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Thromboplastinase, a New Enzyme Which Destroys Thromboplastin. (20515)

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The nature of thromboplastin has long been a subject of investigation. This material is clearly described only in terms of its activity: the initiation of the blood coagulation process. The most active, best characterized thromboplastic preparation to date is the lipoprotein fraction obtained by differential centrifugation according to Chargaff(1). Whether thromboplastic activity is associated with a specific molecule, a type molecule, or a combination of factors awaits clarification. The nature of the active lipid moiety of thromboplastic preparations likewise remains obscure. The relationship between the active materials in thromboplastic suspensions prepared from different tissues, as well as that found in human blood is undetermined. The preparation, preservation, variation in activity and fundamental mode of action of this material consequently remain empirical and ill-understood.

This laboratory recently reported on the marked reduction of thromboplastic potency in tissue suspensions due to a bacterial contaminant(2). The organism was later found to accomplish this potency reduction by virtue of an adaptive enzyme which it elaborated. Bacteriological studies are presented elsewhere (3).

This communication will describe the preparation and properties of the new enzyme, tentatively called "thromboplastinase" which we believe will be a useful tool in the investigation of thromboplastin. Our studies have led to evidence for a structural moiety necessary for thromboplastic action which is common to thromboplastic suspensions of different origins.

Materials. Glassware. All glassware was scrupulously cleaned in detergent, dichromate cleaning solution and then thoroughly rinsed. *Plasma.* Normal human plasma was collected in 1/9 volume oxalate in glass test tubes, centrifuged, and the supernatant plasma removed. *Thromboplastin.* Rabbit brain(4), human placenta(5), or human brain(3) were used as sources of thromboplastin. *Thromboplastinase.* An adaptive enzyme elaborated by a *Bacillus cereus-B. megatherium* intermediate under certain conditions.†‡ This organism grew well in a simple medium of salts and sugar, without elaborating thromboplastinase. However, if the substrate was a biological source medium such as a thromboplastic tissue extract or Brain Heart Infusion, (Difco), the enzyme was formed. Growth and enzyme production proceeded as well when the dialysate of Brain Heart Infusion was used as the medium. The dialysate was therefore employed to avoid the extraneous protein of the Brain Heart Infusion. The following method was then used to obtain crude enzyme.

A Brain Heart Infusion dialysate was prepared by placing a dialysis bag containing 250 ml of 0.9% Brain Heart Infusion into 1 liter of distilled water in a 2 liter flask and autoclaving at 15 lb for 20 minutes. Upon cooling, the sterile dialysate was seeded with a 24-hour (37°C) culture from a Brain Heart Infusion-agar slant and allowed to incubate at 37°C for 24 hours. During the course of this incubation, a progressive increase in enzyme activity of the culture medium was

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† Previously mistakenly reported to be *B. subtilis*.

‡ Obtainable from ATCC as No. 10987 (NR Smith's strain 248).

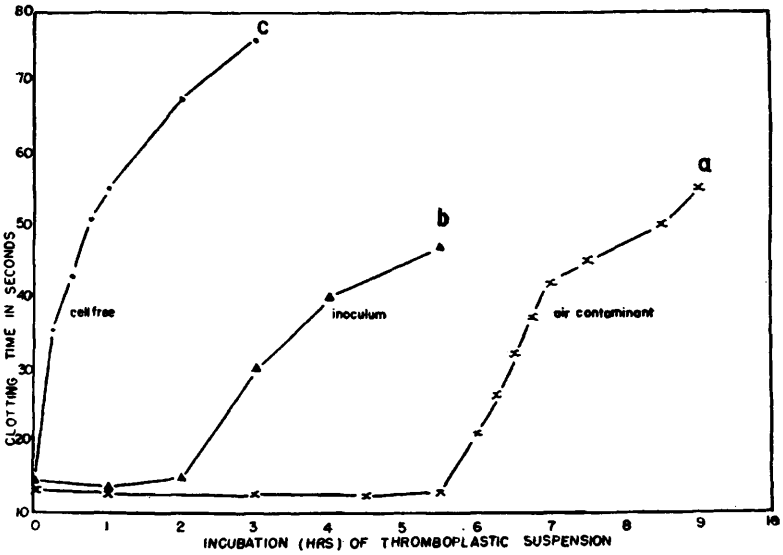

FIG. 1. Time course of thromboplastin destruction by thromboplastinase; a. from air contaminant; b. from an actively growing culture of the organism introduced as such into the thromboplastic suspension; c. from a cell-free crude enzyme preparation.

demonstrable. Incubation for longer periods, or at higher temperatures resulted in decreased activity or decreased elaboration of enzyme. The culture medium (1 liter), rendered cell-free by centrifugation,§ was employed as a crude, active enzyme preparation. This crude cell-free preparation was stable for 2-3 days at 0-5°C. Partial purification and concentration of the enzyme preparation was effected by adsorption at 0-5°C on Ca_3 $(PO_4)_2$ in final concentration of 0.2%. Elution at 25°C with 100 ml of 0.5 M Na Citrate was followed by centrifugation.

After dialysis at 0-5°C, which resulted in no loss of activity, this preparation was lyophilized. The yield was about 25 mg of a fluffy white powder which was stable indefinitely under refrigeration. Aqueous solutions of this powder were stable for a week under refrigeration.

Methods. Assay of enzyme activity: A modified one-stage test. The activity of the enzyme was measured in terms of substrate (thromboplastin) destruction. This was conveniently done by adapting the one stage method of Quick(4) to a test for thromboplastin.

§ Seitz filtration is to be avoided as it causes a partial loss of enzyme activity by adsorption.

When equal parts of normal human oxalated plasma, isotonic NaCl and $CaCl_2$ (.025 M) were mixed at 37°C and the clotting time determined, clot formation was observed after about 2 minutes. The reaction rate was greatly increased (the clotting time was sharply decreased) to about 13 seconds when a suspension of active tissue thromboplastin was substituted for the isotonic NaCl in the above procedure. Less active thromboplastic suspensions gave longer clotting times. The clotting time, as a function of the thromboplastic concentration, is shown in Fig. 2 where the various concentrations of thromboplastin, in percent, were obtained by simple dilution with saline of the thromboplastic stock preparation.

Thromboplastinase action was gauged then by the loss of potency of thromboplastic suspension after the enzyme and substrate were mixed and incubated. The incubating mixture consisted of 4 parts by volume thromboplastin, 5 parts $CaCl_2$ and one part thromboplastinase. The residual thromboplastic potency of the mixture was estimated by using it as a source of thromboplastin in the one-stage test. The calibration curve shown in Fig. 2 was used to translate to percent thromboplastin the clotting times obtained with the

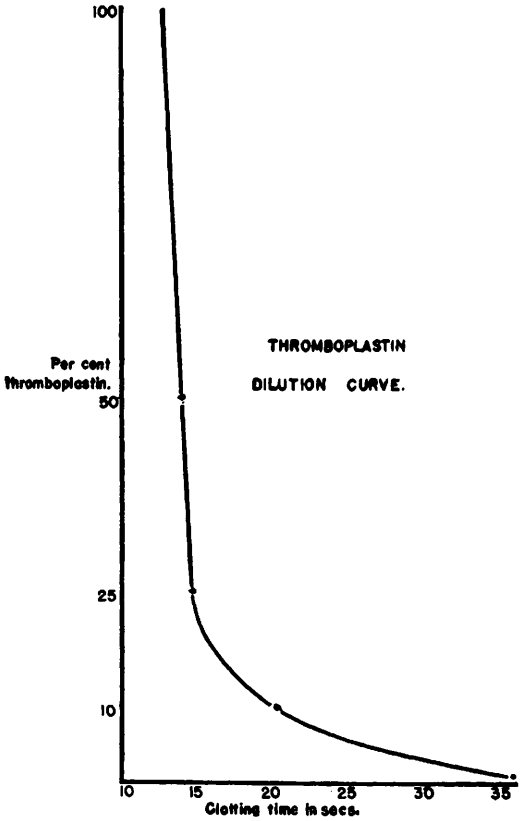

FIG. 2. Clotting time—dilution curve of an acetone-dried rabbit brain thromboplastin. 100% thromboplastin = least concentration which empirically yielded shortest clotting time.

incubated mixture.

To interpret the loss of thromboplastic activity as being due to destruction of thromboplastin, two conditions had to be satisfied: 1) boiled enzyme had to be ineffective and 2) no inhibitor should have been formed as a result of thromboplastinase activity. As will be seen below both these conditions were met. We therefore use Fig. 2 as a calibration curve for translation of clotting time to residual percent thromboplastin and by difference obtain the thromboplastin destroyed. Calibration curves should be set up for each laboratory, since thromboplastic potency is notoriously variable. Preparation of active, stable, reproducible thromboplastic suspensions has been described elsewhere(6).

Results. The time course of thromboplastic destruction by the bacterial enzyme thromboplastinase is shown in Fig. 1. Curve "a" represents that obtained when thromboplastic suspension is allowed to incubate in open vessels. The sudden rise of clotting time (loss of thromboplastic potency) was observed to occur after 6-8 hours. Plasma coagulation factors are not responsible for this clotting time rise(2).

Curve "b" represents that obtained when an actively growing culture of the organism is inoculated into the thromboplastic suspension. Here the "break" in the curve was seen to occur after about 2 hours.

Curve "c" is that obtained when a cell-free preparation of thromboplastinase was added to thromboplastic suspension. It is seen that thromboplastinase action was immediate.

The data summarized below indicate the enzymatic nature of thromboplastinase action. Fig. 3 shows that *the loss of thromboplastic potency caused by thromboplastinase was continuous over a time course.* Fig. 4 shows that *the rate of destruction of thromboplastin was a function of enzyme concentration. Small amounts of enzyme were effective*: using a crude preparation, less than 1 part of enzyme to 10,000 parts of thromboplastic suspension almost completely inactivated the latter in less than 5 minutes. *The active enzyme was recovered in the supernatant of the altered thromboplastic suspension.* In a typical experiment the enzyme concentration was adjusted so as to just give 90-100% inactivation of the thromboplastin in the given incubation time. Then employing high speed centrifugation (R.C.F. 20,000 G) the altered thromboplastic suspensions were separated from the supernatants containing the enzyme This supernatant was then used as the suspending fluid for fresh unaltered thromboplastic pellets obtained by similar centrifugation. Thromboplastic potency of this mixture was then determined after the same incubation period. Suitable controls were employed. The enzyme activity after 3 such recoveries was still high, approximately 98%, 92% and 83% thromboplastin being destroyed respectively.

The enzyme was inhibited by substances in trace amounts. The search for and use of inhibitors of thromboplastinase was complicated by the requirement that these inhibitors must not affect any of the reactions of the blood

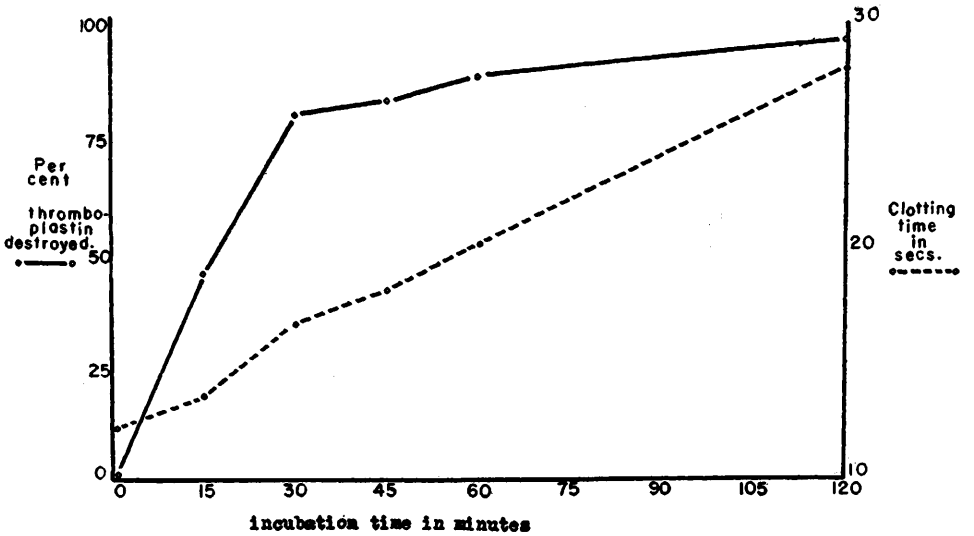

FIG. 3. Action of "thromboplastinase" on rabbit brain thromboplastin over a 120-min. time course. Boiled enzyme was ineffective.

clotting process since this is the means employed at present to assay thromboplastinase activity. Of many substances tested, cupric ion in final concentration of 10^{-4} was found to be suitable and to inhibit completely destruction of thromboplastin by thromboplastinase.

The action of thromboplastinase did not cause a pH change which might account for the loss of thromboplastic potency. When the reaction was allowed to proceed in the absence of added buffer, the pH of the incubating mixture remained at 6.4-6.5 throughout. Moreover, pH titration curves with acid or alkali pre- and post-incubation were not significantly different. *A clotting inhibitor was not formed by the action of the enzyme.* Neither the altered thromboplastic suspension, nor its centrifuged sediment, nor its supernatant showed thromboplastic activity or inhibition of thromboplastic activity when added to fresh thromboplastic suspensions. Thus the conversion of clotting time to percent thromboplastin discussed previously seems to be valid.

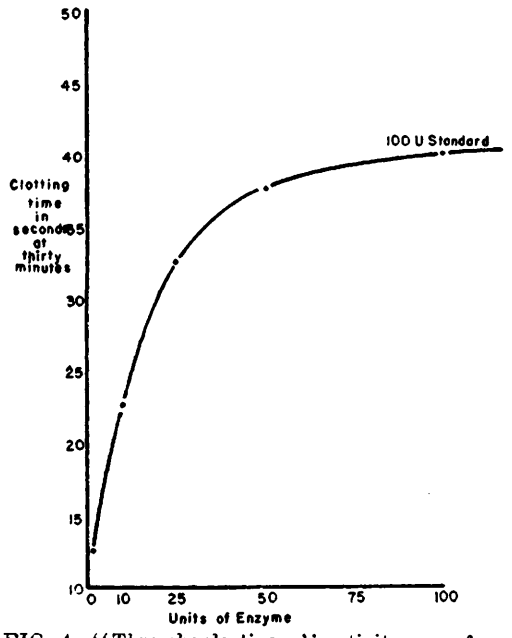

FIG. 4. "Thromboplastinase" activity as a function of concentration.

The data presented thus far were obtained with the enzyme acting on rabbit brain thromboplastic suspensions. In testing thromboplastinase against thromboplastic suspensions of other tissues and other animals, we found that thromboplastinase also decreased the potency of all thromboplastic suspensions tried to date (Table I). Note that Russel-Viper venom, a non-thromboplastic accelerator of blood clotting was not affected by thromboplastinase. The destructive action of thromboplastinase on thromboplastin of whole blood components is reported elsewhere(7).

We have, as yet, found no other enzyme to

TABLE I. Action of "Thromboplastinase" on a Variety of Thromboplastic Substrates.

Source	Clotting times (sec.) T zero	T 60 min.
Rabbit brain	12.6	50
" lung	11.4	82
Beef "	15.0	101
Human placenta	12.1	130
" brain	12.9	83
Rat "	27.9	65
Mouse "	41.1	95
Russel viper venom	20.2	21

cause such a rapid destruction of thromboplastin. Commercial preparations of lipase, rennin, papain, pepsin, trypsin, pancreatin and amylase did not reduce the clotting potency of thromboplastic suspensions. Crude jack bean meal urease caused a slight decrease in thromboplastin potency. Crystalline urease was without effect. Twelve organisms, common air contaminants, other than ATCC No. 10987, grown and tested under similar conditions failed to produce an active enzyme.

The action of the thromboplastinase is apparently highly specific. The enzyme yielded negative tests for lecithinase(8) phosphomonoesterase(9), lipase(10) ATP-ase(11) RNAse (12). Gelatin was not liquefied by the enzyme. No split of phosphorus was observed with cephalin as a substrate. There was no evidence of a nitrogen-split with thromboplastin as substrate using a micro-Kjeldahl procedure for analysis.

Attention was turned to the biochemical changes which bring about or accompany the loss of thromboplastic activity. The substrate employed in these investigations was a "Chargaff preparation" from human placenta. In these experiments, enzyme and substrate were mixed and incubated and aliquots removed over a time course for analysis. One aliquot was analyzed for thromboplastic potency; another was removed simultaneously, and after cold trichloracetic acid precipitation was centrifuged in the cold and analyzed for phosphorus content of the supernatant fluid. Three controls were employed: 1) An incubation mixture having the usual boiled enzyme control; 2) a control in which all factors were kept the same as the test incubation mixture except that copper was added initially to the incubation mixture and 3) a final control in which the copper was added after the reaction had been allowed to proceed to about half completion. These latter two controls were of special significance since it had been found, as mentioned earlier, that copper was an inhibitor of enzyme action. It was of interest then to determine whether chemical changes could be correlated with functional changes in thromboplastin. Results are shown in Fig. 5.

It is seen that with placenta thromboplastin as substrate, an increase in inorganic phosphorus of the supernatant was found, which was correlated with destruction of thromboplastin. The fact that addition of cupric ion to the reaction mixture either initially or during the reaction immediately stopped both loss of thromboplastic potency and the split of phosphorus suggests the identity of these two phenomena.

Further work of this type employing rabbit brain thromboplastin or human brain thromboplastin indicated that analyses for organic rather than inorganic phosphorus was a more reliable index of the chemical action of thromboplastinase. Such analyses were made by digesting the supernatant samples, then analysing for inorganic phosphorus by the method of King(13).

Discussion and conclusions. A previous

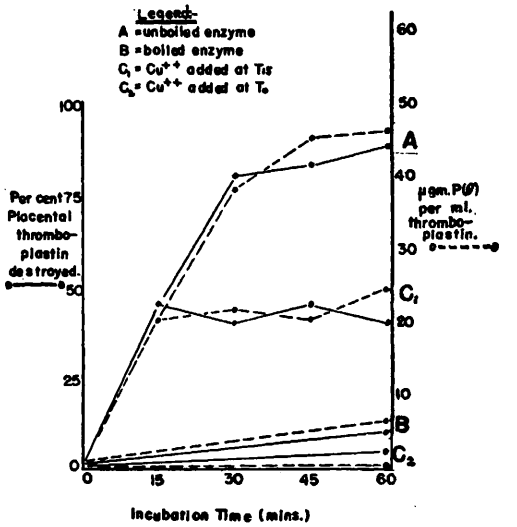

FIG. 5. Loss of thromboplastic activity and split of inorganic phosphorus due to enzyme action.

communication indicating a sudden potency loss of thromboplastic suspensions due to bacterial contamination can now be attributed to the elaboration of the enzyme thromboplastinase by a specific micro-organism. So far as we know, thromboplastinase is the only enzyme capable of destroying tissue thromboplastins of diverse sources, prepared in several different ways. The mode of action of this enzyme is apparently highly specific, involving a split of organic phosphorus seemingly correlated with the loss of thromboplastic potency. A conclusion from such evidence that tissue thromboplastin of different organs or of different species are the same is no doubt premature. It would appear, however, that they do contain a common structural unit, necessary for thromboplastic activity which is susceptible to thromboplastinase cleavage. Analysis of 3 different thromboplastic preparations indicates that this common moiety contains phosphorus. There seems, moreover, to be a quantitative relationship between thromboplastin destroyed and phosphorus liberated as a result of thromboplastinase action. This suggests the possibility of calibrating different thromboplastic preparations in terms of this functional phosphorus rather than in terms of non-specific nitrogen content of the tissue preparation. Further work reported elsewhere(14) indicates that the site of thromboplastinase action is on that portion of thromboplastic lipid which is associated with the inositol phosphatide fraction.

Summary. Thromboplastinase, a new enzyme of bacterial origin has been reported and its preparation and mode of action described. To our knowledge, this is the only enzyme which is apparently specific for thromboplastin. The split of a phosphorus moiety, apparently necessary for thromboplastic activity has been correlated with enzyme action. The theoretical and practical implications of these observations are discussed.

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Reaction of Certain Phytoagglutinins with Flexner-Jobling Carcinoma Cells of the Rat.* (20516)

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The complexity of the surface of all mammalian tissue cells is suggested by our knowledge of the various antigens that make up the surface of human erythrocytes. It is possible that the surfaces of cells vary not only with their histological classification, but also with their state of health. It is not illogical to postulate that the surface of epithelial cells of a carcinoma could be differentiated from the cell surfaces of related normal cells if proper

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