of the "lag phase" is accomplished by substituting convertin for proconvertin.

Additional experiments showed that convertin was unable to form thrombin from prothrombin unless calcium, thromboplastin, and accelerin were also present.

#### CONCLUSIONS

1. The final thrombin yield, in the presence of standard amounts of calcium, thromboplastin, accelerin, and proconvertin, is dependent upon the initial prothrombin concentration.
2. The rate of thrombin formation, in the presence of standard amounts of calcium, thromboplastin, accelerin, and prothrombin, is dependent upon the proconvertin concentration.
3. Proconvertin is activated to convertin by calcium and thromboplastin. The presence of accelerin is not necessary.
4. The "lag phase" in thrombin formation in a mixture containing prothrombin, thromboplastin, calcium, accelerin, and proconvertin can be eliminated by pre-incubation of the proconvertin, calcium, and thromboplastin.

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