10991

Prothrombin, Thromboplastin, and Thrombin: Quantitative Interrelationships.*

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We wish to present data concerning the relative amounts of prothrombin, thromboplastin, and calcium which are needed to form a given amount of thrombin. One obstacle to such a study has always been that prothrombin and thromboplastin are accompanied in nature by antithrombin. Unless this antithrombin is carefully removed, the thrombin is inactivated almost as rapidly as it is formed, and it is impossible to obtain satisfactory data concerning the amount of thrombin produced.

With technics recently developed in this laboratory we can prepare prothrombin and thromboplastin which are highly potent and are free of antithrombin; but while using these preparations we encountered a second obstacle which had not been anticipated. It was found that on incubating prothrombin, thromboplastin and calcium, there is promptly formed a substance which inactivates prothrombin, and thus decreases the eventual yield of thrombin.[1] This destructive agent was not present in the prothrombin, thromboplastin, nor in the calcium. It is evident that either the destructive agent is thrombin itself, or that it is some other substance which is formed under similar conditions.

In the present study the inactivation of prothrombin is still evident, but by making due corrections, it appears that the formation of thrombin from prothrombin and thromboplastin follows simple laws of proportion. This finding eliminates support for the old concept that thromboplastin is a traditional enzyme, capable of converting unlimited quantities of prothrombin into thrombin. This work also supplements older work on prothrombin and thromboplastin which until now has been on a qualitative basis.[2, 3]

Experimental Procedure. The purified thromboplastin solution was prepared from beef lung as previously described.[1] The prepa-

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1 Mertz, E. T., Seegers, W. H., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 657.

2 Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 531.

3 Ferguson, J. H., *Am. J. Physiol.*, 1938, **123**, 341.

ration of thromboplastin thus obtained contains approximately 2.5% organic solids. In the present experiments it was diluted 11-fold with calcium-saline (0.9% NaCl + 0.15% $Ca(NO_3)_2$) prior to use.

In order to determine quantitatively the effect of thromboplastin, it is essential that the prothrombin used be entirely free of thromboplastin. Technically this result is not readily achieved, and most of our prothrombin preparations[4] form a limited amount of thrombin when calcium is added. In the present experiments, however, we used only those samples of prothrombin which did not react with calcium unless thromboplastin was added also.

It was also important for these studies that antithrombin be eliminated as completely as possible from the reacting mixtures. All solutions of prothrombin and of thromboplastin were tested in advance by being mixed with measured amounts of thrombin. These preparations were then accepted for use provided they produced no detectable destruction of thrombin in 10 hours.

The prothrombin was made up in a solution with NaCl (0.9%) and $Ca(NO_3)_2$ (0.15%) which was diluted with 5% of its volume of imidazole buffer[1] before the prothrombin was added. The amount of calcium present was found to be physiologically optimal. An increase

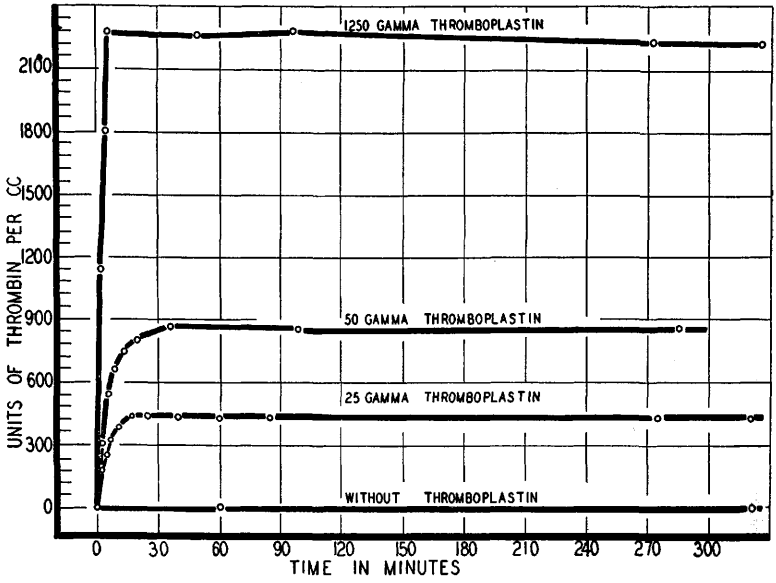

FIG. 1.

Rate of thrombin formation with small and with large quantities of thromboplastin. Initial prothrombin concentration was 2300 units per cc.

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

or decrease of 20% had no effect on the results. The prothrombin solution used contained 4% prothrombin, and had a thrombin titer of approximately 2200 units of prothrombin per cc (*i. e.*, on being diluted 2200-fold, the thrombin formed from it clots fibrinogen in a standard interval of 15 seconds).

Results. Figure 1 shows the result of treating a large amount of prothrombin with calcium and variable quantities of thromboplastin. With 25 gamma of thromboplastin, the thrombin titer rose within 25 minutes to the 400 unit level. It then remained at this low level for many hours. This experiment shows that thromboplastin does not convert unlimited quantities of prothrombin into thrombin.

By increasing the amount of thromboplastin enormously, the relationships are reversed. Under these circumstances, we observed that prothrombin also has a sharply limited capacity to produce thrombin. Thus, on adding 1250 gamma of thromboplastin, there occurred a rapid production of 2300 units of thrombin. Other experiments, not shown on the chart, showed that further increase in thromboplastin did not increase the yield of thrombin beyond this point.

Figure 1 also shows the results obtained with 50 gamma of thromboplastin. The yield of thrombin in this case was almost exactly twice as great as when 25 gamma were used. This suggests a rela-

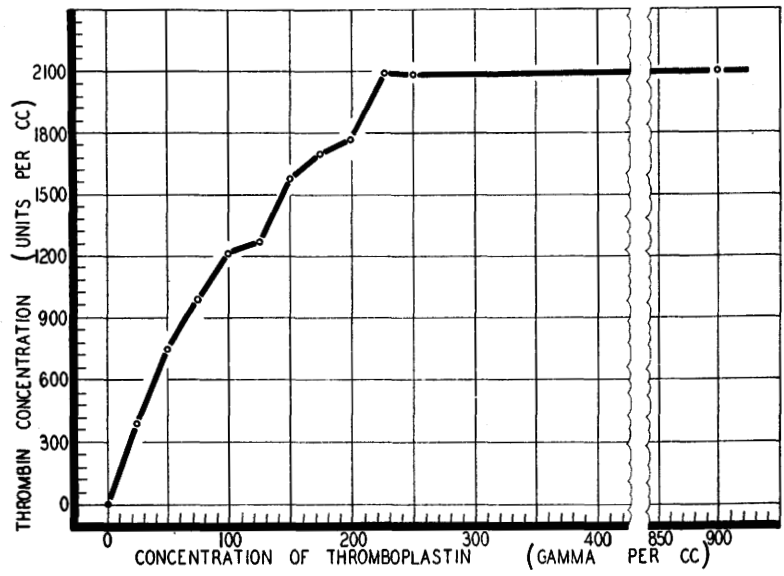

FIG. 2.

Total thrombin production with varying quantities of thromboplastin. In all cases the initial prothrombin concentration was 2100 units per cc. The curve gives the thrombin concentration at the end of 10 hours.

tionship of direct proportionality, and for the purpose of studying this further, a larger series of tests was made, the results of which are given in Fig. 2. Varying amounts of thromboplastin were employed, and in each case, sufficient time was allowed for the thrombin titer to reach its maximum. These maxima are presented in the chart as ordinates; the abscissae correspond in each case to the amount of thromboplastin used. The maximum yield of thrombin, 2100 units per cubic centimeter, was obtained by adding 225 gamma of thromboplastin. The individual protocols of these experiments showed that larger amounts of thromboplastin increased somewhat the reaction rate, but not the amount produced.

The curve leading up to the 2100 unit level followed quite closely the path of a straight line throughout the first half of its course. The second half deviates definitely to the right, giving the inflection point at the top a definitely rounded appearance. Suspecting that this deviation from the course of a straight line might be due to complicating factors, we conducted studies which led to the discovery mentioned above that thrombin itself appears to destroy a part of the prothrombin, and thus prevents the thrombin titer from being as great as might otherwise be the case.

Figure 3 illustrates the degree to which prothrombin inactivation occurs. To a solution containing 2300 units of prothrombin were added 16 gamma of thromboplastin. The thrombin titer rose within

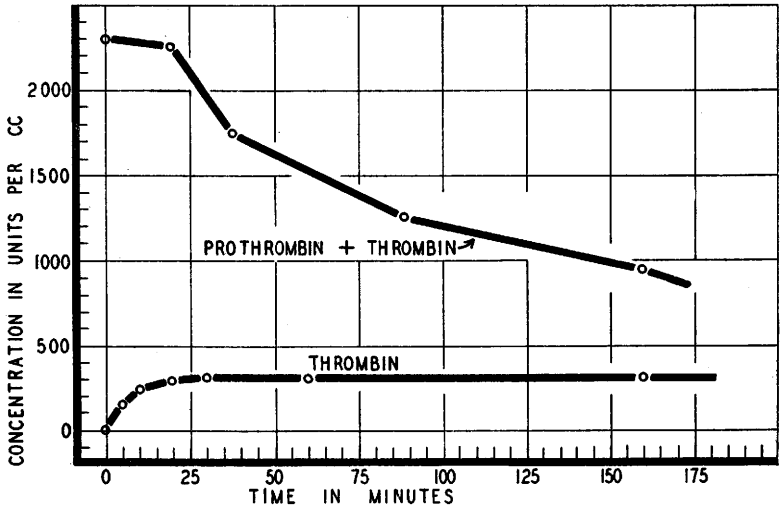

FIG. 3.

Inactivation of unconverted prothrombin. The prothrombin activity was measured as the increment of thrombin produced in 6 minutes by the addition of a large excess (1250 gamma) of thromboplastin.

25 minutes to the 300 unit level, after which the titer remained unchanged. This formation of thrombin involved, theoretically, a corresponding fall in the prothrombin titer but no fall in the combined titer of the two. That the combined titer did fall is shown in the figure and is due to inactivation of prothrombin.[1] This inactivation did not eliminate the supply of prothrombin for many hours, but did reduce the supply markedly during the period illustrated in the figure. In this particular experiment, it is evident that the supply of prothrombin was adequate to exhaust completely the thrombin-forming power of the thromboplastin. In the lower portion of the curve of Fig. 2, there is also an excess of active prothrombin, and the thrombin values approached very closely the maximum theoretical converting power of the thromboplastin used. The same can be said of the horizontal portion of the curve at the top of Fig. 2. In this portion of the curve, thromboplastin was present in such great excess that the reaction was driven rapidly to completion and the time which elapsed was too brief to permit any consequential changes in the reactivity of the prothrombin. However, in the upper half of the ascending curve (1200-2100 units thrombin), the reaction proceeded more slowly and the inactivation of prothrombin was such that the full titer of thrombin could not be obtained. In experiments not recorded here, we have shown that under these conditions, reactive thromboplastin still exists in the mixture. On adding additional quantities of prothrombin, further amounts of thrombin are formed and the entire ascending portion of the curve approximates that of a straight line.

We have conducted many other experiments of this general type, but differing in having a constant quantity of thromboplastin and variable quantities of prothrombin. The curves have all been similar in form to those shown in Fig. 2. There is, in each case, an intermediate zone in which a measurable quantity of prothrombin disintegrates and must be replaced, if one is to obtain the theoretical yield of thrombin. With this correction, the experiments confirm the law of proportionality developed in connection with Fig. 2.

The prothrombin solution used in the above experiments contained 40 mg prothrombin per cc. By correcting Fig. 2 for prothrombin inactivation, it is evident that approximately 0.15 mg of thromboplastin were equivalent to this amount of prothrombin. This implies a utilization ratio of approximately 265 to 1. With further purification of prothrombin and thromboplastin, these data are subject to revision. The figure is one which must be given consideration in conducting further work on the mechanism of thrombin formation.

Finally, we wish to point out that the rule of proportionality per-

mits us, for the first time, to lay the groundwork for a quantitative method for the titration of thromboplastin. By mixing the unknown material with an excess of purified prothrombin, one can bring about the rapid development of thrombin in the solution. The titer of this thrombin solution is directly proportional to the amount of thromboplastin present. This method is analogous to the one developed and used in this laboratory for the titration of prothrombin.

Summary. Thromboplastin is consumed when it reacts with prothrombin in the presence of calcium ion. This finding eliminates support for the old concept that thromboplastin is a traditional enzyme, capable of converting unlimited quantities of prothrombin into thrombin. Evidence is presented which shows that the quantity of thrombin produced from an excess of prothrombin is directly proportional to the quantity of thromboplastin added to the reaction mixture. When, on the other hand, thromboplastin is present in excess, the amount of thrombin formed is proportional to the amount of prothrombin added.

10992

Occurrence of the Pellagra-Like Syndrome in Range Chicks.

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In chick virus transmission experiments it is sometimes necessary to use range chicks hatched away from the laboratory. The presence or absence of borderline nervous symptoms are often neither observed nor detected as part of the experimental procedure. The use of commercial rations is usually unsatisfactory in relieving cannibalism,[1] and other findings such as delayed prothrombin clotting values may occur in supposedly normal chicks.[2, 3]

This note summarizes the results of an investigation of the occurrence of the pellagra-like syndrome in range chicks maintained on an adequate ration.

In over a thousand pullorum-free White Leghorn chicks maintained since hatching (April 12) at a farm range, over 200 presented

1 Bass, C. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 488.

2 Schønheyder, F., *Am. J. Physiol.*, 1938, **123**, 348.

3 Mason, H. C., and Smith, M. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 583.