continued oozing underneath the clot. In fact, the clot on the surface acts as a barrier between the fluid blood and the thrombin being applied. In such instances more satisfactory results are often obtained by applying the thrombin in a fine jet under pressure, thus forcing the thrombin into contact with the tissues. Further studies with this and other methods of application are being made.

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Inactivation of Prothrombin by Purified Thrombin Solutions.*

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In some recent experiments on the conversion of prothrombin into thrombin, we discovered a new reaction in which prothrombin is inactivated. It is our present purpose to present data regarding this inactivation, and to discuss the probable nature of the reaction.

Methods. Thromboplastin: Mix 100 g fresh ground beef lung with 100 cc saline. Allow to stand, with occasional stirring, for 48 hours at 5°. Centrifugalize and dilute the fluid obtained with an equal volume of saline. Any prothrombin present is removed by adsorption (one-sixth volume of $Mg(OH)_2$ suspension[1]) followed by centrifugalization. To 100 cc of the clear adsorbed solution add 100 cc $(NH_4)_2SO_4$ solution (saturated at 5°). Centrifugalize and dissolve the precipitate, containing thromboplastin, in 100 cc saline. Repeat the precipitation, and dissolve the final precipitate in 15 cc saline. Dialyze against saline until free of $(NH_4)_2SO_4$. The entire procedure is carried out in the cold room (5°). The product used in the present series of experiments contained 26 mg organic solids per cc.

To prepare an isotonic buffer solution (pH 7.25) which does not interfere with the action of calcium ion, dissolve 1.72 g imidazole (Eastman Kodak) in 90 cc of 0.1 N HCl, and dilute to 100 cc with H_2O.

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[1] Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

The titration of prothrombin was carried out by the 2-stage method of Warner, Brinkhous and Smith.[1, 2]

Experimental. In Fig. 1 are shown experiments in which prothrombin was treated with thromboplastin and calcium in measured amounts. A solution was first prepared containing 0.9% NaCl and 0.15% $Ca(NO_3)_2$. To 14 cc of this was added 1.0 cc of the imidazole buffer solution. Purified prothrombin[3] was added in solid form in such amounts that each cc contained 2250 units prothrombin (approximately 25 mg). The thromboplastin solution, above described, was then added in measured amounts. By this addition the prothrombin-calcium-buffer mixture was diluted somewhat. The data given in Fig. 1 contain a correction for this dilution.

When 1250 gamma (1.25 mg) thromboplastin were added, the conversion of prothrombin into thrombin was completed within 6 minutes (upper curve), and thereafter the thrombin titer remained constant throughout the 11-hour period of observation, showing that the clotting agents had been prepared free of antithrombin. Data not given in the figure showed that this quantity of thromboplastin was a great excess, for complete conversion of 2250 units of prothrombin could be obtained by use of as little as 200 gamma of this thromboplastin. The additional thromboplastin does not increase the thrombin titer, but merely increases the speed of the reaction.

With the use of a much smaller amount of thromboplastin (25 gamma), 450 units of thrombin were promptly formed, and for several hours thereafter the thrombin titer remained at this same level. This sharply limited capacity of thromboplastin to produce thrombin is proof that thromboplastin is consumed in the reaction. This is an important fact which will be discussed in a later paper.

The above mixture, containing 450 units of thrombin, was allowed to stand for a period of 350 minutes. During this period the prothrombin suffered partial inactivation, for one could no longer raise the thrombin titer to the 2250 unit level by adding thromboplastin in excess. Furthermore, the thrombin which did form under these conditions made its appearance very slowly. Controls not given in Fig. 1, showed that this inactivation of prothrombin had occurred gradually throughout the first 4 hours of the experiment. During the first half hour almost all of the prothrombin

[2] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

[3] Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Biol. Chem.*, 1938, **123**, 751.

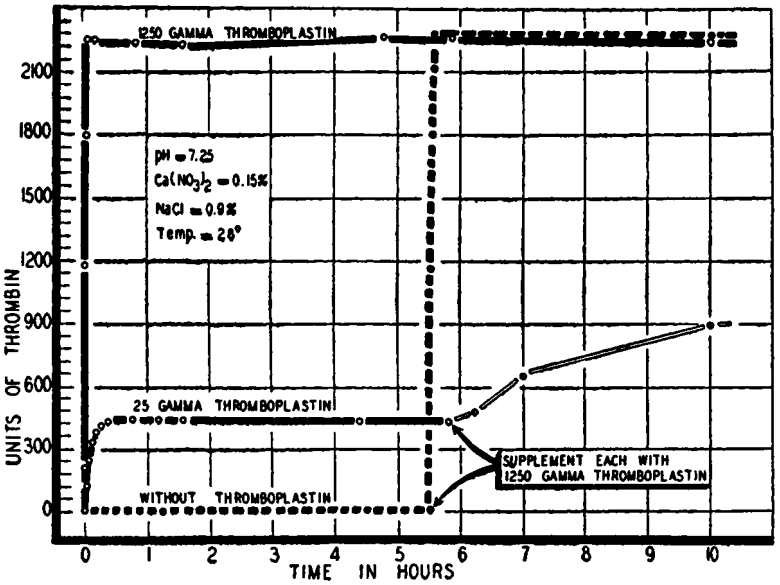

FIG. 1.
Thrombin formation and prothrombin destruction.

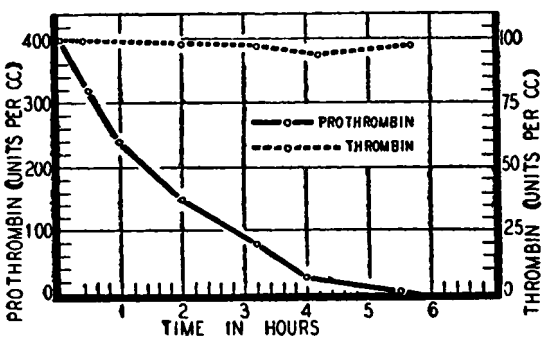

FIG. 2.
Prothrombin inactivation by means of a purified thrombin preparation. The mixture contained 400 units of purified prothrombin and 100 units of purified thrombin. It also contained the imidazole buffer solution (1 volume in 15) and NaCl to the extent of 0.9% and potassium oxalate to the extent of 0.045%. Temperature of experiment 28°.

originally present could be converted into thrombin by the addition of large amounts of thromboplastin. During the following 3.5 hours the excess prothrombin gradually lost its ability to form thrombin, giving finally the weak response described above.

This alteration of prothrombin is clearly not a change which occurs spontaneously in prothrombin, for we have repeatedly found that purified solutions of this material retain their activity for many hours at room temperature. Nor is the inactivation due

to the action of thromboplastin, for when prothrombin and thromboplastin are incubated in the absence of calcium the prothrombin retains its activity for long periods of time. Furthermore, the lower curve of Fig. 1 shows that calcium does not inactivate prothrombin. The two substances were left in contact for over 5 hours, after which thromboplastin was added. A total of 2250 units of thrombin promptly formed, showing that the prothrombin had retained its activity in full.

Since the destruction of prothrombin is due neither to calcium nor to thromboplastin, it would appear that thrombin itself may be the agent responsible. Unless this is true, one must assume that the destructive agent is one which appears simultaneously with the formation of thrombin, and, like thrombin, requires both calcium and thromboplastin for its production.

Fig. 2 shows an experiment which gives further evidence that thrombin is the substance which causes the inactivation of prothrombin. Purified solutions of prothrombin and thrombin were mixed and allowed to stand at room temperature for a period of 6 hours. At intervals the prothrombin titer was determined as the increment of thrombin produced in 6 minutes by adding calcium in optimal amounts (0.15% $Ca(NO_3)_2$) and thromboplastin in excess. As Fig. 2 shows, a marked fall in the prothrombin titer began almost at once, and at the end of 6 hours very little prothrombin activity remained. The thrombin used in this experiment was purified by a technic previously described[4] and the excess thromboplastin used in its formation was largely removed in the process. In the present experiment (Fig. 2) calcium ion was eliminated by including potassium oxalate in the mixture. It is thus clear that the agent which destroys prothrombin is present in the thrombin preparation itself. Since the thrombin was purified by repeated precipitation, it seems likely that the destructive agent is thrombin itself and not some impurity.

We have conducted a few preliminary experiments on heat inactivation of purified thrombin solutions. A temperature of 60° for 30 minutes causes considerable loss in both the clotting power and in the ability to inactivate prothrombin. The loss in each respect cannot yet be said to be quantitatively identical, but they are clearly of the same order of magnitude.

The evidence that thrombin causes inactivation of its own precursor, prothrombin, introduces an apparent anomaly. This con-

[4] Seegers, W. H., Brinkhous, K. M., Smith, H. P., and Warner, E. D., *J. Biol. Chem.*, 1938, **126**, 91.

clusion, however, is somewhat analogous to the recent conclusion of Kunitz[5] that trypsin can convert its precursor, trypsinogen into an inert protein.

It is of interest that in the experiment of Fig. 2 very little thrombic activity was lost during the 6-hour period of incubation. This might be taken to indicate that thrombin acts enzymatically to inactivate prothrombin, but further work is needed before this question can be answered definitely.

Summary. Evidence is presented to show that solutions of purified thrombin contain a substance which reacts slowly with prothrombin, causing inactivation of the latter. The destructive agent is not present in the calcium, prothrombin, or thromboplastin solutions from which the thrombin is produced. The factor is heat-labile and the evidence indicates that thrombin itself is the factor in question.

[5] Kunitz, M., *J. Gen. Physiol.*, 1939, **22**, 293.