Platelet cothromboplastin: a platelet factor(s) related to the blood clotting mechanisms*)

From the Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Michigan, USA

Pyung-Hee Lee,**) Shirley A. Johnson and Walter H. Seegers

A brief review of the literature on Russell's viper venom as related to the clotting of blood was presented by Lee, Johnson, and Seegers (1). From their experiments with the use of purified prothrombin, it was indicated that the venom functions effectively with platelets in the conversion of prothrombin to thrombin. The factor in platelets was found to be different from platelet factor 3, but no further information was available related to its possible identity with previously recognized clotting factors. In the work described in this paper, a concentrate was obtained from platelets and it contains the factor of Lee, Johnson and Seegers (1). It functions with Russel's viper venom in the conversion of purified prothrombin to thrombin and also with tissue thromboplastin in the conversion of prothrombin to thrombin. This latter fact is the basis on which we propose to refer to this factor as *platelet cothromboplastin.* Quantitative assay procedures have been developed, and some of the properties of our concentrates are described.

Materials and methods

All the materials and general methods used in these experiments have been described previously as follows: (a) purified prothrombin (2, 3, 4); (b) purified prothrombin free of Ac-globulin (4); bovine platelet preparations (5); assay for thrombin activity (6); and assay for prothrombin activity (7). Russels's viper venom was purchased from the Burroughs Wellcome & Co., and a solution containing 0.001 mg of dried venom per ml in physiological saline was used.

*) This investigation was supported by a research grant H-2026 (c) form the National Heart Institute, National Institutes of Health. Funds for Research Fellowships in Physiology were provided by Parke, Davis and Company.

**) Fellow of the Presbyterian Church Mission Board of the United States of America. Home address: Department of Physiology, Severence Union Medical College, Seoul, Korea. By coincidence a previous paper on this subject was also by Lee, Johnson and Seegers; however, in that instance the first author was Chen-Yuan Lee of Formosa.

Experimental

Quantitative approximation of platelet cothromboplastin activity. In confirmation of previous work (1), it was found that the yield of thrombin obtained from purified prothrombin is very low in a mixture consisting of purified prothrombin(Ac-globulin not removed) calcium ions and Russel's viper venom. By adding large amounts of platelet material, conversion of the prothrombin to thrombin occurs rapidly. If small amounts of platelet suspension are used the yield of thrombin is incomplete and the amount of thrombin obtained is more or less an index of the amount of platelet material in the reaction mixture. These relationships were exploited for the assay of platelet cothromboplastin activity. We measured the thrombin yield in the following standardized reaction mixture:

Purified prothrombin (3000 units per ml)	1.0 ml
Snake venom solution	0.5 ml
$CaCl_2$ (0.162 M) in imidazole buffer	0.5 ml
Platelet suspensions or fractions	0.5 ml
Physiological saline	0.5 ml

By measuring the thrombin concentration in the reaction mixture described above, the "standard" curves of Fig. 1 were reproduced easily. By definition 0.1 ml of packed bovine platelets, disintegrated with ultrasonic waves, contain 600 units of platelet cothromboplastin activity. In actual practice, the platelets were centrifuged at 3000 g for 20 minutes, then one part of the suspension was mixed with four parts of physiological saline solution. The suspension was frozen, thawed, and subjected to ultrasonic waves (800 kc) for 4 minutes. The various dilutions of the platelet suspension were placed in the reaction mixture described above. Then quantitative thrombin analyses were performed and the curves of Fig. 1 were plotted. Whenever an unknown sample was tested for activity, the yield of thrombin was determined in the standardized reaction mixture. Sometimes this was translated into units of platelet cothromboplastin and sometimes the results were considered directly in terms of thrombin units.

Purification of platelet cothromboplastin. The clotting of blood consists of a large number of chemical interactions and this implies that several substances are involved. Alexander Schmidt postulated the existance of prothrombin and the actual isolation and characterization of such a substance has now been achieved. Heparin and fibrinogen have also been characterized and it should be possible to obtain any hypothetical substance in purified form. However, at first even concentrates suffice to substantiate the view that observed phenomena cannot be without the involvement of one or more molecular entities not previously described. Failure to obtain concentrates of a supposed substance may be due to our inability to apply appropriate manipulations or due to

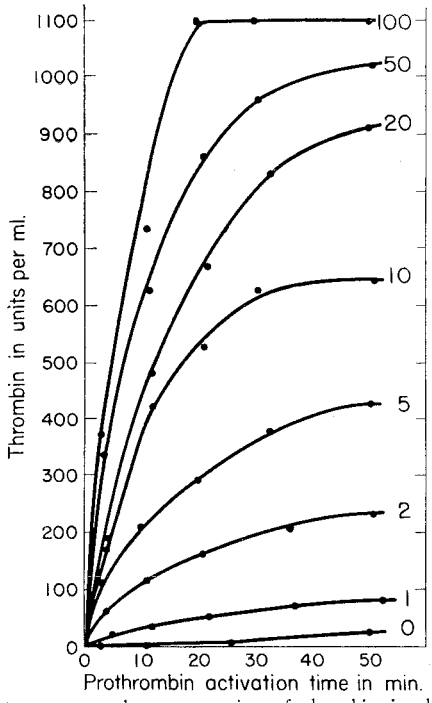

Fig. 1: The top curve represents the concentration of thrombin in the standardized prothrombin reaction mixture when platelet cothromboplastin activity was equivalent to 0.1 ml packed bovine platelets. Before assay, the platelets are broken up with the use of ultrasonic waves. The other curves are obtained with proportionately less platelet factor, each number at the end of the curve indicating the amount. For assay of an unknown sample, the platelet activity is diluted so as to obtain a curve between 5 and 50.

inaccuracy of the supposition. These ideas motivated our attempts to obtain concentrates of platelet cothromboplastin.

In many exploratory experiments, certain properties of the platelet factor were noted. It is apparently adsorbed on charcoal, not on kaolin and not on barium carbonate unless large quantities of the adsorbing agent are used. Ether extraction of platelet suspension neither destroyed nor removed the activity. Fractionation with the use of ammonium sulfate was possible and physiological saline solutions containing the activity could be dried from the frozen state without loss of avtivity during the drying operation. The platelet source materials could be stored in the deep freeze for more than eight months without loss of activity. This preliminary information was obtained and utilized in many purification attempts and the procedure described below is our most successful method and has been repeated many times.

Packed bovine platelets serve as starting material. Any suitable quantity is used; however, the description given below is written in terms of 4 ml of starting material. The platelets are mixed with water to give a 1 : 5 dilution, and frozen in a deep freeze where they may be obtained at any convenient time

later. After thawing the platelets, now extensively broken down, are centrifuged at 2000 g and washed twice with 40 ml cold physiological saline. The supernatant solutions contain the platelet clottable factor (8) and are discarded. The mass of platelet material is suspended in 40 ml of cold saline and forced through a mechanical emulsifier or homogenizer (Catalog No. 70180, Central Scientific Company, 1700 Irving Park Road, Chicago, Illinois). The particles are now degraded by subjecting the suspension to ultrasonic waves (800 kc) for 4 minutes. This treatment usually increases the activity of the preparation. Upon centrifugation in a refrigerated centrifuge at 2000 g for 10 minutes, one finds the desired activity in the supernatant fluid from which impurities can be selectively adsorbed on barium carbonate. For that purpose barium carbonate is first washed with physiological saline solution and then mixed with the preparation in the amount of 100 mg barium carbonate per ml. Centrifugation in a refrigerated centrifuge at 2000 g for 10 minutes removes the adsorbing agent. With ammonium sulfate fractionation, impurities are removed at 25% of saturation and the active material precipitates when the concentration of ammonium sulfate is raised to 50% of saturation. To carry this procedure through, the solution is cooled to 0° C and ammonium sulfate solution, saturated at room temperature, is added dropwise and with stirring so that the temperature remains near zero. The precipitate obtained at 25% of saturation is quite large and is discarded. The precipitate obtained at 50% saturation contains the active material and is dissolved in 10 ml distilled water and dialyzed efficiently against cold distilled water. For this purpose the apparatus described previously is used (3). In a few hours the specific resistance is 2000 ohms or more. Sometimes the pH is considerably below neutrality and when that is observed an adjustment to pH 7.0 is made by adding a little dilute alkali. The solution can then be dried from the frozen state without loss of activity. The average yield is about 20%, and from 1 to 1.5 mg of the dry product are obtained for each ml of packed platelets. Only a portion of the dry material is soluble in water; and since the insoluble material is apparently inactive further purification is achieved by simply adding one ml of water to each 10 mg of dry material. By centrifuging at 106,000 g for 2 hours a clear supernatant solution is obtained. This can again be dried from the frozen state.

Stability Related to pH. An attempt was made to find the limits of acidity and alkalinity within which this factor is stable. The results of these experiments are presented with Fig. 2. The purified material was first dissolved in water. Samples were then adjusted to various desired pH values by adding either 0.01 N acetic acid or 0.01 N sodium hydroxide. After 30 minutes standing at room temperature the solutions were brought to pH 7 by adding either acid or alkali. Each sample was then tested for activity and the results were corrected for volume changes due to the addition of acids or alkali. It was found that the

material is stable in the range pH 4 to 11 and outside of these limits the activity is drastically reduced.

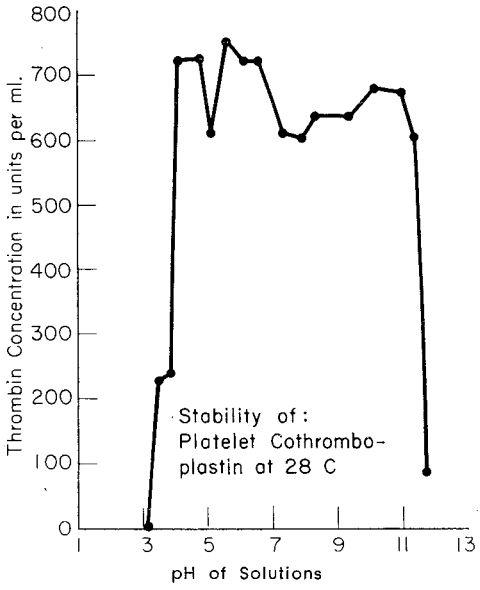

Fig. 2: Loss of platelet cothromboplastin activity observed in acid and alkaline solutions

Temperature and Stability. The object of this work was to find the minimum temperature required for destroying the activity. This study was made with a preparation in water solution. The pH was 7.2. The solutions were rapidly brought to the desired temperature, kept there for 30 minutes, and then rapidly cooled and tested for activity. At 50° C there was very little denaturation, but at higher temperatures there was much loss of activity (Fig. 3). It was interesting that some activity remained even at 60° C. This platelet factor is thus far more heat labile than purified platelet factor 3.

In water solutions at pH 7.2 full activity was maintained for many months in a deep freeze and for 70 days in an ice box. In 50% glycerol solution full activity was also maintained for a period of 70 days, and then no more analysis were done. Bubbling oxygen through these solutions did not destroy the activity. Platelet cothromboplastin is thus relatively stable.

Electrophoresis Experiments. About 45 mg of material were dissolved in 6 ml of veronal buffer of pH 8.6, ionic strenght 0.1. This was dialyzed against a liter of the same buffer overnight. Since the material was not plentiful, only the ascending boundary was formed and studied with the Aminco-Stern electrophoresis apparatus. The solubility of the preparation was found to be low in our solvent. The observed boundary was a sharp single peak which showed no resolution after 90 minutes of electrophoresis. The mobility calculated on the

basis of data at 60 and at 90 minutes of electrophoresis was 4.1×10^{-5} cm/v/sec. We wish to thank Dr. Richard Fenichel for his technical assistance in this phase of our work.

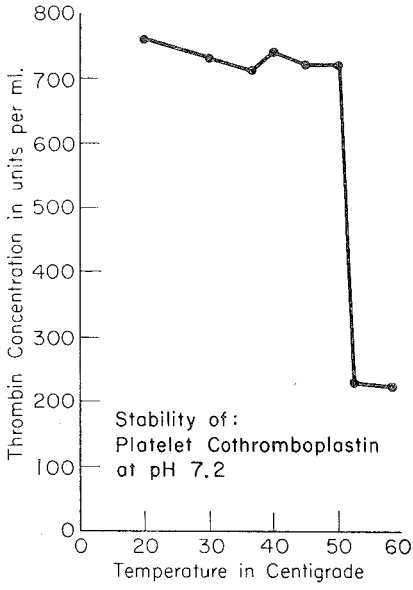

Fig. 3: Platelet cothromboplastin activity is destroyed at temperatures higher than 50° C.

Miscellaneous Observations. For nitrogen analysis (Dumas) the values 7.47% and 7.32% were obtained. Carbohydrate, determined by the anthrone method of Dreywood (9) and with equal quantities of galactose and mannose as standard, yielded 13.2%; 12.0%; 13.2% and 16.0% respectively with four different products. Dr. J. R. Carter of the State University of Iowa kindly examined the material for sulfur by amperometric technic (10). No sulfhydryl could be detected. On the other hand 1.0 micromole of disulfide per 100 mgm of material was found. Quantitative analysis for phosphorus by the method of Milton and Waters (11) indicated 0.14% P. This value is low and minimizes the possibility of a phospholipid being the active component. Infrared absorption properties were examined by mixing with potassium bromide and compressing under high pressure. The absorption bands were distinct and could be interpreted on the basis of carbohydrate and protein being in the preparation. In another kind of experiment, some of the platelet cothromboplastin was placed in saline solutions. An equal volume of ether was added and after vigorous shaking an attempt was made to measure the activity in the ether and also in the aqueous phase. Neither then contained any appreciable amounts of platelet cothromboplastin activity.

Cothromboplastin Activity of Platelet Preparation. The only known activity of the platelet material discussed thus far is related to the conversion of prothrombin to thrombin in combination with snake venom. Apparently purified prothrombin converts to thrombin according to the following equation:

$$\text{Prothrombin} \xrightarrow[\text{Russell's viper venom}]{\substack{Ca^{++} \\ \text{Ac-globulin} \\ \text{Platelet cothromboplastin}}} \text{Thrombin}$$

After several attemps to find other substances with which activity might develop we found that this material is a thromboplastin cofactor. This information was obtained using the materials and technics described by Seegers, Alkjaersig and Johnson (12) in their studies on autoprothrombin I. In the presence of Ac-globulin, calcium ions, and *dilute* lung extract thromboplastin only a small amount of thrombin is obtained from a purified prothrombin substrate; but if purified platelet material is also added the prothrombin substrate is rapidly converted to thrombin (Fig. 4). One activity of this platelet factor can thus be indicated by the following equation in which the word thromboplastin is the activity from lung extracts:

$$\text{Prothrombin} \xrightarrow[\text{Thromboplastin}]{\substack{Ca^{++} \\ \text{Ac-globulin} \\ \text{Platelet cothromboplastin}}} \text{Thrombin}$$

Discussion

In previous work, relating Russell's viper venom to the blood clotting mechanism, emphasis was placed on lipids derived from various sources (13—16). We do not exclude the possibility that a lipid is also present in our preparation, because our purification work does not provide a product consisting of a single substance. It consists mostly of protein and carbohydrate. The phosphorus content is so low that any phospholipid would have to be present in low concentration. We can, therefore, invite attention to the likelihood that the active material is not a lipid.

Of greater interest is the observation that the platelet material is a cothromboplastin; that is, it greatly enhances the activity of dilute thromboplastin solutions in the conversion of prothrombin to thrombin. This is comparable to the chief distinguishing feature in the activity of autoprothrombin I. The latter is found in serum, and since it is also a cothrombinplastin, it should function with

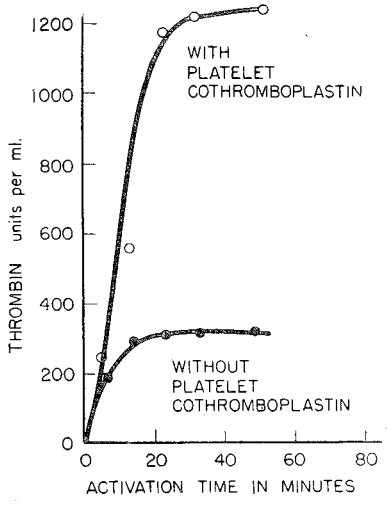

Fig. 4: A purified prothrombin solution is only partly converted to thrombin in association with calcium ions, and diluted lung extract thromboplastin. By supplementing with platelet cothromboplastin, 10 units per ml, the prothrombin substrate is all converted to thrombin. If there is no Ac-globulin in the prothrombin preparation, the platelet cothromboplastin is not effective.

snake venom. By this logic we were led to try the activity of autoprothrombin I in association with the snake venom, and it was found that autoprothrombin I *does* function *with* Russell's viper venom (17) in the activation of purified prothrombin.

In the papers of Hjort, Rapaport and Owren (15) and Rapaport, Aas and Owren (16), they do not as yet consider that proconvertin is prothrombin and that convertin (autoprothrombin I) is derived from prothrombin. By taking that view we can consider their work with snake venom in relationship to ours. Firstly, when they find that a combination of Russell's viper venom and lipid is independent of the proconvertin (prothrombin) or convertin (autoprothrombin I) content of the plasma, we assume their observations are accurate for the conditions of their study. Secondly, this does not exclude that Russell's viper venom functions *with* autoprothrombin I. When two roads lead to the same destination, the predominant use of one does not exclude the other and vice versa. We have found conditions where autoprothrombin I functions *with* the snake venom and where it functions with material in platelets that has the same kind of activity as autoprothrombin I.

Summary

Bovine Platelets contain a factor(s) that greatly enhances the capacity of lung extract thromboplastin to conver purified prothrombin to biothrombin. It also may function with Russell's viper venom in the activation of prothrombin

The activity is destroyed below pH 4 or above pH 11 or at temperatures above 52° C. Concentrates of the activity have been obtained in a fraction of platelets that consists predominately of carbohydrate and protein.

Résumé

Les plaquettes bovines ont un facteur qui accélère l'activité de la thromboplastine tissulaire pour convertir la prothrombine en biothrombine. Ce facteur a la même influence sur le Russell's viper venom. Cette activité est abolie au pH inférieur à 4 ou supérieur à 11, et disparaît également par chauffage audessus de 52° C. Une préparation à haute activité a été obtenue dans une fraction plaquettaire formée essentiellement par des hydrates de carbone et des protéines.

Zusammenfassung

Rinderthrombozyten enthalten einen Faktor (oder Faktoren), der die Fähigkeit von Lungenextraktthrombokinase, gereinigtes Prothrombin in Biothrombin umzuwandeln, stark erhöht. Dieser Faktor entfaltet seine Wirkung auch gemeinsam mit Russel-Vipern-Gift bei der Aktivierung von Prothrombin. Seine Aktivität wird bei einem pH unter 4 und über 11 sowie bei Temperaturen über 52° C zerstört. Die Aktivität konnte in einer vorwiegend aus Kohlehydraten und Eiweiß bestehenden Thrombozytenfraktion konzentriert erhalten werden.

References

(1) Lee, C. Y., Johnson, S. A. and Seegers, W. H.: Clotting of blood with Russell's viper venom. J. Mich. med. Soc. **54:** 801 (1955).

(2) Seegers, W. H., Loomis, E. C. and Vandenbelt, J. M.: Preparation of prothrombin products. Isolation of prothrombin and its properties. Arch. Biochem. **6:** 85 (1945).

(3) Seegers, W. H.: The purification of prothrombin. Rec. Chem. Prog. **13:** 143 (1953).

(4) Ware, A. G. and Seegers, W. H.: Studies on prothrombin: Purification, inactivation with thrombin, and activation with thromboplastin and calcium. J. biol. Chem. **174:** 565 (1948).

(5) Schneider, C. L., Claxton, E. B., Hughes, C. H. and Johnson, S. A.: Bovine platelets in large quantities, properties, and activities concerned with hemostasis. Amer. J. Physiol. **179:** 236 (1954).

(6) Seegers, W. H. and Smith, H. P.: Factors which influence the activity of purified thrombin. Amer. J. Physiol. **137:** 348 (1942).

(7) Ware, A. G. and Seegers, W. H.: Two-stage procedure for the quantitative determination of prothrombin concentration. Amer. J. clin. Path. **19:** 471 (1949).

(8) Ware, A. G., Fahey, J. L. and Seegers, W. H.: Platelet extracts, fibrin formation and interaction of purified prothrombin and thromboplastin. Amer. J. Physiol. **154:** 140 (1948).

(9) Dreywood, R.: Qualitative test for carbohydrate material. Ind. Engng. Chem. Anal. Ed. **18:** 499 (1946).

(10) Carter, J. R.: Amperometric Determination of disulfides in intact proteins. Science **120**: 895 (1954).
(11) Milton, R. F. and Water, W. A.: Methods of quantitative microanalysis. Arnold, London, 1949.
(12) Seegers, W. H., Alkjaersig, N. and Johnson, S. A.: Formation of autoprothrombin I in solutions containing purified prothrombin and purified platelet factor 3. Amer. J. Physiol. **181**: 589 (1955).
(13) Macfarlane, R. G., Trevan, J. W. and Attwood, A. M. P.: Participation of a fat-soluble substance in coagulation of the blood. J. Physiol. (London) **99**: 7 (1941).
(14) Leathes, J. B. and Mellanby, J.: The action of lecithin on the thrombokinase of deboia venom and of brain extracts. J. Physiol. (London) **96**: 39 (1939).
(15) Rapaport, S. I., Aas, K. and Owren, P. A.: The clotting action of Russell's viper venom. Blood **9**: 1185 (1954).
(16) Hjort, P., Rapaport, S. I. and Owren, P. A.: A simple specific one-stage prothrombin assay using Russell's viper venom in cephalin suspension. J. Lab. clin. Med. **46**: 89 (1955).
(17) Seegers, W. H., Johnson, S. A. and Penner, J. A.: Quantitative concepts related to prothrombin and autoprothrombin I activity. Can. J. Biochem. Physiol. **34**: 887 (1956).