Activation of Chymotrypsinogen by Purified Thrombokinase. Phosphatide and Ionic Calcium as Accessory Factors.* (29560)

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Thrombokinase has been isolated from bovine plasma(1) and shown to resemble trypsin in some respects(2). It has long been known that trypsin activates chymotrypsinogen; and it has now been found that purified thrombokinase activates chymotrypsinogen. Moreover, phosphatide and ionic calcium hasten this process, as they do the activation of prothrombin. These results have been quite definite and easily reproducible. However, they have led to new issues which invite further inquiry.

Materials and methods. BTEE.—Benzoyl-L-Tyrosine Ethylester, Mann Research Laboratories, Inc. *TAMe.*—p-Toluenesulfonyl-L-Arginine Methylester HCl, H. M. Chemical Co. Ltd. *Tris*—Tris(Hydroxymethyl)aminomethane. *Purified thrombokinase.*—As described(3), except for step 8, where a broad band of electrophoretic fractions was used. The enzyme was finally concentrated by precipitating at low ionic strength near pH 5.0, and redissolving in 0.05 M tris buffer, pH 7.8. The yield was 23.3 mg from 212 liters of oxalated plasma; and the TAMe esterase activity was 227 units(4) per mg. Crucial experiments were verified with thrombokinase taken only from the 3 peak electrophoretic fractions. The latter material has given a single boundary in the ultracentrifuge. *Step-6 thrombokinase.*—Partially purified thrombokinase, containing appreciable thrombin(2). *Trypsin, chymotrypsin, chymotrypsinogen A.*—Crystallized. Worthington Biochemical Corp. Crucial results were verified with chromatographically homogeneous chymotrypsinogen which contained practically no chymotrypsin. The other chymotrypsinogen preparation, although repeatedly crystallized, did contain a small amount of active enzyme, revealed in Fig. 1. *Phosphatide.* "Inosithin," alcohol-insoluble phosphatides from soybeans. Associated Concentrates, Inc. Chiefly inositol phosphatides and cephalin. *Activation of Chymotrypsinogen.* In 0.05 M tris buffer, pH 7.8 at 30°C. Total volume, 1.0 ml. At intervals, samples were assayed for chymotrypsin. *Assay of chymotrypsin activity.* Hydrolysis of BTEE, measured by increase in optical density at 256 millimicrons(5). The 1 to 21 dilution of the sample and the short time required for assay (11 minutes at most), made continuing activation negligible for present purposes, and optical density had a linear relation to time.

Results and discussion. With chymotrypsinogen constant, the rate of activation was determined by the amount of thrombokinase, as shown in Fig. 1. With no thrombokinase, there was no increase in activity. Under the conditions of the test, thrombokinase showed no activity on BTEE, and did not produce such activity upon incubation alone. As estimated from an empirical chart, the highest activity reached in Fig. 1 was equivalent to 26.7 μg alpha-chymotrypsin per ml. This study was concerned with rate of activation rather than final yield.

Most remarkable was the large quantity of thrombokinase required. Exact comparisons with trypsin have not been possible, because the activity-quantity curves have not been parallel for the 2 enzymes. However, at one level, trypsin caused activation comparable to that caused by 1,000 times its weight of thrombokinase. Two possibilities may be considered: 1. the effect on chymotrypsinogen is due to a small amount of another enzyme contaminating the thrombokinase; 2. thrombokinase activates chymotrypsinogen, but not very efficiently.

Preparations of thrombin have been reported to activate chymotrypsinogen(6), but the present results cannot be ascribed to thrombin. Purified thrombokinase activated chymotrypsinogen faster than a quantity of

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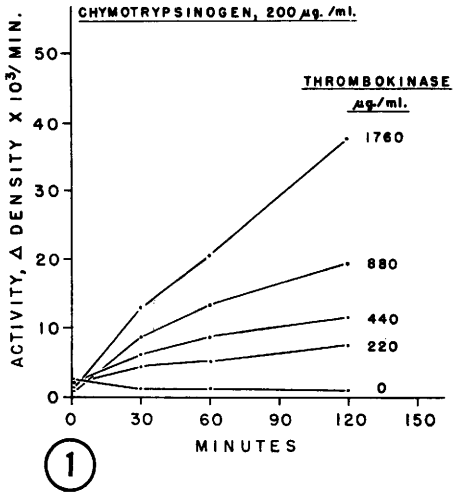
step-6 thrombokinase which brought more than 100 times as much thrombin into the system. As a first approximation, the chymotrypsinogen-activating power of these 2 preparations was proportional to their thrombokinase activity. Nevertheless, the possibility remains that the thrombokinase preparations might contain another activator.



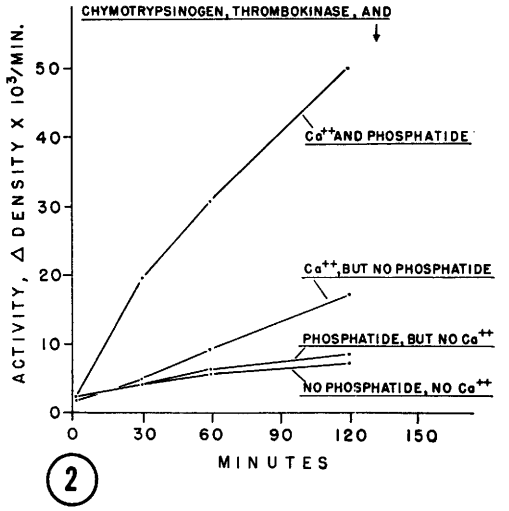


FIG. 1. Activation of chymotrypsinogen by purified thrombokinase. Each mixture contained 200 µg chymotrypsinogen per ml and amount of thrombokinase shown on chart.

FIG. 2. Phosphatide and ionic calcium as accessory factors. All mixtures contained 200 µg chymotrypsinogen and 220 µg thrombokinase. Calcium chloride, when present, was 0.0025 M. When phosphatide was added, the amount was 100 µg. In all cases, thrombokinase was the last reagent added.

On the other hand, if the results of Fig. 1 are due to thrombokinase *per se*, consideration must be given to its low activity. In the first place, the tests were performed without any attempt to minimize proteolytic complications. Perhaps such measures would increase apparent rates. Second, the concentrations of thrombokinase indicated in Fig. 1, although very large, are only 44 times those used to activate prothrombin in presence of ethylenediamine tetra-acetate (EDTA)(3). This difference might reflect the better adaptation of thrombokinase to prothrombin. Third, the efficiency of thrombokinase, even in the activation of prothrombin, depends on the presence of accessory factors.

It is therefore of interest that ionic calcium and phosphatide also function as accessory factors for purified thrombokinase when the substrate is chymotrypsinogen. As shown in Fig. 2, phosphatide had little or no effect unless ionic calcium was included. This resembles the relation established for prothrombin activation. Calcium had a definite effect without phosphatide; the analogous effect on prothrombin activation has not been beyond the limits of experimental error(7). The present effect of calcium was not attributable to magnification of assay values, since the calcium content of the BTEE assay medium, which was routinely 0.048 M, was increased less than 0.3% by the experiment. This interpretation was verified by tests in which calcium was added to chymotrypsin before assay. In other control experiments, phosphatide plus calcium seemed to augment the assay of chymotrypsin in some instances, but not enough to account for the large effect of the pair during the activation process. Moreover, the effect of phosphatide plus calcium, present continuously during activation, was much greater than their effect when added to the activating mixture shortly before assay. While it is thus certain that the overall activation process was accelerated, the mechanism is still not defined, and might include stabilization effects. Without thrombokinase, phosphatide plus ionic calcium failed to activate chymotrypsinogen.

In an earlier study(8) phosphatide had

no effect on the activation of chymotrypsinogen by trypsin, in the presence of ionic calcium. If this were to hold true over a wide range of conditions, it would offer a possible distinction between activation by trypsin and by thrombokinase. Further tests are now indicated, for experience has emphasized the variability of the phosphatide effect with changing conditions.

Phospholipids augment other enzyme reactions(9-11). The present case may be the first in which phosphatide accelerates the enzymatic activation of a zymogen other than a clotting factor.

Summary. Highly purified thrombokinase, derived from bovine plasma, activated chymotrypsinogen. Chymotrypsin was assayed by the hydrolysis of BTEE, which was not split by thrombokinase under the conditions used. Activation of chymotrypsinogen may have been due to thrombokinase, *per se,* or to an impurity, since a large quantity of thrombokinase was required. However, phosphatide accelerated the process, provided that ionic calcium was included. Without thrombokinase, phosphatide plus ionic calcium failed to activate chymotrypsinogen.

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Anticomplementary Effects and Complement Activity of Human Sera. (29561)

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The relationship between low complement (C′) activity and anticomplementary action of serum has been a source of considerable confusion. Pronounced anticomplementary action of fresh human serum is observed with a small percentage of sera, chiefly from patients with conditions associated with hyperglobulinemia(1). When unheated, these pathological sera are not anticomplementary since they invariably possess C′ activity. Anticomplementary action of such sera becomes manifest, however, only after the serum has been heat inactivated, usually at 56°C or above for 30 minutes. With the standard sheep cell hemolytic system, C′ activity can be found in most fresh unheated mammalian sera, although wide variations exist among different species. In humans, C′ levels below normal have been found among patients with acute glomerulonephritis(2), systemic lupus erythematosus(3), and even from a healthy person(4). In these individuals slight anticomplementary action may be independent of C′ titer, however, as exemplified in this

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